A STUDY OF THE INTERACTION BETWEEN SUSCEPTIBLE AND RESISTANT GRAPEVINES AND PHYLLOXERA

A thesis submitted in fulfilment of the degree of Doctor of Philosophy

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by

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ABSTRACT

This thesis comprises a study of the interaction between grapevine roots and the gall-forming pest grape phylloxera (*Daktulosphaira vitifoliae* Fitch).

A study of the interaction with the susceptible *Vitis vinifera* cv. Shiraz has shown that on primary roots phylloxera feed within the root cortex. Nodosity formation is accompanied by inhibition of endodermis differentiation and accumulation of starch to a concentration approximately ten times that in uninfested roots. Comparisons of nodosities with uninfested roots by SDS-PAGE analysis of proteins revealed higher concentrations in nodosities but no significant differences in composition; HPLC analysis of free amino acids showed more than double the amino nitrogen content in nodosities; Northern blot analysis, targeting various genes including those proposed to be defence-related, revealed no significant change in gene expression with the exception of the VvTL2 gene which was upregulated in leaves of SRU-1 phylloxera infested vines.

The interaction of phylloxera with several rootstocks or resistant vine species (Schwarzmann, Ramsey, *V. riparia*, Börner, *V. rotundifolia*) was compared with the interaction with *V. vinifera*. Tissue culture-based cocultivations allowed assessment of resistance to VWL-strain phylloxera, and allowed biotype comparisons of the strains VWL-1 and SRU-1. VWL-1 phylloxera established successful nodosities on *V. vinifera* and Ramsey, with poorer performance on Ramsey. VWL-1 phylloxera could not survive on the other varieties, and did not even attempt to feed on *V. rotundifolia*. Feeding sites on Ramsey roots showed accumulation of an autofluorescent compound, possibly indicating a defence response. In Ramsey no starch accumulated within the gall tissue. In *V. riparia,* nodosities were initiated and starch accumulation was evident, but was accompanied by tissue browning and oxidation of phenolics. SRU-1 phylloxera survived on Schwarzmann and accumulation of an autofluorescent compound at the feeding site did not appear to adversely affect this strain of phylloxera.

Primary root anatomy was not significantly different between the vine varieties examined, however the more resistant varieties have higher phenolic content in the outer layers of the root cortex. Qualitative HPLC analysis of root free phenolics revealed no significant difference between varieties, except for one compound unique to the immune species *V. rotundifolia*. Resistance to phylloxera appears to be associated with rapid oxidation of phenolics, and immunity of *V. rotundifolia* with non-preference.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the university library, being available for loan and photocopying.

Alison Kellow.

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PUBLICATIONS ARISING FROM THIS RESEARCH

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Kellow, A.V., Corrie, A.M. and van Heeswijck, R. (1999) Surface sterilisation of phylloxera eggs for investigating grapevine-phylloxera interactions in tissue culture. Australian Journal of Grape and Wine Research. **5**, 27-28.

Kellow, A.V., Sedgley, M., McDonald, G. and van Heeswijck, R. (2000) Analysis of the interaction of phylloxera with susceptible and resistant grapevines using *in vitro* bioassays, microscopy and molecular biology. Eds Powell, K.S. and Whiting, J. Proceedings of the International Symposium on Grapevine Phylloxera Management. 21-30.

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DEFINITIONS OF TERMS USED IN THIS THESIS

amyloplast

Plastid containing starch grains.

immune

A grapevine which is completely unaffected by phylloxera. Phylloxera are completely unable to establish feeding sites or initiate gall formation.

nodosity

Phylloxera-induced gall formed on a primary root of a grapevine.

phylloxera strain

A homogeneous phylloxera population showing distinct genetic characteristics (determined by PCR based DNA typing).

phylloxera biotype

A homogeneous phylloxera population (or strain) showing distinct biological characteristics in terms of its response to and effect on various grapevine species or hybrids.

phylloxera susceptible

A grapevine which is unable to withstand infestation with phylloxera. Phylloxera are able to reproduce freely, successfully initiating both nodosities and tuberosities, and the vine eventually declines and dies.

phylloxera resistant

A grapevine which is able to negatively affect the phylloxera population. Phylloxera are able to survive and reproduce only at very reduced rates or not at all. Few nodosities are initiated and these are often aborted.

phylloxera tolerant

A grapevine which is able to withstand infestation with phylloxera. Phylloxera are able to reproduce but generally only initiate nodosities.

primary roots

Roots showing only primary anatomy. In tissue cultured vines this is the only root type present.

secondary roots

The storage root system; roots more than one year old, with secondary thickening.

tuberosity

Phylloxera-induced gall formed on a secondary root of a grapevine.

variety

Not used here in the botanical sense, but rather a term used in this study to refer to a particular grapevine species, accession or hybrid.

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ADPG	adenosine diphosphate-glucose
AMPS	ammonium persulphate
ATP	adenosine triphosphate
AU	absorption units
β-Me	β-Mercaptoethanol
°C	degrees Celsius
cDNA	complementary DNA
CHS	chalcone synthase
cm	centimetre
CO_2	carbon dioxide
cv.	cultivar
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAA	formaldehyde 5 parts, acetic acid 5 parts, alcohol (ethanol) 90 parts
fw	fresh weight
g	gram(s)
GABA	γ-amino butyric acid
GMA	glycolmethacrylate or 1,4-hydroxyethyl methacrylate
G-1-P	glucose-1-phosphate
GUS	β-glucuronidase
HPLC	high pressure liquid chromatography
HR	hypersensitive response
hr	hour(s)

IAA	indole-acetic acid
J2	stage two juvenile nematode
kg	kilogram(s)
kV	kilovolts
L	litre(s)
M	molar
mg	milligram(s)
µg	microgram(s)
ml	millilitre(s)
µl	microlitre(s)
mm	millimetre(s)
min	minute(s)
mw	molecular weight
NaDIECA	sodium diethyldithiocarbamate
PAL	phenylalanine ammonia-lyase
PAS	periodic acid-Schiff's reagent
PAS/TBO	periodic acid-Schiff's reagent/ Toluidine blue O
PBS	phosphate buffered saline
pers. comm.	personal communication
PPO	polyphenol oxidase
PR	pathogenesis-related
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
RT	room temperature
SAR	systemic acquired resistance
SD	standard deviation

SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
sec	second(s)
sp.	species (singular)
spp.	species (plural)
SRU-1	phylloxera strain isolated from Schwarzmann leaf galls, Campbell's vineyard, Rutherglen, isolate no. 1. (Corrie *et al.* 1997a)
StSy	stilbene synthase
SSC	sodium chloride/ sodium citrate
TE	tris/EDTA
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
TBO	toluidine blue O
UDP-glucose	uridine diphosphate glucose
vol.	volume
v/v	volume/volume
w/v	weight/volume
V	volts
VR	*Vitis rotundifolia* × *V. vinifera*
VWL-1	phylloxera strain isolated from *V. vinifera* root galls, Brown Brothers' Whitlands vineyard, King Valley, isolate no. 1. (Corrie *et al.* 1997a)

1. INTRODUCTION

1.1 Grapevine phylloxera

1.1.1 Biology of phylloxera

Daktulosphaira vitifoliae Fitch or 'grape phylloxera' is a cecidogenic (gall-forming) aphid belonging to the Order Homoptera, Family Phylloxeridae. Its host range is limited to members of the genus *Vitis* which comprises species native to America and Asia, and the European *Vitis vinifera* L. (Viala and Ravaz 1901). Grape phylloxera is a native of the north-eastern United States of America where its natural hosts are some of the American *Vitis* species (Helm 1983).

Phylloxera live in pouch-like galls on the leaves of some American *Vitis* species, and overwinter on the roots where galls may form as simple swellings (Coombe 1963). The roots of these American vines show varying degrees of tolerance or resistance to phylloxera, and many species are able to survive infestation without being adversely affected (Viala 1901).

The response to phylloxera of *V. vinifera,* the vine species involved in most commercial grape production, is quite different from that of American *Vitis* species. On *V. vinifera*, phylloxera are generally unable to form leaf galls, but the roots have no effective defence mechanism and become heavily infested, with the formation of many well developed root galls. The formation of galls on secondary roots of susceptible vines leads to the decay of the root system and eventual death of the vine.

Lifecycle of phylloxera

The generally accepted lifecycle of grape phylloxera is summarised by Coombe (1963) (figure 1.1). The basic lifeform known to occur in Australian vineyards is the asexual root dwelling form, called the 'radicolae'. Phylloxera dwelling on roots produce eggs which hatch to highly mobile first instar insects, often referred to as 'crawlers'. These undergo three moults to produce fourth instar insects which mature to adulthood and produce eggs. This lifecycle takes, on average, three weeks on a compatible host.

Phylloxera may also develop into a winged form (alate). Presence of alates, according to the accepted lifecycle, implies at least initiation of the sexual cycle. Alates have been reported in Australian vineyards, but completion of the sexual cycle under field conditions in Australia remains in some doubt (King and Buchanan 1986). It is clearly the radicolae which are responsible for economically significant damage to *V. vinifera* vines.

Recently, populations of phylloxera that exhibit a significantly different lifecycle have been described by Downie and Granett (1998) on *Vitis arizonica* Engelm. in Arizona. In these populations there is no winged form, nor a root dwelling form, and sexual forms are produced by 'gallicolae' (phylloxera living in leaf galls). It is unlikely that such strains occur in Australia, however this example serves to illustrate that the lifecycle of this insect might vary considerably more between populations than has been acknowledged to date.

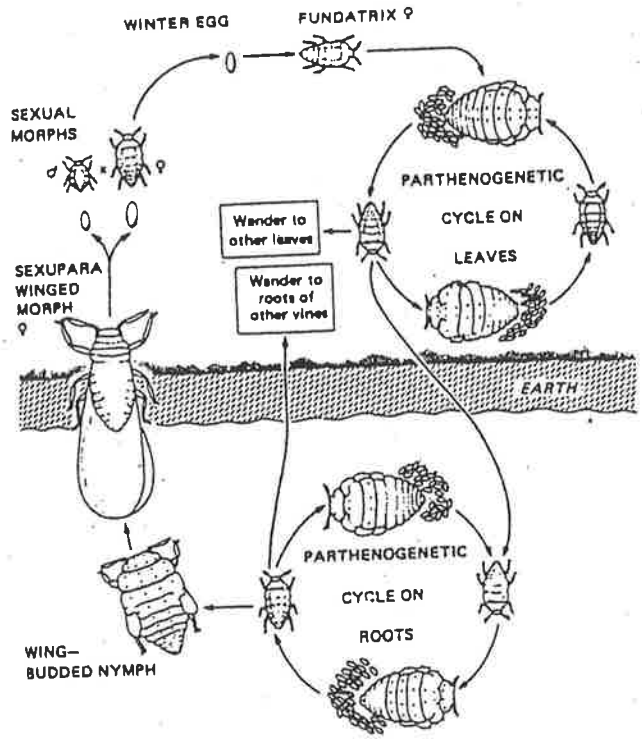


Figure 1.1 Lifecycle of phylloxera (from Coombe 1963).

Presence of biotypes

Phylloxera populations can be grouped into strains and biotypes according to their genetic differences and biological response to, and effect on, different grapevine species and/or varieties. Phylloxera strains are defined here as homogeneous populations with distinct genetic characteristics, and biotypes as homogeneous populations with distinct biological phenotypes. In Australia, strains appear to be a subset of biotypes, however strictly speaking, the definitions are independent of one another.

Börner (1914) investigated and confirmed the differential response of two phylloxera isolates to various vine types. Despite this, and other suggestions of the existence of biologically distinct phylloxera strains (de Castella 1936), subsequent observation of variation in vine response to different phylloxera isolates was often attributed to

environmental factors (Coombe 1963, King *et al.* 1982, Whiting *et al.* 1987, Buchanan 1990). Prior to 1985, the decline of vineyards in California prompted renewed investigation into the biological characteristics of phylloxera strains (Granett *et al.* 1985, King and Rilling 1985, 1991).

The term 'biotype' was coined by Granett *et al.* (1985) who described 'biotype B' as being biologically distinct from a previously characterised isolate of phylloxera, 'biotype A'. Biotype B has increased fecundity and shorter generation time on the rootstock ARG1 (*V. vinifera* cv. Aramon × *V. rupestris* cv. Ganzin). Most importantly, it is able to colonise secondary roots and thus overcome the tolerance of this rootstock in the field, which biotype A is unable to do. Distinct biotypes have now been characterised for a range of additional phylloxera strains, including strains 1, 2, 3 and 4 from California (Fong *et al.* 1995, de Benedictis *et al.* 1996), a German strain, and Hungarian strains Hung 1, 2 and 3 (Kocsis *et al.* 1999).

In Australia, four genetically distinct populations of phylloxera have been identified (Corrie *et al.* 1997a, Corrie *et al.* unpublished). Their biotyping was carried out in 1997-98 (in parallel with the work conducted in this study). Two main biotypes have been described (Corrie *et al.* unpublished): one similar to biotype B which includes strain VWL-1, and a second one distinct from either biotype A or B. This biotype, which includes strain SRU-1, is less damaging to *V. vinifera* than VWL-1 and has higher survival and developmental rates on the rootstocks Schwarzmann and ARG1 than on *V. vinifera*. Australian biotypes of phylloxera are discussed further in Chapter six.

1.1.2 History of the spread of phylloxera

Phylloxera was initially introduced to new viticultural areas via the importation of infested American vine species. This was followed by spread on infested vine propagation material during the rapid expansion of vineyards worldwide in the nineteenth century (Ordish 1972). Phylloxera was first reported in France in 1868, California in 1873 (Lider 1958), and in Australia in 1877 (Buchanan and Hardie 1978). In Australia, infestations occurred in Queensland, New South Wales and Victoria (Helm 1983), with the latter two states still containing regions of infestation known as Vine Disease Districts (figure 1.2). These areas constitute less than 2% of the current grape growing regions in Australia.

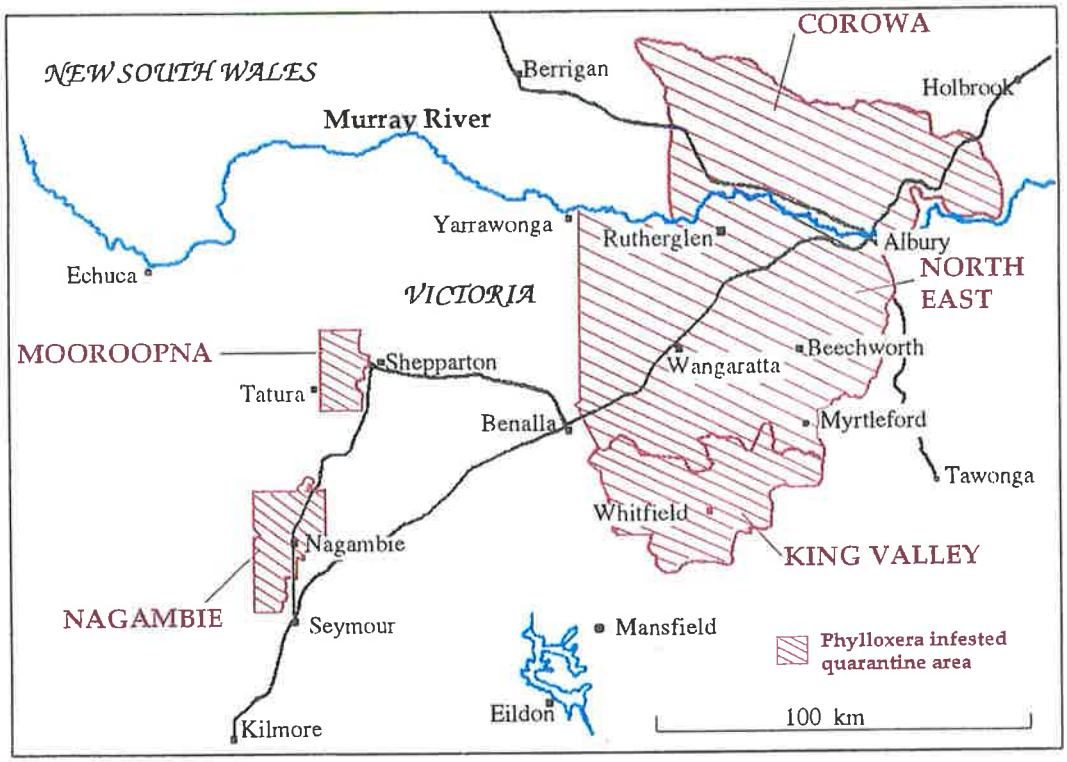


Figure 1.2 Vine Disease Districts in Australia, 1999. From 'A guide to the identification, prevention and control of grape phylloxera.' Agriculture Victoria. A Vine Disease District also occurs at Orchard Hills, south-west of Sydney, New South Wales.

Over one million hectares of French vineyards were destroyed by phylloxera towards the end of the nineteenth century (Ordish 1972). In Australia, the severity and extent of damage was limited, probably due to the institution of quarantine regulations, and the relative isolation of vineyards and viticultural regions compared with Europe (Buchanan 1990).

Much time and effort was invested in phylloxera eradication programs in France and California over the last two decades of the nineteenth century. Methods tried included fumigation of the soil with carbon bisulphide, flooding, and surrounding the rootzone of infested vines with sand (Viala and Ravaz 1901, Lider 1958). No eradication measure proved effective, nor has a successful means of chemical control yet been developed.

1.1.3 The use of rootstocks for control of phylloxera

Once it became apparent that there was no available method for eradicating phylloxera, other means of maintaining commercial production of grapes in infested areas were sought. Laliman, in 1869, first suggested grafting *V. vinifera* onto the roots of various tolerant or resistant American *Vitis* species (Lider 1958). French viticulturists developed grafting techniques, and tested a wide range of species and hybrids for suitability as phylloxera-resistant rootstocks.

Grafting proved successful, and many American *Vitis* species and hybrids, showing varying degrees of resistance or tolerance (see 'Definitions', page X) to phylloxera, are currently used as rootstocks (Viala and Ravaz 1901, Boubals 1966a, Pongrácz 1983). The most common rootstock parent species worldwide include *V. rupestris* Scheele, *V. riparia* Mischx., and *V. berlandieri* Planch. (Pongrácz 1983). In Australia the most commonly used rootstocks are Ramsey (*V. champini* Planch. - this species is now thought to be a natural hybrid between *V. mustangensis* Buckley and *V. rupestris* (Moore 1991) and Schwarzmann (*V. riparia* × *V. rupestris)*, both of which are considered to be highly resistant to phylloxera (Whiting *et al.* 1987, May 1994), although Ramsey is most often used for nematode control.

At least some accessions of *V. cinerea* Noronha (e.g. 'Arnold') are thought to be immune to phylloxera (Davidis and Olmo 1964, Granett *et al.* 1987, Becker 1988). Hybrids of *V. cinerea* with *V. riparia* have been developed, some of which have also been rated as immune, including the rootstock Börner (Becker 1988).

V. rotundifolia subgenus *Muscadinia* Mischx. is also generally considered to be immune to phylloxera (Boubals 1966a, Granett *et al.* 1987), but is not suitable for use as a rootstock because of graft incompatibility with *V. vinifera* (Viala and Ravaz 1901, Davidis and Olmo 1964). Hybrids of this species with *V. vinifera* (called VR hybrids) have been developed (e.g. VR 043-43, VR 039-16) (Davidis and Olmo 1964, Firoozabady and Olmo 1982), but their long-term phylloxera resistance is questionable (Davidis and Olmo 1964, Granett *et al.* 1987, May 1994). While all VR hybrids tested were reported as immune by Firoozabady and Olmo (1982), earlier tests by Davidis and Olmo (1964) showed the formation of galls on both primary roots (roots without secondary thickening) and secondary roots (woody roots with secondary thickening) of at least some of these hybrids. Granett *et al.* (1987) subsequently demonstrated that the hybrid VR043-43 is immune to biotype A phylloxera, but only tolerant to biotype B. The use of different biotypes of phylloxera in earlier trials may account for these apparent discrepancies, however other environmental factors such as temperature and/or soil type may have also played a role. Much earlier, Viala and Ravaz

(1901) warned that the *V. vinifera* parentage in VR hybrids always introduces an element of 'negative resistance' (i.e. susceptibility) and did not recommend their use.

Phylloxera appears to be able to adapt to rootstocks with *V. vinifera* in their parentage, and to overcome their tolerance. This was most dramatically illustrated in California with the emergence of Biotype B which overcame the resistance and tolerance of ARG1 (Granett *et al.* 1985). It is now recommended that hybrids including *V. vinifera* should not be used in phylloxera infested soils (Granett *et al.* 1996).

V. vinifera cultivars and *Vitis* rootstocks may either be accessions of natural species or, alternatively, cultivars and hybrids resulting from breeding. In order to simplify their description in this thesis the term 'varieties' (not used in its botanical sense) will be used to describe all vine types examined.

1.1.4 Susceptibility, tolerance, resistance and immunity

The terms used to describe the resistance of grapevines to phylloxera can vary according to the author, and may therefore seem confusing. The following definitions (see also 'Definitions', page X), based on King and Rilling (1991), will be used in this thesis: *Susceptibility* - the grapevine is unable to tolerate infestation by phylloxera and death of the vine eventually results; *Tolerance* - the grapevine is able to indefinitely withstand infestation by phylloxera and phylloxera populations are able to feed and reproduce unaffected by the vine. Feeding sites are established only on primary roots; *Resistance* - the grapevine is able to withstand infestation and exerts a negative effect on the growth and/or reproduction of the phylloxera population; *Immunity* - phylloxera are unable to establish infestations on the grapevine. Vines classed in-between susceptibility and immunity tend to show a combination of resistance and tolerance, making strict definitions difficult to apply.

The following review provides a background for the research conducted in this study into the interaction between the roots of *Vitis* species and hybrids with phylloxera, firstly outlining what is currently known about the interaction between grapevines and phylloxera, then the interactions of other plants with similar plant-parasitic pests. The grapevine-phylloxera interaction is very complex, and a number of hypotheses have been proposed for mechanisms of gall induction and host plant resistance. It is still unclear what the bases for these phenomena are. It is clear, however, that galls, which are modified plant organs, provide phylloxera with their nutritional requirements. In this respect some parallels can be drawn between the grapevine-phylloxera interaction, and interactions between plant roots and gall-forming nematodes. This chapter will include some examples of plant-nematode interactions for comparison. Finally, a discussion of defence mechanisms of grapevines, to

both phylloxera and other pathogens will lead to some suggestions on the type of resistance mechanisms which might be operating in rootstock species.

1.2 Grapevine root-phylloxera interactions

A relatively comprehensive review of published studies of the phylloxera-grapevine interaction was published by Miles (1989). To date, most phylloxera research has focused on assessment of the resistance of rootstocks for practical application in the vineyard. This has generated considerable information on the degree to which rootstocks are able to resist phylloxera gall formation, and the associated effects on scion yield. Comparatively little, however, is known about the physiological or biochemical interactions between plant and insect that are responsible for these observed effects.

1.2.1 Response of susceptible vines to phylloxera attack

Susceptible grapevines, including *V. vinifera*, respond differently to phylloxera feeding than do resistant or tolerant American *Vitis* species. They form root galls of two types: nodosities, which are fleshy galls on the primary root system (figure 1.3a) and tuberosities, which are swellings on the secondary roots (figure 1.3b). Leaf gall formation is common on American *Vitis* spp. , but they are not found on *V. vinifera* in Australian vineyards, although they may occur under artificial conditions (e.g. in tissue culture). King and Rilling (1985) found that a strain of phylloxera collected from Germany produced leaf galls on *V. vinifera* cv. Müller-Thurgau, while on the same cultivar, a New Zealand strain did not. Formation of leaf galls may therefore be dependent on interactions between the biotype of phylloxera, the variety of grapevine and environmental conditions.

Formation of galls on roots

The induction and formation of insect-induced galls is not well understood. They are believed to result from injection by the insect of some inductive agent affecting the metabolism of a plant growth regulator, possibly indole-acetic acid (IAA), which in turn affects the root in a manner dependant on a concentration gradient (Forrest 1987). This causes inhibition of growth close to the feeding site of the insect, and stimulation of growth at some distance from this site.

Phylloxera-induced root galls are superficially simple structures, and general models of gall formation might provide at least a basis for understanding their induction and function. Petri (1907) described phylloxera as feeding from the cortex of primary roots rather than from the phloem (figure 1.4). While this observation needed to be confirmed, it supports the possibility of injection of gall-inducing agents directly into the tissues which become affected. Miles' review of aphid feeding processes (1987) included a report of the presence

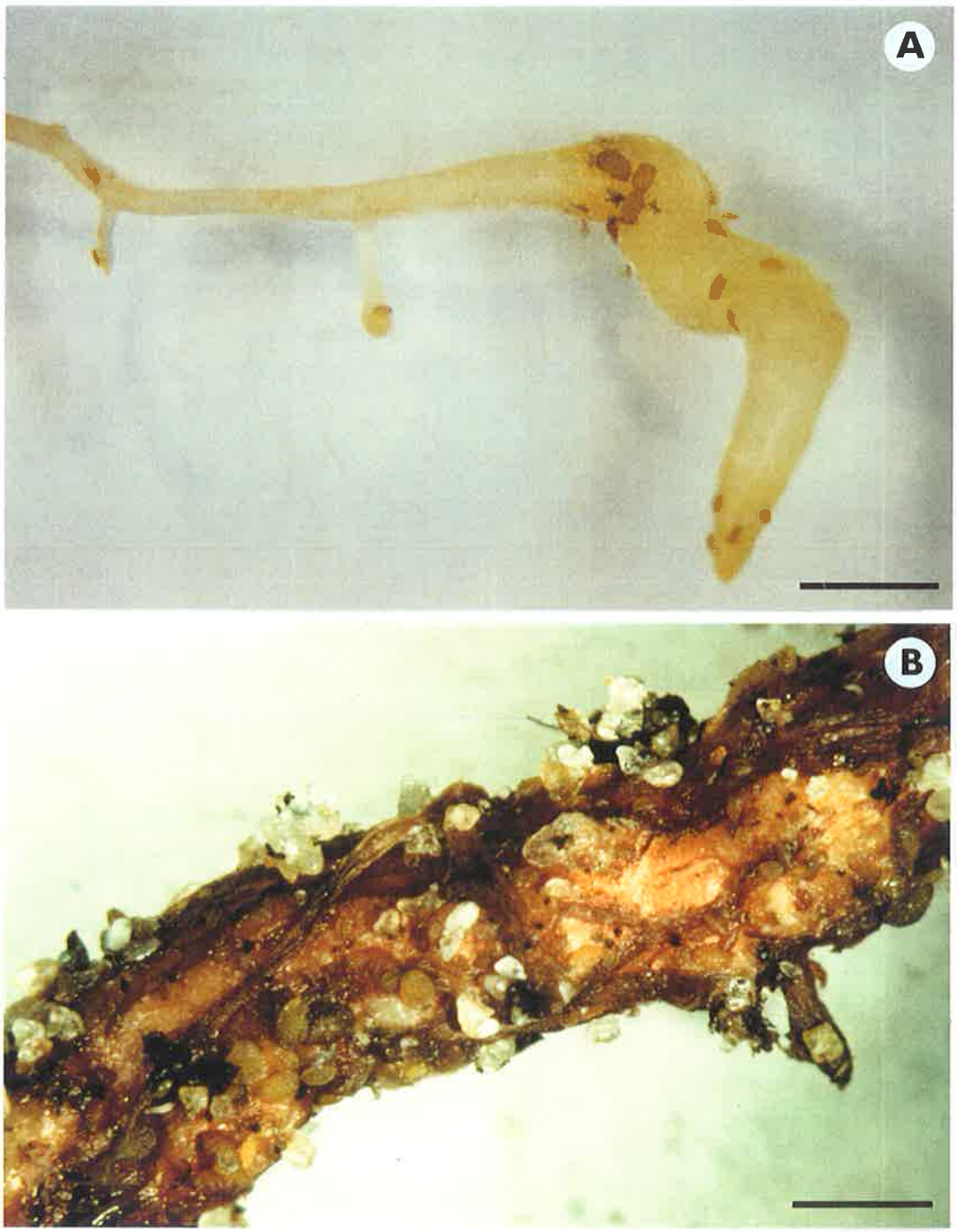


Figure 1.3 a) Nodosity on fibrous root of *V. vinifera* b) Tuberosity on mature root of *V. vinifera* (photograph from 'A guide to the identification, prevention and control of grape phylloxera' Agriculture Victoria). Scale bar equals approximately 2mm.

of the auxin IAA in the saliva of phylloxera. While artificial injection of roots with IAA produced swellings similar in appearance to tuberosities (Granett 1990), these swellings did not attract phylloxera, and it is possible that they did not function as metabolic sinks, as true galls do. It is also likely that the concentration of IAA in phylloxera saliva is not adequate for the induction of galls (Miles 1987), although it may be involved in triggering further changes in concentration of endogenous plant hormones.

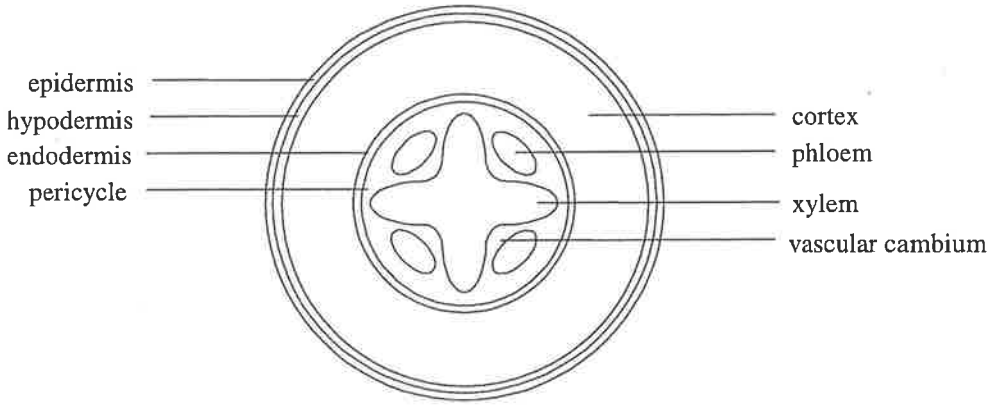


Figure 1.4 Diagrammatic representation of a transverse section through a primary dicotyledon root showing location of the major tissue types (adapted from Esau 1977).

Niklowitz (1954) published a detailed anatomical study of phylloxera-induced gall formation in *V. vinifera* using light microscopy. He proposed that the hormones involved are of plant, not insect origin. Galls are clearly initiated very rapidly; formation of nodosities involves changes in the endodermis within 24 hours of initiation, followed by hypertrophy of cells on the side of the root distal to the feeding site. This causes the nodosity to assume a hook-like shape. The site of phylloxera attack is generally close to the root meristem in the zone of elongation. Further away from the root tip, tissues are already differentiated and are less responsive to gall induction. After three days, starch deposition has commenced on the side of phylloxera feeding. The angle of the hook may reach as much as 90° within five days of initiation of feeding. Nuclei surrounding the feeding site are enlarged compared with normal root cell nuclei.

The mechanism by which phylloxera attack causes decline and death of susceptible vines is ill-defined. Phylloxera feeding and gall formation do not appear to be sufficient to cause vine death. It is, however, a fundamental characteristic of phylloxera root galls that they eventually decay, beginning as early as 15 days after initiation on susceptible vines (King *et al.* 1982), although more commonly after several weeks (Niklowitz 1954). In a heavy infestation, this would lead to significant loss of either primary roots, or, in the case of

tuberosities, of secondary root vascular tissue, and thus decrease the performance of the grapevine. On a susceptible vine, necrosis of the surface of secondary roots may occur faster than roots can be replaced, and it is probably this loss which causes vine death. Events leading to the necrosis of phylloxera galls have not been described. Necrosis may, for example, result from a delayed hypersensitive response (HR) by the root as a form of defence, or from an early natural senescence brought on by the high metabolic activity in gall tissues.

It has been suggested that the lack of an early defence response in susceptible vines results in secondary pathogen invasion of the root vascular tissue through punctures made by the feeding insect, and it is this which causes the death of the vines (Miles 1989). A study by Omer *et al.* (1995) has shown that such invasion is almost certain to occur, and at the very least will increase the damage to vines.

Phylloxera nutrition

The dietary requirements of phylloxera are unknown. Granett (1990) showed that tuberosities (galls on secondary roots) are preferred by phylloxera as feeding sites over morphologically similar swellings resulting from the application of IAA. This suggests that galls are specialised to suit the needs of phylloxera. The only published study assessing the composition of galls with a view to understanding phylloxera nutrition is that of Sobetskiy and Derzhavina (1973). They studied the chemical composition of exudate from leaf galls of the *V. rupestris* × *V. riparia* hybrid 101-14. The exudate was presumed to be representative of 'sap' which would be ingested by phylloxera. This was compared with extract obtained from entire leaf galls or uninfested leaves. The exudate had a lower amino nitrogen content, much higher levels of phosphates and carbohydrates, and lower levels of phenolics (although the latter was higher in the whole gall compared with the leaf). The authors hypothesised that starch present in the galls would be hydrolysed at the stylet tip by enzymes present in the saliva, but were unable to identify any potential products of this hydrolysis. There have been no similar studies of root gall composition. Nedov *et al.* (1992) reported the existence of protease activity in the saliva of phylloxera, and suggested that phylloxera are able therefore to ingest and digest proteins.

The small size of phylloxera (100-300μm long) makes nutritional studies difficult. At present it is not possible to conduct excised-stylet sap studies, nor to raise phylloxera on an artificial diet. It is thus not surprising that more research has not been carried out in the area.

1.2.2 Response of resistant grapevines to phylloxera attack

Resistant and/or tolerant grapevines respond to phylloxera in ways which limit the formation of tuberosities or nodosities. The mechanisms by which they do this are largely unknown, although secondary roots of vines which are strongly tolerant have been shown to seal off the wound site with a layer of corky tissue, preventing further damage to the root system (Boubals 1966a, King and Rilling 1991). The extent to which the wound site is sealed off appears to be related to the degree of tolerance. Vines which are completely susceptible show no localised wound response (as observed by light microscopy).

Phylloxera are still able to initiate nodosities on the primary roots of tolerant vines (Granett *et al.* 1983). The extent of nodosity formation is related to the degree of resistance of the vine. Any nodosities which do form on resistant vines tend not to have the typical 'hook' shape (King *et al.* 1982, Forneck *et al.* 1996), suggesting they are less sensitive to any gall-inducing agents of the phylloxera. The roots of vines which are considered immune do not form galls at all. In some cases superficial necrotic lesions have been seen at attempted feeding sites on *V. rotundifolia* (Boubals 1966a, King *et al.* 1982).

Assessment of the degree of grapevine resistance to phylloxera

Assessment of the degree of resistance of rootstocks to phylloxera is of twofold importance. Firstly, on a practical level, it is essential that rootstocks with effective and stable resistance or tolerance are recommended for use in phylloxera infested vineyards. Secondly, the degree of resistance of different rootstocks must also be determined to enable investigations into the mechanism of resistance, and ultimately an understanding of the potential durability of that resistance.

Researchers have used a range of criteria in assessing resistance to phylloxera. Viala and Ravaz (1901) seem to use a historical perspective (although they do not state what criteria they use) on vine performance in phylloxerated soil. Assessment of the root's response to attack was favoured by some researchers. Boubals (1966a) stated that for secondary roots there were only two forms of resistance. One is an active 'repulsion' of phylloxera by the root, the other development of a corky layer between the feeding site and the stele. In the second case, the rapidity of formation and continuity of the corky layer was responsible for the degree of resistance and this was his criteria for assessment. A similar approach was taken by King and co-workers (King *et al.* 1982, King and Rilling 1991). This method of assessment does not take into account the interaction of phylloxera with the primary root system. Davidis and Olmo (1964) assessed resistance by counting numbers of tuberosities and nodosities formed, but also warned against relying solely on this method as a measure of

resistance, as lesions may be equal in number but vary in depth and therefore severity according to the resistance or tolerance of the roots.

In contrast to methods where the response of the grapevine to phylloxera is assessed, Granett and co-workers (Granett *et al.* 1983, Granett 1990, de Benedictis and Granett 1993) use the ability of phylloxera to settle and reproduce, especially on secondary roots, as indicators of susceptibility, tolerance or resistance of the vine.

In general, ratings given to the same rootstock by different research groups are similar (table 1.1), but there is some degree of variation both within and between groups. For example, note the variable ratings of *V. vinifera* and ARG1 given by King *et al.* (1982) and King and Rilling (1991), and the difference in rating for rootstock 41B given by Boubals (1966a) and de Benedictis and Granett (1993). Given that some of these studies may have used different biotypes of phylloxera, the variation in results potentially reflects variation in the aggressiveness of phylloxera biotypes (see section 1.1.2.).

Table 1.1 Summary of resistance classifications given by different researchers.

vine variety	Viala	Boubals	King *et al.*	Granett	de Benedictis
V. vinifera	0-1	3	1-7; avg. 4-5	S	V
V. rupestris	18	0,1,2	-	-	-
V. riparia	18	0,1,2	-	-	-
V. berlandieri	17	0,1,2	-	-	-
V. champini	14				
V. cinerea	15	1	-	-	-
V. rotundifolia	19	0	-	-	-
140-Ruggeri	17	1	-	-	II/III
ARG-1	*	2	1-7; avg. 3-4	R or S	III/IV
41B		1			IV/V

*Ä rating is not given, although this hybrid is referred to as resistant in the text.

Key:

Viala:	0 - 20 susceptible to immune. (Viala and Ravaz 1901)
Boubals:	3 - 0 susceptible to immune. (Boubals 1966a)
King *et al.*:	1 - 7 susceptible to immune. (King *et al.* 1982, King and Rilling 1991)
Granett:	S, R or I susceptible, resistant or immune. (Granett *et al.* 1987)
de Benedictis:	V - I suitable to unsuitable host. (de Benedictis and Granett 1993)

Many of these resistance ratings have been based on the interaction between phylloxera and secondary roots. Only very recently have Grzegorczyk and Walker (1998) used a tissue culture-based system to observe the interaction between phylloxera and primary roots. The authors advocate the amenability of this method for determination of resistance of different species, rootstocks and accessions within each group, but their assay was limited in two respects. First, their experiment was limited by the use of mixed populations of phylloxera

containing various (unpublished) proportions of one or more biotype. As pointed out by King and Rilling (1985, 1991) the use of different biotypes can lead to differences in resistance ratings, so use of homogeneous phylloxera populations is important for such assays.

Second, Grzegorczyk and Walker (1998) indicate that the tissue culture-based bioassay may be overly sensitive, that is grapevines which appear sensitive in culture may be resistant or tolerant in field situations. King and Rilling (1991) were able to demonstrate, however, that it is possible for some phylloxera populations to give rise to tuberosities, but not nodosities, on some rootstocks. This means that tissue culture results (or, for that matter, results from potted vine rootstock trials, where only primary roots are observed) should only be used to predict the performance of rootstocks in field situations with these caveats in mind.

1.3 Plant interactions with sucking pests

1.3.1 Gall-forming aphids

Over 700 species of gall-forming aphids are known (Forrest 1987). Galls can be caused by either phloem feeders or parenchyma feeders. Most aphid galls occur on leaves or aerial parts of plants and vary in complexity from simple rolled leaves, to complex pouches.

Clearly, the primary function of aphid galls (where physical protection of the insect is not involved) is to provide nutrition to the insect. To do this the gall must be a strong sink for photoassimilates and nitrogenous compounds. A correlation has been noted between sink strength and reproductive performance of several species of aphids on *Pistacia palaestina* (Burstein *et al.* 1994). In these interactions, the aphids which induced the strongest sinks were able to alter vascular tissue morphology at distances of up to 50cm from the gall - illustrating the remarkable influence a gall-forming aphid can have over its host plant. The importance of sink formation for aphid fecundity was also illustrated by the example of the gall-forming aphid *Pemphigus betae* on narrow-leaved cottonwood (*Populus angustifolia*) (Larson and Whitham 1991). In this case, galls which drew the most assimilates from neighbouring leaves supported the highest number of progeny (up to a 65% increase), and removal of nearby female catkins (a competing sink) also increased success in gall induction by 31%.

It has been suggested that suitability of hosts, or of gall formation sites within a host is based on the chemical composition of those plants or plant tissues. For example, suitability of *Populus angustifolia* leaves for galling by *Pemphigus betae* was shown to be inversely proportional with their phenolic content (Zucker 1982). However, Burstein and Wool (1993) found that the gall-forming aphid *Smynthurodes betae* was unable to detect optimal

sites for gall formation on its host *Pistacia atlantica*, or even to select a host preferentially over a non-host species, *Pistacia palaestina*. These examples suggest that generalisations cannot be made regarding the ability of gall-forming aphids to select hosts, or suitable galling sites within these hosts.

1.3.2 Plant-parasitic nematodes

Plant-parasitic nematodes are root pests, many of which have highly specialised relationships with their hosts. They infest almost every commercially important plant species known, and cause enormous economic damage. As such important pests, they have been the subject of extensive research, as reviewed by Sijmons (1993). Plant-parasitic nematodes fall into five main groups on the basis of feeding behaviour: migratory or sedentary ectoparasites; migratory or sedentary endoparasites; and migratory ecto/endoparasites (Hussey 1989, Sijmons *et al.* 1994).

While phylloxera feeding structures do not appear to be as anatomically specialised as those induced by some nematodes, they do induce root galls. They feed from the root cortex, as do some ectoparasitic nematodes, and cause accumulation of starch, as does the false root-knot nematode *Nacobbus aberrans*. In this respect there exist similarities between two otherwise unrelated groups of organisms, and further investigation of grapevine-phylloxera interactions should be conducted in the light of recent knowledge gained about plant-nematode interactions.

Sedentary endoparasites, including root-knot (*Meloidogyne* spp.), cyst (*Heterodera* or *Globodera* spp.) and false root-knot (*Nacobbus* spp.) nematodes form specialised feeding sites in roots and are able to gain nutrition from relatively few cells. The specialised feeding structures formed by endoparasitic nematodes have been reviewed by Jones (1981) and include: root-knot nematode-induced giant cells, formed by repeated mitosis without cytokinesis; cyst or false root-knot nematode-induced syncytia formed by digestion of cell walls at pit fields and subsequent fusion of protoplasts; and nurse cells induced by the citrus nematode *Tylenchulus semipenetrans*. Cellular features common to most of these specialised feeding structures are enlarged nuclei, loss of primary vacuolation, and an increase in the number of organelles, all implying increased metabolic activity. While IAA has long been associated with gall formation on plants, this has only recently been directly demonstrated in nematode-induced galls through the use of β-glucuronidase (GUS) reporter gene fusion constructs (Hutangura *et al.* 1999). The induction of galls by the root-knot nematode *Meloidogyne javanica* in white clover (*Trifolium repens* cv. Haifa) was shown to be associated with accumulation of flavonoids and induction of the GH3 promotor,

suggesting accumulation of IAA, throughout the gall, particularly at the early stages of induction of giant cell formation.

Many features of nematode feeding sites indicate the presence of mechanisms to enhance unloading of solutes from the phloem and/or transport to the feeding site. Plasmodesmata are abundant in pit fields between syncytial cells and sieve elements; a feature which would facilitate unloading of solutes from the phloem. Jones (1981) also refers to evidence of metabolite leakage from root-knot nematode-infested roots which suggests that the apoplastic barrier provided by the endodermis, which normally prevents such leakage, may have been lost. Giant cells have been demonstrated to contain proton pumps in their plasmalemma which act in symport with amino acid porters (Sijmons *et al.* 1994), as well as sufficient plasmodesmata to allow symplastic transport of necessary nutrients (Williamson and Hussey 1996). Where giant cells abut vascular elements the walls of the giant cells typically have ingrowths indicative of transfer cells (Jones and Payne 1977). It seems likely that all of these features play a role in nematode nutrition.

The false root-knot nematode, *Nacobbus aberrans*, induces syncytia in the stele, which are accompanied by the proliferation of abnormal sieve elements (Jones and Payne 1977). Plasmodesmata are abundant between syncytia and the new phloem, indicating a symplastic transport pathway. There is massive accumulation of starch throughout the surrounding stele and cortex of the root, which is thought to either indicate enhanced sucrose unloading, or possibly induction of 'leaky' phloem (where solutes are able to passively leak out of phloem). The amount of starch present decreases during nematode reproduction, the most metabolically active phase, suggesting its importance in nematode nutrition (Jones and Payne 1977).

In feeding sites of the non-galling plant parasitic nematode *Criconomella xenoplax,* plasmodesmata connecting single 'food cells' to neighbouring cells are modified to enhance movement of solutes (Hussey *et al.* 1992). The migratory ectoparasite *Xiphinema index* (the dagger nematode) also feeds from a single cell within the root cortex, preferably in the elongation zone, and injects saliva which causes induction of binucleate cells immediately surrounding the food cell (Weischer and Wyss 1976, Sijmons 1993).

These features indicate that nematode-induced galls or feeding sites function as specialised sinks providing nutrients to the pests. Gall formation is likely to result from altered concentrations of plant growth regulators, although this may not fully account for formation of specialised feeding structures (Jones 1981). Plant-parasitic nematodes secrete various compounds into their host plant (mainly proteins and carbohydrates), and while these remain largely uncharacterised, it is likely that they play a role in both feeding site induction, and in

pre-digestion of food (Sijmons *et al.* 1994, Williamson and Hussey 1996). Ding *et al.* (1998) have cloned a cDNA encoding a secretory cellulose-binding protein from the J2 stage of *Meloidogyne incognita* which they propose plays a role in either a complex cellulase system allowing penetration into the root, or the early induction of giant cells. cDNAs encoding cellulases have also been cloned from cyst nematodes (Smant *et al.* 1998) and root-knot nematodes (Rosso *et al.* 1999).

1.3.3 Resistance mechanisms to plant-parasitic pests

Effective resistance to plant pests requires either a constitutive resistance mechanism, either chemical or physical, or an inducible resistance mechanism whereby the plant is able to detect pest attack and respond accordingly. In the case of aphid or nematode attack, plants might respond either to wounding, to injected saliva or to release of plant cell wall fragments by salivary hydrolases (Ryan *et al.* 1981, Campbell 1986, Zacheo *et al.* 1997). When investigating possible resistance mechanisms it is important to keep in mind that the many and varied defence responses a plant may show following attack may or may not be involved in conferring resistance to that pest (Harrewijn 1990, Dixon and Paiva 1995).

Aphids, unlike most insect pests, are often able to detoxify, and/or to safely sequester low levels of certain toxic compounds (Mullin 1986). Plants therefore have needed to evolve more specific resistance mechanisms against aphids than against many other insect pests. The particular compounds an aphid will be exposed to is determined in part by its method of feeding. Phloem feeders will be exposed to only a limited range of allelochemicals including flavonoids and glycoalkaloids. Parenchyma feeders may be exposed to a wider range of compounds including flavonoids and other phenolics, alkaloids, terpenoids, glucosinolates, cyanogenic glycosides and saponins (Mullin 1986).

Not all resistance involves toxic compounds, however. For example, a single dominant gene, *Nr*, confers resistance to the leaf aphid *Nasonovia ribis nigri* on lettuce (*Lactuca sativa*) (Eenink 1982a, 1982b, van Helden *et al.* 1993). The mechanism by which this gene affects resistance is not known, however it appears to prevent the aphid from feeding and does not involve toxic antibiosis (van Helden *et al.* 1993).

Endoparasitic nematodes have much greater physical contact with the root than do aphids with their hosts. This means that the host may be able to recognise and respond more readily to the presence of the parasite (Curtis 1996). Often, resistance to nematodes results from an active response to infection such as an induced hypersensitive response (HR) rather than from the presence of a constitutive nematicidal compound (Mote *et al.* 1990). An HR involves a complex cascade of reactions in plant cells, the most characteristic of which is

cell death. Other cellular mechanisms involved in HR include expression of pathogenesis-related proteins, rapid influx of calcium, generation of reactive oxygen intermediates, production of phytoalexins and cross-linking of cell wall components (Gilchrist 1998). HR is involved in the resistance of tomatoes carrying the *Mi* gene to root-knot nematodes (Rossi *et al.* 1998).

Plants possess a wide variety of resistance mechanisms to nematodes, which may be either broad or narrow in their specificity, and which have a range of inheritance patterns, suggesting the involvement of single or multiple, dominant or recessive genes (reviewed by Sijmons 1993, Williamson and Hussey 1996). Multiple resistance mechanisms may even occur within a host, as seen with the *Me* genes which confer resistance to root-knot nematodes in *Capsicum annum* (Bleve-Zacheo *et al.* 1998). The *Me1* gene acts via a delayed defence response whereby giant cells are unable to form, whereas *Me3* involves a rapid HR response preventing invasion of the root by juvenile nematodes (J2s). A number of nematode resistance genes have been mapped in a range of plants including tomato, potato, sugar beet, soybean and wheat (reviewed by Williamson and Hussey 1996). At least two nematode-resistance genes have been cloned, $Hs1^{pro\text{-}1}$ from sugar beet, *Beta vulgaris*, which confers resistance to the beet cyst nematode *Heterodera schachtii* (Cai *et al.* 1997) and the *Mi* gene from tomato which also confers resistance to the potato aphid *Macrosiphum euphorbiae* (Rossi *et al.* 1998)(see 'Gene-for-gene interactions', page 21).

While progress is continually being made in this area, the precise mechanisms of resistance of plants to sucking pests such as aphids and nematodes in most cases remains unknown. A range of possible mechanisms is summarised in the following section.

Physical resistance mechanisms

In many plant species a physical barrier to feeding may present a major defence against aphids (Klingauf 1987). Such barriers include cell wall lignification and suberisation (Panda and Khush 1995), and external features such as leaf hairs and trichomes (Webster *et al.* 1994, Panda and Khush 1995, Bosland and Ellington 1996).

The build up of callose, a β-1,3-glucan, in phloem around an aphid wound site may serve to block the phloem and thus deprive a phloem-feeding aphid of a food source. Callose synthesis in melon, *Cucumis melo*, was shown to be positively correlated with resistance to the melon aphid *Aphis gossypii* (Shinoda 1993). Aphids whose saliva contains glucanases may be able to overcome this mechanism (Dreyer and Campbell 1987). Callose deposition may also be involved in resistance of the grass species *Aegilops variabilis* to the root-knot nematode *Meloidogyne naasi* (Balhadère and Evans 1995).

The presence of pectinases in saliva is essential for aphid stylet penetration between plant cell walls (McAllan and Adams 1961). Dreyer and Campbell (1987) have developed a pectin/pectinase based model for resistance to phloem-feeding aphids based on aphid stylet penetration patterns. They use as examples the increased pectinase activity in greenbug, *Schizaphis graminum*, being correlated with increased virulence of the aphid, and synthesis of less readily degraded pectins conferring greater resistance in sorghum.

There are few examples of physical resistance to plant-parasitic nematodes, which tend to attack root tips. The failure of xylem of resistant plants to respond to syncytium induction by cyst nematodes (Sijmons 1993) may be considered a physical resistance mechanism. Lignification and later suberisation appears to be involved in resistance of wild barley, *Hordeum chilense,* to *Meloidogyne naasi* (Balhadère and Evans 1995).

Biochemical resistance mechanisms

The majority of studies on plant resistance mechanisms involve the search for biochemical compounds whose presence is correlated with resistance. Most of these are secondary metabolites which are synthesised via complex pathways, with equally complex regulation.

Phenylpropanoid pathway

The phenylpropanoid pathway includes synthesis of many plant secondary metabolites including phenolics, flavonoids and stilbenes (Appendix one). Many components of the phenylpropanoid pathway have been implicated in plant defence against both pathogens and insects (Rhodes and Wooltorton 1978), either through a direct antibiotic effect (e.g. as phytoalexins), or through strengthening of the plant cell wall (e.g. lignin) (Baron and Zambryski 1995).

Mullin (1986) suggested that a build up of phenolics may provide a particularly effective defence mechanism against aphids as, unlike hard-bodied chewing insects, they are unable to incorporate and sequester phenolics into their cuticles.

The functions of products of the phenylpropanoid pathway in plant-insect interactions are believed to include formation of hydrogen or covalent bonds between phenolics and dietary proteins or digestive enzymes in the insect (Appel 1993). Oxygen radicals produced during oxidation of phenolics could also disrupt membrane integrity of the insect gut lining and gut metabolism. The presence in aphid saliva of polyphenol oxidases (PPOs) which have the potential to detoxify these phenolics (Peng and Miles 1988) suggests that they play a key role in plant-aphid interactions.

Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL) is the first enzyme of the phenylpropanoid pathway, and has a key regulatory role in determining flux through that pathway. Its level of synthesis is important in determining the overall rate of production of phenylpropanoid pathway products (Bate *et al.* 1994). There is more than one PAL gene, encoding different isoforms of the enzyme in many plants, including grapevines (Sparvoli *et al.* 1994). As in other plant species, grapevine PAL genes are likely to be subject to tissue- specific and temporal regulation (Dixon and Paiva 1995). Patterns of PAL gene expression may also depend on the developmental stage of a particular tissue (Melchior and Kindl 1991).

PAL gene expression is induced in response to many forms of stress including mechanical wounding and pathogen attack, the enzyme accumulating around the site of wounding or infection (Rhodes and Wooltorton 1978, Dixon and Paiva 1995). Production of other enzymes of the phenylpropanoid pathway, including stilbene synthase, has been shown to be co-ordinately regulated with PAL expression (Melchior and Kindl 1991, Boss *et al.* 1996).

PAL gene expression has also been correlated with aphid and nematode resistance in plants. In lettuce, the speed and quantity of PAL induction upon attack by the lettuce root aphid *Pemphigus bersarius* was greater in resistant than in susceptible varieties (Cole 1984). Mote *et al.* (1990) found that two novel PAL isozymes appeared in roots exclusively of resistant tomato on infection with the root-knot nematode *Meloidogyne incognita*. In addition, the speed and quantity of PAL induction was far greater overall in resistant than in susceptible tomato. The induction of PAL gene expression, as well as other phenylpropanoid pathway genes was upregulated in resistant, but not in susceptible lines of soybean following infestation with *Heterodera glycines* or *M. incognita* (Edens *et al.* 1995). In alfalfa (*Medicago sativa*) resistant to the root lesion nematode *Pratylenchus penetrans* (a migratory endoparasite), constitutive expression of several phenylpropanoid pathway genes, including PAL, is elevated when compared with levels in susceptible plants, and although downregulated 48 hours after infection, this timing is significantly later than in susceptible plants (Baldridge *et al.* 1998). Also, levels of medicarpin, a product of the phenylpropanoid pathway shown to reduce nematode motility *in vitro*, is consistently elevated in the most resistant alfalfa plants.

Down-regulation of PAL gene expression was reported in compatible interactions between *Meloidogyne incognita*, *Heterodera schachtii* and transgenic *Arabidopsis* and tomato (Goddijn *et al.* 1993). In susceptible soybean, PAL enzyme activity decreased in response to *Heterodera glycines* or *M. incognita* (Edens *et al.* 1995). This downregulation was proposed to be necessary for establishment of successful feeding sites.

Tannins

Condensed tannins, also known as proanthocyanidins, form as a result of condensation of single catechins, products of the phenylpropanoid pathway, to form oligomers. Tannins are very complex phenolic components of woody tissue which have the ability to react with and precipitate proteins (Haslam 1981, Ferreira *et al.* 1992). Research into the biological role of condensed tannins has been limited because of their complexity. Their ability to react with other compounds suggests an active role in plant-pest interactions, and possibly in aphid resistance. For example, the level of condensed tannins in phloem sap of peanut (*Arachis hypogeae*) was negatively correlated with fecundity of the aphid *Aphis craccivora* on this plant (Grayer *et al.* 1992). The concentration of tannins in the leaves of some cotton varieties was negatively correlated with the population size of some aphids (mainly *Aphis gossypii*) (Mansour *et al.* 1997).

Chlorogenic acid and *iso*-chlorogenic acid

Chlorogenic acid and *iso*-chlorogenic acid are both products of the phenylpropanoid pathway. Chlorogenic acid production is induced upon wounding in many plants (Rhodes and Wooltorton 1978, Dixon and Paiva 1995), and its presence has been found to correlate with aphid and nematode resistance in a number of plant species. In lettuce resistant to the lettuce root aphid, *Pemphigus bursarius*, *iso*-chlorogenic acid levels were greater than in susceptible varieties (Cole 1984). Chlorogenic acid content increased in roots of tomato infected with root-knot nematode (*M. incognita*) (Mote *et al.* 1990). The speed and quantity of the increase was significantly higher in resistant than susceptible varieties.

A direct role for chlorogenic acid in resistance is suggested by the finding that in artificial diets chlorogenic acid prevented reproduction of greenbug (*Schizaphis graminum*) (Todd *et al.* 1971). Riley and Kolattukudy (1975) suggest that chlorogenic acid may also play a role in resistance to pests by providing a reservoir of compounds necessary for rapid synthesis of suberin. Matern and Kneusel (1988) suggested that it may be the action of phenolases converting chlorogenic acid to the corresponding toxic *ortho*-quinone which is responsible for its role in resistance (Devlin and Gustine 1992).

Oxidation of phenolics

Recently, the role of oxidation in plant defence responses to pathogens and insect pests has received increased attention. Oxidation is required for activation of many toxic phenolic compounds and is also involved in HR (Devlin and Gustine 1992, Appel 1993, Calderón *et al.* 1994, Bi and Felton 1995, Zacheo *et al.* 1997). Factors responsible for HR may be

activation of NADPH-oxidase, and accumulation of free radicals as suggested for cereals attacked by *Meloidogyne naasi* (Balhadère and Evans 1995).

Miles and Oertli (1993) proposed a redox (reduction, oxidation) hypothesis as the basis of many plant defence mechanisms against insect pests. In this model, plant phenolics interact with plant and insect polyphenol oxidases to form toxic quinones. Lignins are synthesised later to seal off the feeding site. Bi and Felton (1995) proposed three major mechanisms for the role of oxidation in plant defence: oxidation of secondary compounds such as phenolics to toxic quinones; depletion of antioxidants such as ascorbic acid; and cellular damage caused by reactive oxygen species such as peroxide and oxygen radicals. For example, increased lipid peroxidation and increased oxygen radical formation is proposed to be involved in resistance of alfalfa to aphids (reviewed by Bi and Felton 1995).

Other biochemical mechanisms of resistance

Several other classes of compounds have been associated with plant resistance to aphids. These include lectins, hydroxamic acids and alkaloids.

Lectins are carbohydrate-binding proteins, many of which have antibiotic properties against insects (Cole 1994b, Gatehouse *et al.* 1996, Sauvion *et al.* 1996). Snowdrop (*Galanthus nivalis*) lectin (GNA), and a chitin-binding lectin present in wild brassica (*Brassica fruticulosa, B. spinescens)* have both been correlated with increased resistance to aphids (green peach aphid *Myzus persicae,* and *Brevicoryne brassicae* respectively) (Cole 1994b, Gatehouse *et al.* 1996).

The concentration of a hydroxamic acid, DIMBOA-glucoside, is negatively correlated with performance of the cereal aphid *S. avenae* on wheat (Givovich *et al.* 1994). Alkaloids found in aphid-resistant lupin (*Lupinus angustifolius*) repress reproductive rate, reduce fecundity, and decrease feeding of *Myzus persicae* (Berlandier 1996).

Proteinase inhibitors have produced anti-metabolic effects on a range of insects and nematodes (Ryan 1990). For example, a trypsin inhibitor in transgenic potato plants has been shown to reduce fecundity and growth of the nematodes *Globodera pallida* and *Meloidogyne incognita* (Vrain *et al.* 1995a,b in Zacheo *et al.* 1997). Transgenic *Arabidopsis* expressing a modified rice cystatin Oc-IΔD86, a cysteine proteinase inhibitor, completely inhibited reproduction of both *Heterodera schachtii* and *M. incognita* (Urwin *et al.* 1997). Little is known about the involvement of these proteinase inhibitors in natural resistance mechanisms.

The nutritional status of host plants has also been cited in several cases as an important factor determining the resistance of plants to parasitic pests. Klingauf (1987) and Harrewijn

(1990) both stated that low nitrogen status of plants may be correlated with resistance to aphids. Resistance in wheat to the cereal aphid *Sitobion avenae*, which is manifested as inhibited reproductive capacity, was proposed to be related to poor nutritional status of the host plants (Caillaud *et al.* 1994), although a precise mechanism was not proposed. Hedin and Creech (1998) reported that the increase in the amino acid content of cotton roots observed when infested with root-knot nematodes was greater in susceptible than resistant varieties.

In some cases, plant resistance thought to be based on a physical barrier has, on further investigation, been associated with some biochemical component. A mechanical barrier to phloem penetration in wheat was proposed by Dreyer and Campbell (1987), but Caillaud *et al.* (1995) showed later that there was no mechanical barrier, and the resistance mechanism seemed to be interference with 'initiation of a sap ingestion event' in the sieve element.

In another case, Cole (1994a) reported an apparent physical barrier in the phloem as responsible for resistance of wild *Brassica* spp. to cabbage aphid *Brevicoryne brassicae*. Shortly afterwards, a chitin binding lectin was isolated which specifically occurred in resistant strains of *Brassica* (Cole 1994b). The lectin was proposed as playing a key role in aphid resistance in this instance.

Gene-for-gene interactions

The *Mi* gene from tomato is one of the best examples of characterisation of a resistance mechanism to both aphids and nematodes (Rossi *et al.* 1998). Both the Mi gene and $Hs1^{pro-1}$ mentioned earlier (Cai *et al.* 1997) encode proteins which have features similar to proteins encoded by disease resistance genes cloned from other plants, including putative membrane spanning regions and leucine-rich repeats suggesting a role for protein-protein interactions, possibly in recognition of elicitor compounds. The *Mi* gene confers resistance, in an isolate-specific manner, to both potato aphid and root-knot nematode. Because of the isolate-specific nature of the resistance conferred by this gene, and its homology to other R genes, a gene-for-gene hypothesis involving *avr* genes in the aphids and nematodes is proposed. The *H1* gene from potato, which confers resistance of potato to *Globodera rostochiensis* also has a gene-for-gene relationship involving an *avr* gene (Janssen *et al.* 1991). The *Mi* gene appears to be triggered by attempted giant cell induction, while the *H1* gene is triggered by the invading juvenile nematode (Williamson and Hussey 1996).

Several other proposed gene-for-gene systems for sucking insect resistance are cited in Rossi *et al.* (1998) including genes for resistance to the rose leaf curling aphid (*Dysaphis devecta*) on apple, and hessian fly (*Mayetiola destructor*) on *Triticum*.

1.4 Grapevine defence mechanisms

Mechanisms of defence of grapevines against both fungal pathogens and insect pests have received relatively little attention compared with those of model plants such as *Arabidopsis*, tomato and potato. Most research on grapevine defence to date has been directed at the phytoalexins such as stilbenes and their derivatives, which are abundant in *Vitis* spp. and have been implicated in many resistance mechanisms. Other products of the phenylpropanoid pathway have also been implicated in grapevine resistance mechanisms to phylloxera. More recently, research has begun to focus on the production of pathogenesis-related (PR) proteins.

1.4.1 Grapevine defence against other pathogens and pests

Role of the phenylpropanoid pathway in grapevine defence

The phenylpropanoid pathway was discussed in section 1.3.3 and Appendix one. Various phenolic compounds which are products or intermediates of this pathway have been proposed to be involved in resistance of grapevines to a number of fungal pathogens. Stilbene production can be induced in grapevines by pathogen invasion, fungal elicitors, UV irradiation or wounding (Langcake and Pryce 1976, 1977, Hoos and Blaich 1988, Melchior and Kindl 1991, Keller *et al.* 1997). The intensity and speed of stilbene production has been shown to be correlated with resistance of *Vitis* spp. to grey mould, *Botrytis cinerea* (Langcake 1981, Stein and Blaich 1985, Sbaghi *et al.* 1995), and to downy mildew, *Plasmopara viticola* (Langcake 1981, Dercks and Creasy 1989). Resveratrol, a stilbene-phytoalexin found in grapevine, is the precursor for production of the many oligostilbenes including pterostilbene (Langcake *et al.* 1979), stilbenoids such as ampelopsin A and hopeaphenol (Reniero *et al.* 1996) and the phytoalexins known as viniferins (Langcake and Pryce 1977, Pryce and Langcake 1977, Mattivi and Reniero 1992, Korhammer *et al.* 1995).

Stilbene synthase and PAL are both elicited in grapevine cell cultures in response to a *Botrytis* cell wall glucan, indicating their involvement in a defence response to pathogens (Liswidowati *et al.* 1991). Transfer of a grapevine stilbene synthase gene (*vst1*) (which encodes an enzyme which synthesises resveratrol) to a variety of plants has enhanced their resistance to a range of fungal pathogens. In rice, resistance to the rice blast fungus *Pyricularia oryzae* was increased (Stark-Lorenzen *et al.* 1997); in potato, resistance to *Botrytis cinerea* was enhanced (Hain *et al.* 1993), and in tomato, resistance to *Phytophthora infestans* was enhanced (Thomzik *et al.* 1997).

Peroxidase-mediated oxidation of resveratrol is responsible for the necrotic HR in response to an elicitor from *Trichoderma viridae* in cell cultures of *V. vinifera* (Calderón *et al.* 1994).

The level of inducible peroxidase activity is greater in cell cultures derived from leaves or stems of *Vitis* cultivars resistant to fungal attack (cvs. Pollux and Sirius) than cultures derived from sensitive ones (cvs. Riesling and Optima) (Hoos and Blaich 1988). Both peroxidase and PPO activities increase in roots of grapevines on attack by the dagger nematode *Xiphinema index* (Sopp *et al.* 1998). Enzyme activities increase to the greatest extent in the most resistant varieties Börner and *V. cinerea*, and this increase is associated with an HR in these varieties. Possibly one of the only examples of correlation of a constitutive characteristic with resistance is the level of a 4-hydroxystilbene-oxidising isoperoxidase, which is over-expressed in downy mildew-resistant hybrids of *Vitis* spp. (Calderón *et al.* 1992). These reports support the potential role of peroxidase in HR-related defence mechanisms in grapevine.

It is likely that stilbene-based resistance mechanisms of grapevine involve a balance of different biochemical reactions including induction of the synthesis of resveratrol and other stilbenes, and peroxidase-mediated oxidation of these compounds. Other products of the phenylpropanoid pathway have also been implicated in resistance mechanisms of grapevine callus. Dai *et al.* (1995c) show that callus derived from downy mildew-resistant *Vitis* species (*V. riparia* and *V. rupestris)* have higher constitutive levels of gallocatechin derivatives, but also accumulate flavonoids in response to downy mildew infection. Also, cell walls become suberised (suberin is a polymer of fatty acids covalently linked to ferulic and p-coumaric acids, products of the phenylpropanoid pathway (Riley and Kolattukudy 1975)) to a greater extent in callus derived from resistant species than from susceptible *V. vinifera*.

Pathogenesis-related (PR) proteins and systemic acquired resistance (SAR) in grapevine defence

PR proteins have been proposed to play a role in the resistance of many plant species to pathogens (reviewed by Stintzi *et al.* 1993). They are classed into five groups numbered PR-1 to PR-5. They include enzymes such as chitinases and glucanases (belonging to the group PR-2), and are often associated with HR. PR proteins are generally inducible on pathogen attack, elicitation or wounding. Some PR-5 proteins (neutral, osmotin-like proteins) from tobacco have been found to be expressed constitutively in root cortex. PR proteins have also been shown to be constitutively expressed in mature grapevine tissue including leaves and berries (Renault *et al.* 1996, Busam *et al.* 1997b, Tattersall *et al.* 1997, Jacobs *et al.* 1999). Several PR proteins, including β-1,3-glucanases and chitinases are inducible in grapevine (*V. vinifera*) leaves on treatment with salicylic acid, and especially by

infection with *B. cinerea* (Derckel *et al.* 1996, Renault *et al.* 1996, Busam *et al.* 1997b, Derckel *et al.* 1999).

A thaumatin-like PR-5 protein (VvTL1) has been found to accumulate in berries of *V. vinifera* in co-ordination with ripening, and possibly plays a role in resistance of ripe berries to powdery mildew (*Uncinula necator*) (Tattersall *et al.* 1997). Jacobs *et al.* (1999) showed the upregulation of both chitinases and β-1,3-glucanases in leaves and pre-veraison berries of a susceptible grapevine (*V. vinifera*) following infection with powdery mildew. They also noted the upregulation of transcription of another thaumatin-like protein, VvTL2, in response to powdery mildew. Expression of these proteins in response to infection does not, however, afford complete protection to this pathogen (Jacobs *et al.* 1999). The exact role, if any, of these PR proteins in grapevine defence remains unknown.

Systemic acquired resistance (SAR) involves signalling within a plant in response to infection by a pathogen, which effectively results in increased resistance of tissues remote from the point of infection. SAR occurs in a wide range of plants (Chen *et al.* 1993, Hunt and Ryals 1996), although it is not commonly reported in *Vitis* spp.. While there is little information on the operation of SAR in grapevine, studies by Busam *et al.* (1997a, b) have shown the potential for *V. vinifera* to respond to SAR activators, as well as to accumulate PR proteins in tissues remote from infection. In response to SAR elicitors (salicylic acid, 2,6-dichlorisonicotinic acid or yeast cell wall preparations), cell cultures of *V. vinifera* cv. Pinot Noir showed a transient increase in accumulation of PR proteins (chitinases), as well as stilbene synthase, and caffeoyl-coenzyme A 3-O-methyltransferase (correlated with SAR in other species) (Busam *et al.* 1997a). Chitinases were also shown to be upregulated in leaves of *V. vinifera* cv. Pinot Noir adjacent to leaves infected with downy mildew (Busam *et al.* 1997b). Interestingly, this response was not seen in resistant *V. rupestris*. In this case, spread of infection was halted, with associated development of necrotic spots.

It is likely that a combination of PR proteins and products of the phenylpropanoid pathway play major roles in grapevine defence, and that other mechanisms do not. For instance, Kortekamp *et al.* (1997) showed that callose deposition, a common defence mechanism in other plants, played no apparent role in the resistance of *Vitis* spp. to downy mildew. The effectiveness of grapevine resistance mechanisms is almost invariably proportional to the speed and intensity of response to pathogen invasion. The quicker a variety is able to respond, the more resistant it is.

1.4.2 Resistance of grapevines to phylloxera

The response of the leaves of some grapevine species to infection by fungal pathogens is very similar to the response of the roots of the same species to phylloxera. For example, in varieties susceptible to downy mildew, such as *V. vinifera,* there was no localised defence response to infection (Boubals 1966a, Dai *et al.* 1995a, b). Vines resistant to downy mildew, such as *V. rupestris,* showed a rapid build up of condensed tannins upon infection, followed by isolation of the infection site by lignification. *V. rotundifolia*, which is immune to downy mildew, exhibited a rapid hypersensitive response. These responses can be compared to those of grapevine varieties to phylloxera described in sections 1.2.1 and 1.2.2. It is possible therefore that the resistance mechanisms of grapevines to some fungal pathogens are similar to those for resistance to phylloxera.

The relative efficacy of different grapevine defence mechanisms against phylloxera will at least partly depend on the location of the feeding site. As mentioned in section 1.3.3, the biochemical content of parenchyma cells is significantly different to that of phloem. For example, products of the phenylpropanoid pathway are known to be compartmentalised in vacuoles in the cells of *Vitis* species (Hrazdina 1992). The location of phylloxera feeding sites was investigated by Petri (1907), who described it as a parenchyma feeder. This has not been confirmed using more accurate modern techniques. Because of the implications of this for the potential resistance mechanism, the location of the feeding site needed to be confirmed.

Root anatomy

In the grapevine-phylloxera interaction, penetration of the root is essential for phylloxera feeding and subsequent anatomical changes are required for nodosity formation, both suggesting that root anatomy could play a role in grapevine resistance to phylloxera. The anatomy of grapevine primary roots is typical of perennial dicotyledons (figure 1.4) although there is little published information specifically on grapevine root anatomy. A review published by Pratt (1974) cited several studies which implicate certain primary root characteristics such as a long root cap, small cortical cells, and rapid differentiation of the vascular bundle as being characteristics which favour resistance to phylloxera. Foex (1876 in Niklowitz 1954) suggested that the arrangement of medullary rays in secondary roots was correlated with resistance[1] of American vine species. It was hypothesised that the position of the rays in resistant species prevented spread of necrotic lesions throughout the root. Davidis and Olmo (1964) stated that secondary root anatomy correlates with resistance of

[1] According to the definitions used in this thesis, these reports are describing secondary root tolerance, rather than resistance.

VR hybrids to phylloxera. This was based on the observation that for a number of these hybrids, those whose root anatomy bore the strongest resemblance to *V. rotundifolia* showed the highest degree of resistance to phylloxera.

Biochemistry of gall formation

Nysterakis (1946 cited in Granett 1990) suggested that resistant grapevine varieties would not respond to IAA, suggested to be the gall-inducing component of phylloxera saliva. Denisova (1965) also felt that the inability of phylloxera to induce galls on certain vine species was involved in their resistance. However, neither Nysterakis nor Denisova presented data to support their theories. Tests using artificial application of IAA did not support Nysterakis' hypothesis, as all varieties were equally responsive (Granett 1990).

The work of Denisova (1965) also indicated that there may be chemical differences between phylloxera-induced root galls of resistant (*V. riparia* × *V. rupestris*) and susceptible (*V. vinifera* cv. Shasla) varieties of grapevine. Phenolic compounds isolated from root galls of the resistant hybrid were identified as caffeic acid and quinic acid (breakdown products, or precursors of chlorogenic acid), and glycosylated quercetin. These were proposed to be inhibitors of gall induction. In root galls of the susceptible variety, free quercetin and chlorogenic acid were identified, both of which were proposed to be enhancers of gall induction. The relevance of these chemicals to gall formation remains unknown, since insufficient data is given to support the conclusions on their biological functions. Denisova's conclusions are contradicted by Miles (1990), who stated that IAA activity is in fact enhanced (not inhibited as suggested by Denisova) by caffeic acid. The possible role of these phenolic compounds (caffeic acid, quinic acid and glycosylated quercetin) in the resistance of grapevines to phylloxera has not been further assessed.

Evidence which indicates the importance of gall formation in the susceptibility of a vine to phylloxera was presented by Sobetskiy and Derzhavina (1973). They noted that the phenolic content of the exudate from the feeding site of phylloxera in leaf galls, thought to be the actual food of the phylloxera, is significantly lower than that of the sap from the entire gall. If the same were true for root galls, and if phenolic compounds are involved in the defence of grapevines against phylloxera, formation of specialised feeding sites within galls may allow phylloxera to overcome what might otherwise be an effective defence mechanism.

Inducible defence responses

There are several responses shown by resistant grapevines to phylloxera attack, some of which may be as possible resistance mechanisms. As noted in section 1.2.2, susceptible,

resistant and immune varieties show differences in the rate and extent of wound periderm formation in secondary roots; a response thought to be the most important in determining tolerance to phylloxera attack (Boubals 1966a). Susceptible varieties (e.g. *V. vinifera*) show no localised wound response, and extensive damage occurs to the vascular tissue (Boubals 1966a, King and Rilling 1991). In resistant and/or tolerant varieties (e.g. *V. rupestris*, *V. berlandieri*) a layer of wound periderm effectively seals off the gall site from the rest of the root, and is believed to prevent further damage to the root system, including prevention of subsequent entry of pathogenic micro-organisms (Miles 1989).

Denisova (1965) noted distribution of a 'tannic' material in primary roots following phylloxera feeding. In the resistant variety *V. riparia* × *V. rupestris*, it was closely distributed around the vascular bundle, and localised near the site of penetration. In the susceptible variety (*V. vinifera*), it was evenly distributed throughout the gall.

In *V. rotundifolia*, an immune species, an HR leading to necrosis has been observed which appears to prevent phylloxera from feeding (Boubals 1966a). The mechanism by which an HR is elicited in this case remains unknown.

1.5 Conclusions

From the above review it is clear that little is definitively known of the physiological, biochemical or molecular responses of grapevines to phylloxera infestation, or of their mechanisms of resistance. A number of lines of research appear open to investigation. These include:

- Confirmation that the root dwelling form of phylloxera feeds from parenchyma tissue and not from phloem.
- Documentation of the interaction of phylloxera with *V. vinifera*: processes involved in gall formation, including physical, molecular and biochemical responses of the root to phylloxera, and the possible regulatory role of plant hormones and secondary metabolites.
- Elucidation of the role of preformed (constitutive) physical or chemical factors in phylloxera resistance and/or tolerance, and identification of key chemical compounds.
- Elucidation of the role of induced defence responses such as production of physical barriers (e.g. suberin and lignin) or their precursors, including studies on the importance of their rate of formation.

- Elucidation of the role of induced defence responses such as production of secondary plant metabolites (e.g. stilbenes, phenols, flavonoids), and identification of key chemical compounds.

The research described in this thesis begins to investigate some of these aspects of the grapevine-phylloxera interaction. Chapter two describes the development of methods for cocultivation of phylloxera with grapevines in both tissue culture and pots containing potting mixture. Chapter three describes the development and optimisation of methods for analysis of uninfested and infested roots, including histochemistry, molecular and HPLC techniques.

Chapter four documents the interaction between phylloxera strain VWL-1 and the susceptible species *V. vinifera* cv. Shiraz. This study includes anatomy and histochemistry of phylloxera feeding and nodosity formation, biochemistry of uninfested roots and nodosities and expression of various gene transcripts in uninfested roots and nodosities. Chapter five documents the interaction of phylloxera strain VWL-1 with a number of resistant species and rootstocks and compares the interaction to that with *V. vinifera* as a susceptible standard. This includes assessment of resistance to VWL-1 in tissue culture followed by comparative primary root anatomy, histochemistry, and HPLC analysis of free phenolics in uninfested roots as well as histochemistry and HPLC analysis to detect changes in free phenolic compounds in response to phylloxera feeding. Chapter six documents the interaction between phylloxera strain SRU-1 (an unusual biotype) and *V. vinifera* cv. Shiraz and the rootstock Schwarzmann. It focuses on a comparison of this strain of phylloxera with VWL-1, as well as on the histochemical and biochemical response of these varieties to SRU-1 phylloxera infestation.

Chapter seven presents the conclusions which can be drawn from this study considering all the results together, and proposes possible roles for various components of the interaction in the susceptibility or resistance of the various vine types. Directions for future research are proposed.

2. DEVELOPMENT OF METHODS FOR COCULTIVATION OF POTTED OR MICROPROPAGATED GRAPEVINES WITH PHYLLOXERA

2.1 Introduction

In order to study any aspect of the interaction of a pest and its plant host, cocultivation of the two organisms must be carried out in a controlled environment. In most experimental systems such cocultivations use either potted or micropropagated (*in vitro*) plants.

Most methods for cocultivation of grapevines with phylloxera published at the time of this study were developed for rootstock trials or for determining differences in biotypes of phylloxera. In some potted vine trials, containers were used which allow the observation of the roots without disturbing the vine (Firoozabady and Olmo 1982, King *et al.* 1982, King and Rilling 1985, 1991). These trials assessed either the resistance of different vine types or the number of nodosities and tuberosities formed by different phylloxera biotypes.

Excised secondary root pieces have also been used for cocultivation (Granett *et al.* 1985, 1987, de Benedictis and Granett 1993, Omer *et al.* 1995a, Corrie *et al.* unpublished). The root pieces are placed in petri dishes, allowing the experimenter to control environmental conditions, and to observe the exact numbers and behaviour of phylloxera. Such a system is useful for various experiments, and has been used to assess the biotype of phylloxera strains as well as to determine the population's response to preformed tuberosities. The major disadvantage of using excised secondary roots is contamination by fungi, as it has not proved possible to surface-sterilise secondary root pieces. In addition, the phylloxera inoculum in these experiments has not been sterilised before use. Fungal contamination can severely hamper both collection of data, and survival of phylloxera.

In vitro cocultivations are used by many researchers for studying the interactions of plants and pests. They offer many advantages, especially control of environmental conditions and elimination of secondary pathogens. For grapevines, tissue culture cocultivations have been established with the lesion nematode *Pratylenchus vulnus* (Palys and Meredith 1984), the dagger nematode *Xiphinema index* (Bavaresco and Walker 1994) and grapevine aphid *Aphis illinoisensis* (Webb *et al.* 1994).

At the time of inception of this study, only two reports of cocultivation techniques for grapevine and phylloxera had been published using *in vitro* methods (Askani and Beiderbeck 1991, Martinez-Peniche 1994). Grapevines were grown on sloped agar with exposed roots, or as hairy root cultures. Phylloxera thus had free access to the roots, and it was possible to observe their behaviour without disturbing the interaction. Problems were

encountered, however, with condensation in the culture vessels, and there was little mention of phylloxera establishment and survival rates.

This chapter describes optimisation of methods for cocultivation of grapevines with phylloxera, using both potted and micropropagated vines and discusses possible reasons for the success or failure of each. The end result is a relatively straightforward and effective experimental system for working with phylloxera and grapevines in controlled experiments. Successful methods will be noted and will be referred to in subsequent chapters.

In all cases this study focused on the interaction between phylloxera and primary roots. All cocultivation methods were designed to generate infested primary roots for further analysis. A further aim of this work was to establish whether resistance ratings obtained in a bioassay based on primary roots could be related to published resistance ratings for a range of varieties.

All cocultivations with phylloxera were carried out at Agriculture Victoria, Rutherglen, which lies within the Vine Disease District of Rutherglen, Victoria (figure 1.2). Quarantine regulations prohibit the transport of live phylloxera out of phylloxera infested regions and strictly regulate the movement of grapevine material. Cocultivations were therefore assessed in Rutherglen, and root material harvested and rendered inviable either by freezing in liquid nitrogen or by fixing for microscopy before being transported (frozen material in a cryoshipper (Taylor Wharton)) to The University of Adelaide for further analysis.

2.2 Selection of grapevine varieties for cocultivation

Grapevine cultivars or rootstocks (all of which will be termed 'varieties' for the remainder of this thesis) selected for study are listed in table 2.1. The use of some varieties such as *V. rotundifolia* and Börner, which are not yet commercially available in Australia, was confined to tissue culture. Other varieties were used in both tissue culture and potted vine cocultivations.

Vitis vinifera cv. Cabernet Sauvignon and cv. Shiraz were selected as the susceptible standards. 140-Ruggeri, Ramsey, Schwarzmann and *V. riparia* were selected because of reported resistance or high resistance to phylloxera (May 1994, Whiting *et al.* 1987, Pongrácz 1983), while some accessions of *V. cinerea* have even been reported as immune (Becker 1988, Granett *et al.* 1987, Davidis and Olmo 1964). Börner, a recently released rootstock, and *Vitis rotundifolia* were both selected because of reported immunity to phylloxera (Boubals 1966a, Becker 1988).

Table 2.1 Varieties of grapevines, accessions and sources of each used in the study.

Vine variety	Parentage	Clone/Accession	Source[1]
***V. vinifera* cv.**	*Vitis vinifera* L.	LC 10/A.S.80.5397	Nuriootpa
Cabernet		LC 10/A.S.80.5397	Waite
Sauvignon			
***V. vinifera* cv.**	*V. vinifera* L.	PT23/A.N.61.0020	Waite
Shiraz		PT10/unknown	Ag. Vic., Irymple
		12/BVRC-12-C12A	RVIA
ARG1	*V. vinifera* L. cv. Aramon ×	unknown	Waite
	V. rupestris Scheele cv. Ganzin		
Schwarzmann	*V. riparia* Mischx. × *V. rupestris*	WA/A.S.74.2257	Waite
	Scheele	WA/A.V.70.2252	Ag. Vic, Irymple
		WA/R317A	RVIA
Ramsey	*V. champini* (*V. mustangensis* ×	A11V2/I.V.63.2065	Waite
	V. rupestris (Moore 1991))	R315A/I.C.74.8257	RVIA
140-Ruggeri	*V. berlandieri* Planch. ×	Q45-3A	Nuriootpa
	V. rupestris Scheele	Q45-3A	Ag. Vic., Irymple
V. riparia	*V. riparia* Mischx.	unknown	Waite
V. cinerea	*V. cinerea*	*V. cinerea* 55 ×	CSIRO Merbein
		V. cinerea 194-1	
Börner	*V. cinerea* Noronha cv. Arnold ×	unknown	CSIRO Merbein
	V. riparia Mischx.		
V. rotundifolia	*V. rotundifolia* Planch. (Small)	Ex CSIRO/	Nuriootpa
	subgenus *Muscadinia*	I.W.56.6001	

[1] Sources of grapevine material: Nuriootpa: Nuriootpa Research Centre, SARDI, SA; Waite: The University of Adelaide, Waite Campus; CSIRO Merbein: CSIRO Plant Industries, Horticultural Centre, Merbein, Victoria; Ag. Vic., Irymple: Sunraysia Horticultural Centre, Agriculture Victoria, Irymple, Victoria; RVIA: Riverland Vine Improvement Association, Monash, SA.

2.3 Potted vine-based cocultivation trials

One of the analytical methods chosen for this study required extraction of RNA from the vine roots. Preliminary trials indicated that several grams of root material would be needed to obtain sufficient quantities of RNA for analysis. Generation of large quantities of infested root material was the primary goal of the potted vine cocultivations.

Factors considered important for cocultivation of phylloxera with potted grapevines include vigour of the grapevines, potting mix type and cultivation (temperature, watering regime, pest control) conditions. As well as optimising these, it was necessary to find a means of effectively isolating infested from uninfested control vines.

2.3.1 Pot trial one - summer 1996-1997

Grapevines were initially propagated at The University of Adelaide. Grapevine cuttings of four varieties (*V. vinifera* cv. Cabernet Sauvignon, 140-Ruggeri, Schwarzmann accession WA/A.S.74.2257 and ARG1) were struck using a method based on that published by Williams and Antcliff (1984). Each cutting was scored vertically 5-10cm from the base, the end trimmed and the cutting dipped for 20 seconds in a solution of 2000ppm IBA in water. The cuttings were planted in a callusing bed maintained at approximately 25°C in a 4°C coldroom. The bed was watered twice weekly until the cuttings had developed roots (four to six weeks). Cuttings were transplanted to 17.5cm pots in a potting mixture of equal parts peat, perlite and coarse, even-grained river sand (steam sterilised). Four cuttings were planted in each pot, and these were maintained in a glasshouse at 20°C, with incandescent lights maintaining a 16 hour photoperiod. When shoot growth was well established (after approximately ten weeks) the vines were transported to Agriculture Victoria, Rutherglen, where they were placed in a glasshouse at 20-25°C and watered by hand as required.

In order to keep uninfested control vines under identical conditions (i.e. in the same glasshouse) as infested vines, aphid-proof cages of $1m^3$ were constructed from flyscreen frames fitted with fine voile fabric. (Similar aphid-proof cages were used by Askani and Beiderbeck (1991).) 16 pots were placed in each cage in a random block design (figure 2.1a).

The strain of phylloxera selected for infestation was SRU-1 (Corrie *et al.* 1997a) originally sourced from leaf galls of Schwarzmann vines in Campbell's vineyard, Rutherglen and maintained on roots of potted *V. vinifera* vines in the glasshouse at Agriculture Victoria, Rutherglen. Inoculum consisted of nodosities collected from these *V. vinifera* vines. The total inoculum was 0.3g nodosities per pot which contained on average 11.25 ± 3.77 adults and 10.00 ± 4.08 eggs. Each vine was upturned out of its pot and inoculated so that nodosities were placed directly in contact with vine roots.

Over a period of four weeks the vines declined in health due to heavy infestation with two-spotted mite. No sprays for insect pests were applied in case of adverse affects on the phylloxera population, and predatory mites were ineffective.

This trial was abandoned after one month due to the very poor health of the vines. *V. vinifera* roots were badly degraded, and no nodosities were obtained. 140-Ruggeri vines had some healthy roots but no nodosities had developed. The healthiest of the ARG1 vines had a total of four small nodosities. Schwarzmann was the least affected by mites and ten vines

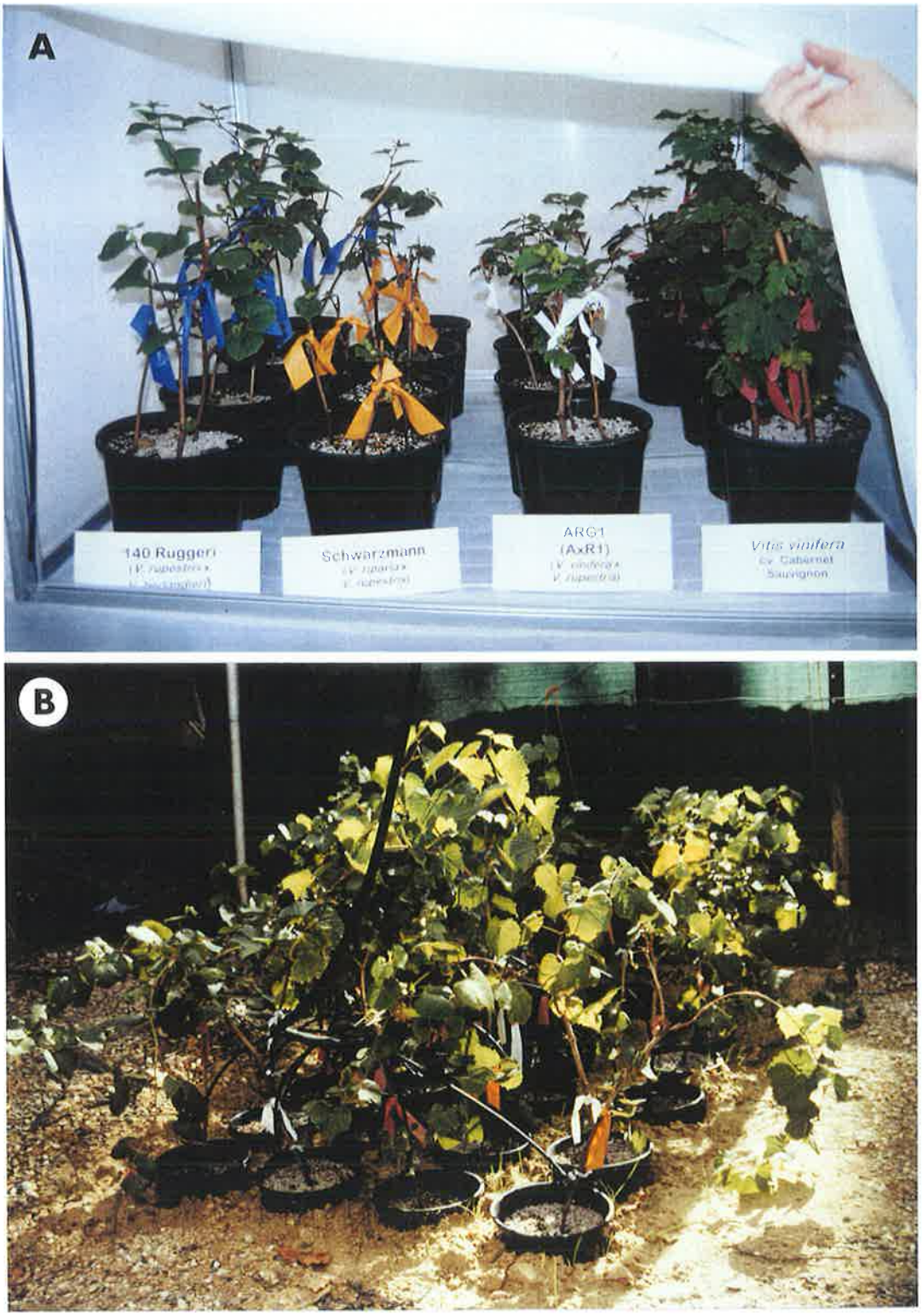


Figure 2.1 Potted grapevine cocultivation trials. a) Aphid-proof cages at the beginning of pot trial one. b) Sand-filled pits used to insulate pots during pot trial four.

had nodosities on the roots. These were mostly small but hook shaped; typical of susceptible vine roots (King *et al.* 1982). Infested root material was not harvested.

2.3.2 Pot trial two - summer 1996-1997

Pot trial one was repeated using the same vine varieties (except ARG1) and accessions. Vines were struck from cuttings at The University of Adelaide, then transported to Agriculture Victoria, Rutherglen where they were repotted into a mixture of equal parts peat, perlite and coarse sand (steam sterilised). Cages and vines were sprayed with Torque™ for two-spotted mite two weeks prior to phylloxera infestation. Predatory mites were subsequently applied to the vines on a fortnightly basis to avoid two spotted mite infestation throughout the course of the trial. At the outset, all vines had very healthy, vigorous root systems. They were inoculated with phylloxera strain SRU-1 freshly collected as leaf galls directly from Schwarzmann vines in Campbell's vineyard, Rutherglen, as insufficient glasshouse root material was available[2]. Three to six leaf galls, each containing up to 200 eggs, were used per pot.

Mites were effectively controlled, but the vines suffered from waterlogging and excessive air humidity, and declined in health. After four weeks there was no evidence of phylloxera infestation. The vines were repotted with an extra 10% perlite to help alleviate waterlogging. They were reinoculated as before, and watering was reduced. After six weeks only a very few nodosities were found on *V. vinifera*. Poor establishment of phylloxera, even on susceptible vines, resulted in the termination of trial two without harvesting of root material. It was decided at this point that the use of aphid-proof cages and the glasshouse would be abandoned as they appear to limit air flow around the plants, exacerbating problems of waterlogging and high humidity.

2.3.3 Pot trial three - summer 1996-1997

One year old rootlings of *V. vinifera* cv. Shiraz accession PT10 and Schwarzmann accession WA/A.V.70.2252 and rooted cuttings of *V. vinifera* cv. Cabernet Sauvignon (as for pot trial two) were potted in potting mix of 1 part commercial potting mix: 1 part perlite. They were infested with phylloxera strain SRU-1, originally collected from leaf galls on Schwarzmann vines at Campbell's vineyard Rutherglen, and maintained on potted Schwarzmann vines grown in a shadehouse at Agriculture Victoria, Rutherglen (leaf galls were no longer present in the field). Inoculated vines were maintained in a shadehouse, with no aphid-proof cages, and watered by hand as required. Care was taken to avoid waterlogging. Uninoculated

[2] King and Rilling (1985) found no significant difference in vine response when using leaf galls or nodosities as inoculum.

vines were maintained on separate benches in the same shadehouse. Tanglefoot™ was used on benches to minimise movement of crawlers between pots. Uninoculated vines were checked carefully for signs of infestation before harvesting.

Phylloxera infestation was apparent approximately two weeks following inoculation, at which time some infested root material was harvested from *V. vinifera* cv. Shiraz and Schwarzmann, with the remainder being harvested at the same time as uninfested root material, four weeks later. Harvested root material was either snap frozen in liquid nitrogen, embedded in cryo-mounting medium or fixed in FAA for microscopy (Appendix three). Leaf material was harvested from *V. vinifera* cv. Cabernet Sauvignon six weeks after inoculation. Leaf material was snap frozen in liquid nitrogen for later analysis by northern blot hybridisation.

It was noted that while nodosities on Schwarzmann vines were generally smaller than those on *V. vinifera*, they were present in approximately equal numbers on both varieties[3].

2.3.4 Pot trial four - summer 1997-1998

In order to overcome the problems experienced in the first season (1996-97), a number of new approaches were adopted for potted vine cocultivations in the 1997-98 season.

The unusual nature of the SRU-1 strain had not been acknowledged prior to the commencement of this study. This was in spite of previous observations of the different interaction of rootstocks with this strain compared with strains collected at other sites in Victoria (Buchanan 1990). In addition, blocks of own-rooted *V. vinifera* have maintained good health and productivity for 20 years in the area from which SRU-1 was isolated (M. Campbell, Campbell's Winery, Rutherglen, pers. comm.). During the course of this work, observations were also made in Campbell's vineyard and Stanton and Killeen's vineyard, Rutherglen, of heavy infestations of phylloxera on Schwarzmann primary roots alongside *V. vinifera* vines with almost no signs of infestation. The pattern of infestations observed in pot trials two and three spurred on the efforts to biotype SRU-1 strain phylloxera. SRU-1 was subsequently shown to be significantly different to biotype A or B as defined by Granett *et al.* (1985), as well as distinct from strains isolated elsewhere in Victoria (Corrie *et al.* unpublished). SRU-1 showed a preference for primary roots of Schwarzmann or ARG1, and overall survived equally well, if not better on Schwarzmann roots than on *V. vinifera*. Compared to other strains tested in Victoria, it was relatively non-aggressive on any vine type.

[3] An observation consistent with those made in rootstock trials conducted at Stanton and Killeen's vineyard in Rutherglen (personal observation).

Phylloxera strain VWL-1, isolated from *V. vinifera* roots at Brown Brother's Whitlands vineyard in the King Valley, Victoria, was subsequently used as the experimental phylloxera strain. This strain is genetically distinct from SRU-1 (51% similarity (Corrie *et al.* 1997a)), very aggressive, and appears to be similar in biotype to biotype B (Granett *et al.* 1985, Corrie *et al.* unpublished). It was chosen in order to generate more typical differential responses from susceptible or resistant vines, and because infestation of experimental vines would be facilitated by the strain's aggressiveness.

Vine vigour, and health of the root system were also felt to be of paramount importance. It was found in the 1996-97 season (e.g. trial three versus trial two) that one year old rootlings, rather than rooted cuttings, had higher vigour and were able to establish a good root system more quickly. One year old rootlings of *V. vinifera* cv. Shiraz accession 12/BVRC-12-C12A, Ramsey accession R315A and Schwarzmann accession WA/R317A were purchased from the Riverland Vine Improvement Association, SA for the 1997-98 season. Fifty rootlings of each variety were potted in polythene nursery bags in a potting mix designed for optimal drainage, of two parts peat: three parts coarse sand: four parts perlite (steam-sterilised). Once shoot growth had commenced (after approximately six weeks) they were transported to Agriculture Victoria, Rutherglen.

All vines were transplanted to 17.5cm pots containing the same potting mix, and watered with drip irrigation. The plants were maintained in shadehouses which provided conditions more suitable for grapevines than the glasshouse, but did not allow temperature control. The construction of new shadehouses at Agriculture Victoria, Rutherglen in 1997 enabled an internal quarantine system to be established, whereby uninfested vines were maintained in a separate shadehouse, under similar conditions to those which were to be infested with phylloxera. Throughout the trial all vines were sprayed weekly with either Tilt™ (0.01%v/v) or Dipel™ (0.1%w/v) alternately to control pathogens and pests. Predatory mites were released fortnightly to control two-spotted mite.

Vine inoculations were carried out fortnightly by burying a 3-8cm piece of heavily infested root (collected fresh from Brown Brothers' Whitlands vineyard, King Valley) in the pot adjacent to the roots of the vine. Thirty vines of each variety were inoculated. After six weeks there was little evidence of infestation on the susceptible vines.

The 1997-98 Summer (December 1997 - February 1998) in Rutherglen had a mean maximum of 31.5°C (Agriculture Victoria, Rutherglen weather station records). Elevated temperature (above 25°C) has been shown to have an adverse affect on the survival of phylloxera (Buchanan 1990). The optimum temperature for establishment of feeding sites is

18-24°C (Turley *et al.* 1996) and at 32°C phylloxera do not survive to adulthood (Granett and Timper 1987). Potting mix temperature was tested during the day and found to range from 32-37°C. Inoculated pots were therefore subsequently insulated by burying in pits dug in the floor of the shadehouse and filled with moist sand (figure 2.1b). This reduced the potting mix temperature to approximately 25°C. Inoculations were then continued on a fortnightly basis for a further six weeks. (In a nearby trial, 50% of pots were wrapped with aluminium foil to reduce the effects of radiant heat. This lowered the potting mix temperature by approximately 5°C.)

Two weeks after the pot temperature had been reduced, roots of susceptible vines inoculated with VWL-1 were observed to be heavily infested. The degree of infestation was highly variable between pots; *V. vinifera* vines were generally heavily infested (more than 100 nodosities per pot), while Ramsey was lightly infested (mean number of nodosities 3.07±2.25 per pot) with relatively small nodosities. No nodosities, or other signs of attempted feeding were found on Schwarzmann vines.

After a further four weeks, nodosities from infested vines and root tips from uninfested vines were harvested and snap frozen in liquid nitrogen or fixed in 3% (v/v) glutaraldehyde (Appendix three) for microscopy.

2.3.5 Pot trial five - summer 1997-1998

Ten Schwarzmann vines (accession WA/R317A) were propagated as for pot trial four. Once at Agriculture Victoria, Rutherglen they were maintained at 20-25°C in a glasshouse separate from all other grapevines. They were inoculated with SRU-1 phylloxera collected fresh as Schwarzmann nodosities from the Department of Agriculture rootstock trial block at Stanton and Killeen's vineyard, Rutherglen (Leaf galls were not present in the field, and this strain was no longer maintained in the glasshouse). This cocultivation was conducted in order to generate infested root material for analysis to complement earlier analytical work resulting from pot trial three. Uninfested control vines were not included. The glasshouse was selected in order to achieve temperature control. Humidity was kept to a minimum and high levels of infestation were achieved within two to three weeks. Infested root material was harvested and snap frozen in liquid nitrogen as for pot trial four.

2.3.6 Discussion of pot cocultivation trials

Parameters of key importance to good infestation are a vigorous root system, and adequate control of pot temperature. The first relies on an appropriate watering regime and low relative humidity. The use of aphid-proof cages is detrimental to grapevine health and is not

recommended. Controlled drip irrigation is preferable in order to prevent over-watering. Effective pest and pathogen control is also essential for grapevine health, particularly in a glasshouse. Glasshouses tend to have high relative humidity, which over a period of several months is highly detrimental to grapevines. They are therefore only recommended for short term cocultivations. Shadehouses provide ideal conditions for grapevines, and are recommended as long as measures are taken to control pot temperature, if necessary. Burying pots is very effective but relatively labour intensive. Wrapping pots in aluminium foil also helps control temperature, and is recommended if the ambient temperature is not above 30°C. Light intensity was not measured in these trials. While it is possible that reduced light intensity in aphid-proof cages may have contributed to the poor health of the vines, other factors were probably at least equally significant. The reduced light intensity in shadehouses, over that which would exist in the vineyard does not appear to be detrimental to the health of vines. Under optimal conditions, pot-based cocultivations are an effective means of generating infested root material for analysis, but infestation rates are highly variable, so they are not ideal for bioassays or rootstock screening.

2.4 Tissue culture-based cocultivation trials

2.4.1 Introduction

At the initiation of this study, conditions had been established for micropropagation of a number of grapevine varieties (Chée and Pool 1985, Gray and Benton 1991, Mauro *et al.* 1995) and phylloxera had been cocultivated with both whole plantlets and excised root cultures (Askani and Beiderbeck 1991, Forneck *et al.* 1996). Tissue culture-based cocultivations present many advantages over other methods. The primary one is the ease of control of experimental conditions. Temperature, light regime and relative humidity, all of which may affect phylloxera behaviour, can be kept more uniform across the experiment. Importantly, secondary pathogens are eliminated. Control of these variables ensures that observed vine responses result only from phylloxera attack.

Cocultivation trials presented here served two main purposes. Firstly, cocultivation conditions were optimised for maximum survival of phylloxera, and to allow observation of phylloxera behaviour during the course of the cocultivation. The method for sterilisation of eggs was also improved to allow determination of the egg hatch rate, and thus the exact inoculum provided to each cocultivation. Secondly, cocultivations were used to determine resistance ratings for all vine varieties used based on the interaction with primary roots[4].

[4] In tissue culture, vine roots do not develop the secondary anatomy associated with mature roots, but retain primary anatomy as is seen in the primary root system of field grown vines.

This was of interest in order to see whether results obtained may be comparable to those from potted vine cocultivations or from field trials where infestation of secondary roots plays an important role in the grapevine-phylloxera interaction. As previously discussed (section 1.1.4) any reduced performance of phylloxera seen on varieties in tissue culture must result from vine resistance, not tolerance.

The following sections present the tissue culture methods developed and tested in chronological order. They document the optimisation of a cocultivation system best suited to the purpose of studying the interaction of phylloxera with a range of grapevine varieties.

2.4.2 Establishment of grapevines in tissue culture

All varieties of grapevine were established in tissue culture by propagation from single nodes according to standard procedures (B. Stummer, University of Adelaide, pers. comm.). The leaves were trimmed off green shoots harvested from vineyard grown vines, and shoots cut into nodal sections. Nodes were rinsed in 70% (v/v) ethanol for one minute, then sterilised in 1% (v/v) sodium hypochlorite (0.3% (v/v) active chlorine) containing one drop of Tween-20/L, by shaking in the solution for 20 minutes. Nodes were then rinsed four times in sterile distilled water. The ends were trimmed from each node and they were planted upright in 8ml agar medium in a 30ml polycarbonate vial.

The initial medium was half strength Murashige and Skoog salts and vitamins (1/2 MS) (Murashige and Skoog 1962), 2% (w/v) sucrose, 0.8% (w/v) Bactoagar (Difco) supplemented initially with 1μM BAP (and 1μM NAA for *V. rotundifolia* to enhance root initiation (Gray and Benton 1991)). Cultures were incubated in a growth cabinet at 23-25°C, with a photoperiod of 16 hours. Once shoots and roots had developed, all vines were transferred to 250ml polycarbonate jars with Breather Caps™ (Sarstedt) containing 50ml of the same medium, without BAP, but with 1μM NAA for *V. rotundifolia*.

All *in vitro* cocultivations with phylloxera were subsequently carried out in a culture room maintained at 23°C with a 12 hour photoperiod and photon irradiance around 40μmol quanta $m^{-2}s^{-1}$.

2.4.3 Sloped medium cultures (Phytatrays™)

Micropropagated grapevines used for cocultivation with phylloxera have previously been grown on sloped agar in Magenta® vessels (Forneck *et al.* 1996). Phytatrays™ (Sigma) were trialed here as alternative culture vessels as they have the advantage of being relatively broad and shallow (114×86×65mm), for easier access to the plantlet when the vessel is open.

The phytatray is also transparent, allowing continuous observation of phylloxera behaviour and gall development.

Three varieties were selected for these trials: *V. vinifera* cv. Cabernet Sauvignon, 140-Ruggeri and *V. rotundifolia*, selected as being representative of susceptible, resistant, and immune vines respectively.

Agar culture medium (40ml each phytatray) was sloped at an angle, so that the medium covered half of the base of the phytatray (figure 2.2, 2.3a). This allowed the vine roots to grow across the surface of the container, and gave phylloxera free access for feeding[5]. Plants were transplanted to phytatrays and transported to Rutherglen when they had developed roots.

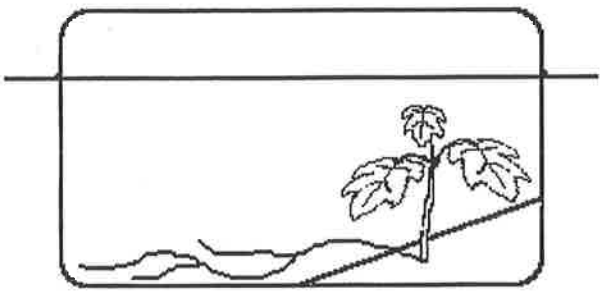


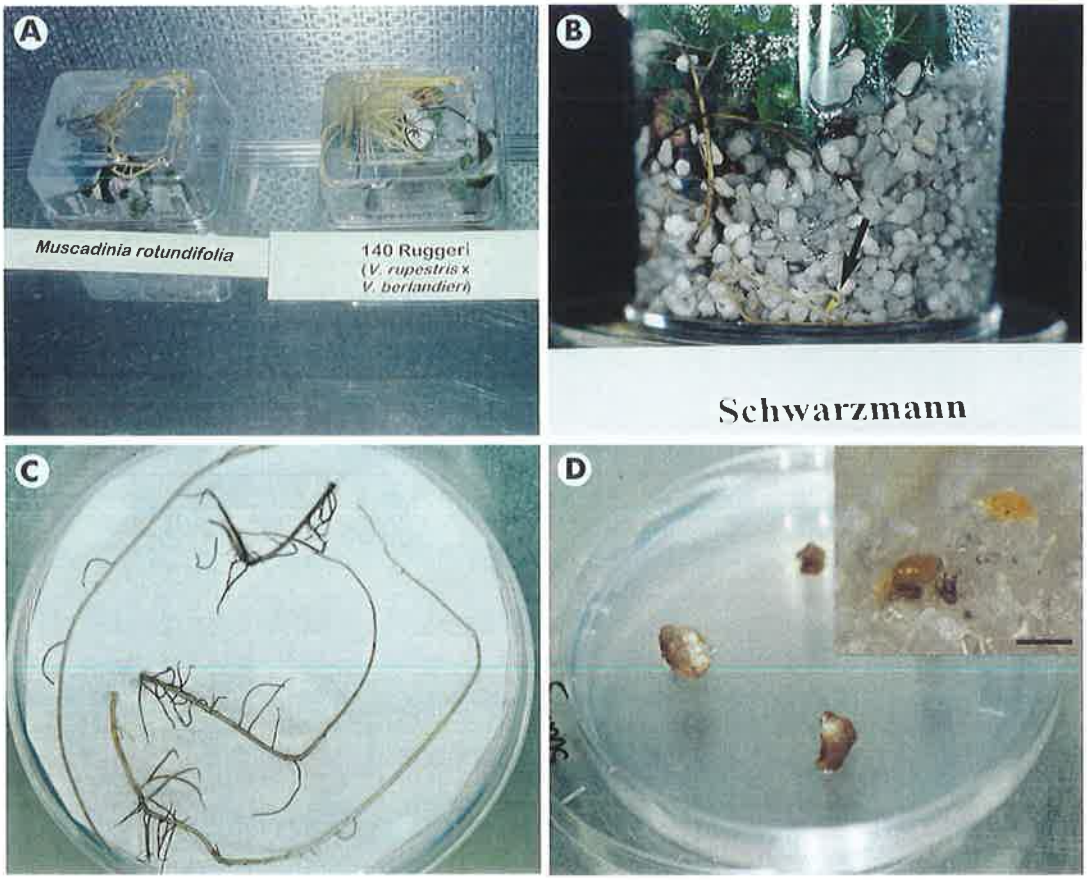
Figure 2.2 Illustration of a grapevine grown on sloped medium in a Phytatray™.

Phytatray™ trial one

SRU-1 phylloxera originally isolated from Schwarzmann leaf galls at Campbell's vineyard, Rutherglen, and maintained on glasshouse grown *V. vinifera* vine roots at Agriculture Victoria, Rutherglen were used as inoculum. Three thousand eggs were harvested and stored on wet filter paper in sealed petri dishes until used (up to two days). Eggs were sterilised using egg sterilisation method one (section 2.4.8), and placed directly onto the roots of *in vitro* plants using an autoclaved No.00 artist's brush. Sterilisation and infestation were carried out in a non-certified biohazard hood, which was the only 'sterile' facility available at the time.

Eight replicates of each vine variety were infested with 100 eggs each. Phytatrays were arranged on one shelf in a culture room in a random block design. Plants were maintained at 23°C for 21 days. After this period, significant levels of fungal and bacterial contamination were present (3/8 *V. vinifera* cv. Cabernet Sauvignon, 7/8 140-Ruggeri and 5/8 *V. rotundifolia* badly contaminated). Because of this, only the infestation parameters for SRU-

[5] Phylloxera are unable to penetrate, or move across agar based medium, and therefore must have access to the roots above or away from the medium to allow feeding.


A
Muscadinia rotundifolia
140 Ruggeri
(V. rupestris x
V. berlandieri)
B
Schwarzmann
C
D

1 on the susceptible variety (*V. vinifera* cv. Cabernet Sauvignon) were recorded (table 2.2). The hatch rate was less than 75%, and only a small number of phylloxera could be located within the culture vessels.

Table 2.2 Survival of SRU-1 phylloxera and nodosity formation per Phytatray on *V. vinifera* cv. Cabernet Sauvignon in Phytatray trial one after 21 days cocultivation. Each phytatray was inoculated with 100 surface-sterilised eggs.

unhatched eggs (±SD)	dead phylloxera (±SD)	surviving phylloxera (±SD)	feeding phylloxera[1] (±SD)	galls initiated (±SD)	phylloxera not accounted for[2] (±SD)
28.57 ± 13.41	9.00 ± 7.51	1.57 ± 1.51	1.57 ± 1.51	1.28 ± 1.50	60.57 ± 14.28

[1]Number of feeding phylloxera is also included in the number of surviving phylloxera. Survivors are not always feeding hence the separation of the parameters.

[2]More than 50% of the phylloxera introduced could not subsequently be located in the culture vessel. In one case a crawler was observed on the outside of the vessel, indicating that insects may have escaped after hatching.

Phytatray™ trial two

Three thousand eggs were harvested from leaf galls of an SRU-1 phylloxera population originally collected from Schwarzmann leaf galls at Campbell's vineyard, Rutherglen, and maintained on Schwarzmann vines grown in a shadehouse at Agriculture Victoria, Rutherglen. Eggs were harvested in batches of 300, sterilised using egg sterilisation method two (section 2.4.8) and vines inoculated, using an autoclaved artist's brush, within 30 minutes of harvesting eggs. Sterilisation and vine inoculation were carried out in a new, certified laminar flow cabinet. Ten replicates of each vine variety were inoculated with 100 eggs each. For replicates one to eight, eggs were placed directly onto the roots of the plants as for Phytatray trial one. Replicates nine and ten were infested by placing the nylon filter on which the eggs were collected after rinsing, directly into the phytatray adjacent to the vine roots.

Phytatrays were arranged on one shelf in the culture room in a random block design. Vines were maintained at 23°C for 21 days. After this period fungal contamination was assessed. 1/10 *V. vinifera* cv. Cabernet Sauvignon, 3/10 140-Ruggeri and 2/10 *V. rotundifolia* were contaminated. Infestation parameters of phylloxera on all vine types were also recorded and are given in tables 2.3 and in Chapter 6, table 6.1.

Only the *V. vinifera* data is presented in table 2.3 in order to illustrate the maximum potential survival and gall initiation rates using this method. See Chapter 6, section 6.2 for a discussion of the results of this trial in terms of assessing the relative resistance of these varieties.

Table 2.3 Survival of SRU-1 phylloxera and nodosity formation on *V. vinifera* cv. Cabernet Sauvignon in Phytatray trial two after 21 days cocultivation. Each Phytatray was inoculated with 100 surface-sterilised eggs. Replicates nine and ten were infested using whole sterilisation filters.

rep.	unhatched eggs	dead phylloxera	surviving phylloxera	feeding phylloxera	galls initiated	phylloxera not accounted for
1	1	17	6	6	6	76
2[1]	-	-	-	-	-	-
3	0	5	2	1	2	93
4	5	26	2	1	1	67
5	0	33	1	0	0	66
6	0	15	2	1	2	83
7	0	53	15	2	5	32
8	2	14	0	0	0	84
9	8	28+17[2]	0	0	0	47
10	9	21+16[2]	0	0	0	54
mean ±SD	2.77 ± 3.63	27.22 ± 15.94	3.11 ± 4.83	1.22 ± 1.92	1.78 ± 2.28	66.89 ± 19.68

[1] Too contaminated to score.
[2] In replicates 9 and 10 dead crawlers were counted as those on roots (first number) and those still on sterilisation filter (second number) so that the actual number which may have potentially been in contact with the roots is indicated.

Discussion of Phytatray™ trials

Contamination was reduced in Phytatray trial two over trial one. For trial one, no reliable laminar flow cabinet was available for inoculating the vines. Using egg sterilisation method one, inoculation takes at least 30 minutes for each vine and exposure to a non-sterile environment was sufficient to cause a high degree of microbial contamination. This problem was largely remedied before trial two by the purchase of a new laminar flow cabinet. As all vines were uncontaminated prior to the trial, the remaining contamination must occur in the infestation process during which the roots are repeatedly touched with the brush used to apply the eggs, although the brush was sterilised by autoclaving prior to use.

The percent egg hatch in trial one was estimated by counting unhatched eggs. Difficulty in locating unhatched eggs means that percent egg hatch (75-80%) is probably over-estimated. In trial two, percent egg hatch was assessed accurately in replicates nine and ten as approximately 95%. Lower hatch rate in trial one was probably caused by the eggs drying out during the extended period between harvesting and sterilisation (up to 48 hours).

The mortality rate of crawlers (first instar) was high in both trials, ranging from 25-36% in those cultures where fungal contamination was not a factor. Most dead crawlers appeared to have drowned in condensation collected on the walls and in the bottom of the phytatrays. It

was necessary to resolve this problem before attempting further tissue culture cocultivations in order to obtain a high enough survival rate of crawlers to provide meaningful infestation data.

Of the 100 eggs introduced to each phytatray, many phylloxera could not be located at the end of the cocultivation period. It is possible that a proportion of these actually escaped from the phytatrays by crawling up the walls, and out through imperfectly sealed lids. It may also be partly due to difficulty in locating both dead crawlers and unhatched eggs. These can become transparent, making them very difficult to see even using a dissecting microscope. It was not possible to enhance the visibility of dead crawlers, but unhatched eggs were made easily detectable by placing the sterilisation filter into the culture vessel. When removed at the end of the cocultivation period, unhatched eggs which remain on the filter are easily counted. This practice did not increase the level of contamination, and was henceforth adopted for all inoculation of micropropagated vines. Despite this, at least 50% of insects in cultures were still not located (see phytatray trial two, replicates nine and ten). The use of phytatrays was discontinued because of the likelihood that these vessels allowed crawlers to escape, and because of problems with excess condensation.

2.4.4 Perlite-based medium cultures

Because of the problem with crawlers drowning in condensation in the phytatray trials discussed above, several methods were investigated for controlling the accumulation of condensation in tissue culture vessels containing agar-based medium. These included improved ventilation of vessels, improved temperature control in tissue culture cabinets, including a 24 hour photoperiod to minimise temperature fluctuations, and spreading a thin layer of plaster of Paris on the surface of the medium to absorb excess moisture. None proved effective owing to the very high moisture content of the medium itself. Consequently, it was decided to investigate the use of a perlite-based medium which had previously been successfully used for maintenance of phylloxera populations in tissue culture at Agriculture Victoria, Rutherglen.

The infestation parameters (hatch, survival, feeding site initiation rates) were unknown for this system, and it had not yet been tested as a cocultivation method for screening rootstock resistance nor for determining biotype differences between phylloxera strains.

The medium consisted of 7g of Perlite, autoclaved 40 min. at 121°C, mixed with 30ml 1/2 MS, 2% (w/v) sucrose, 0.4% (w/v) Bactoagar, in 250ml polycarbonate jars, with Breather Caps™ (Sarstedt), which were then re-autoclaved for 20 min., 121°C. This resulted in a relatively dry, loose culture medium. Established plantlets were transferred to this medium

with roots intact and after two to three weeks ten replicates of each variety were inoculated with 100 surface-sterilised phylloxera eggs each, as described in egg sterilisation method two (section 2.4.8).

After 30 days cocultivation (e.g. figure 2.3b), plants were uprooted gently from the medium and the roots spread out. Nodosities and other feeding sites and phylloxera were counted (table 2.4) and photographed. Egg hatch rate was approximately 87%, and phylloxera of all developmental stages were present. On *V. vinifera*, feeding sites other than nodosities included the base of the stem, or splits along it, splits in larger roots, and in one case, a stem gall. It was not possible to accurately assess the number of eggs produced, as egg masses may have been dislodged during uprooting and remained hidden in the culture medium. Feeding phylloxera were generally well attached to the root so their numbers could be relatively accurately assessed. Once the roots had been assessed, infested roots (nodosities), uninfested roots from infested plants, and roots from uninfested plants were removed and snap frozen in liquid nitrogen or fixed in 3% (v/v) glutaraldehyde (Appendix three) for microscopy.

Table 2.4 Survival of VWL-1 phylloxera life stages and nodosity formation per plant on *V. vinifera* cv. Shiraz in perlite-based medium cultures. Each plantlet was inoculated with 100 surface-sterilised eggs.

		mean no. of live phylloxera at each developmental stage after 30 days cocultivation						
eggs hatched (±SD)	**nodosities formed (±SD)**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults[1]**	**nymphs with wing buds**	**total no. survivors (±SD)**
86.63 ± 5.93	4.43 ± 3.87	2.29	2.29	3.14	0.71	6.43	2.28	17.14 ± 7.51

[1] Egg laying adults were counted separately from other 4th instar insects.

Plants with well established root systems can be successfully transplanted into perlite-based medium, and indeed thrive under these conditions. Phylloxera are able to move freely through the medium, avoiding the condensation which collects only on the walls of the culture vessel. The result is a high survival rate of phylloxera combined with healthy vines, making it an ideal cocultivation system. Because this system provides suitable conditions for the survival of phylloxera (approximately 17% survival in a compatible interaction), it provides the most satisfactory means of assessing the resistance of primary roots to the phylloxera strains used.

The major disadvantage of this method is that it is not possible to observe the behaviour of the phylloxera and the development of nodosities during the cocultivation period. Nor is it possible to measure fecundity, an important parameter of other bioassay systems (Granett *et al.* 1983, 1985, Grzegorczyk and Walker 1998).

This assay is destructive in that the vines must be uprooted to count feeding sites and cannot be replaced without further disruption of the phylloxera population. As cocultivation is stopped at the time of assessment, it is important to select this time carefully. This would ideally be before the second generation has hatched, as this can confuse the results obtained. The generation time depends on both the susceptibility of the grapevine variety and biotype of the phylloxera, generally being the shortest in the most compatible interaction.

While limited in some respects, 'perlite cocultivations' remain the most reliable way of determining infestation parameters with primary roots of grapevines, while eliminating all unwanted variables. The relatively high survival rate of the insects in these cultures also make them a good source of infested, or at least 'challenged' roots for further analysis. Root material collected from these cultures has been the basis of many of the analyses described in Chapters 4, 5 and 6.

2.4.5 Hairy root cultures

In an attempt to overcome some of the limitations of the perlite-based medium cultures, the established method of excised root bioassays using secondary roots (Granett *et al.* 1983) was modified for roots from micropropagated vines. Previous work by Askani and Beiderbeck (1991) and Martinez-Peniche (1994) described conditions for cocultivation of phylloxera with isolated roots in culture. Various studies had shown that it was necessary to transform roots with *Agrobacterium rhizogenes* in order to achieve growth of these roots following excision from the plant (Mugnier 1988, Gribaudo and Schubert 1990, Guellec *et al.* 1990, Askani and Beiderbeck 1991, Nakano *et al.* 1994).

In order to establish a cocultivation system similar to those described in published reports, a transformation system was initiated for two grapevine varieties, *V. vinifera* cv. Cabernet Sauvignon and Schwarzmann accession WA/A.S.74.2257, using two *A. rhizogenes* strains, A4 and NCIB 8196 (K568 from the Clare Collection, Department of Environmental and Molecular Ecology, The University of Adelaide). Strain K599 from the Clare Collection was also tested in a preliminary trial (data not shown), but resulted only in a necrotic response on *V. vinifera* cv. Cabernet Sauvignon.

A. rhizogenes was cultured at 28°C on YEP 30 medium (1% (w/v) yeast extract, 1% (w/v) peptone, 0.5% (w/v) NaCl, 1.5% (w/v) agar). Grapevines were micropropagated as

described in section 2.4.2. Shoots with five to ten nodes were excised from plantlets. Leaves or lateral shoots were trimmed from the lower nodes, leaving them on the apical three to four nodes only. The shoots were inoculated by wounding the stem in each internode with a scalpel blade dipped in a colony of *A. rhizogenes*. Each variety was inoculated with each strain of *A. rhizogenes*, with ten replicates per treatment. Control shoots were wounded in a similar manner with a sterile scalpel blade (a disarmed strain of *A. rhizogenes* was not available). Cocultivation was under the same conditions used for micropropagation of grapevines, with two shoots in each 250ml vessel.

Ten days following inoculation, shoots were transferred to 1/2 MS medium containing 250mg/L cefotaxime (Claforan™, Faulding Roussel) to control *A. rhizogenes* growth. This transfer was repeated fortnightly for six weeks, during which time the response of the vines was recorded (table 2.5).

Potentially transformed hairy roots emerging from wound sites of plantlets inoculated with *A. rhizogenes* strain A4 were excised and transferred to root growth medium 'RGM' (R. Heath, Agriculture Victoria, Plant Sciences and Biotechnology, La Trobe University, Victoria, pers. comm.) (half strength Gamborg B5 nutrients (Sigma), 2% (w/v) sucrose, pH 5.6, 1.5% (w/v) agar) with 250mg/L cefotaxime. These were incubated at 25°C in the dark for ten days, at which time growth was assessed. Non-transformed roots excised from uninoculated plantlets were also transferred to the same medium and growth after ten days was assessed.

There was no significant difference between the growth rate for potentially transformed, and non-transformed roots, the average being 1.15mm/day. This finding is contradictory to results reported previously (Mugnier 1988, Gribaudo and Schubert 1990, Guellec *et al.* 1990, Askani and Beiderbeck 1991, Nakano *et al.* 1994) but may be due to the choice of *Agrobacterium* strains. Strain 15834 is reportedly most suitable for grapevine transformation (Gribaudo and Schubert 1990, Guellec *et al.* 1990, Askani and Beiderbeck 1991) and should be trialed in future experiments. (For this study strain 15834 was obtained from the American Type Culture Collection, but for unknown reasons, could not be revived from the freeze-dried culture and hence was not included in the trial.) The finding that non-transformed excised roots survive in culture led to further development of the excised root culture method.

Table 2.5 Response of *V. vinifera* cv. Cabernet Sauvignon or Schwarzmann vines to wounding (control) or inoculation with one of two strains of *A. rhizogenes*.

	Response of vines over six weeks following inoculation				
Vine type/ *A. rhizogenes* strain	**week 2**	**week 3**	**week 4**	**week 5**	**week 6**
***V. vinifera* /control**	4/10 shoots dead; 6/10 showing no response	transfer to cef. medium	8/10 shoots dead; 2/10 producing anthocyanins at wound site	transfer to fresh cef. medium	all shoots dead or dying
***V. vinifera* /strain K568**	pinkish bacterial overgrowth on all shoots	transfer to cef. medium	anthocyanins and callus forming on all internodes of all shoots	transfer to fresh cef. medium	as for week 4
***V. vinifera* /strain A4**	6/10 shoots showing no response; 4/10 beginning to form callus at basal internodes	transfer to cef. medium	callus forming on all shoots at all internodes; 4/10 shoots with roots at basal internodes	transfer to fresh cef. medium	9/10 shoots dying, but continuing production of new roots from callus on internodes
Schwarzmann /control	6/10 shoots showing no response; 4/10 dead at shoot tip	transfer to cef. medium	9/10 showing no response; 1/10 with slight necrosis and swelling at wound site	transfer to fresh cef. medium	all shoots growing healthily with no further signs of response to wounding
Schwarzmann /strain K568	all shoots showing white or pinkish bacterial overgrowth	transfer to cef. medium	all shoots showing slight necrosis and callusing at wound sites	transfer to fresh cef. medium	as for week 4
Schwarzmann /strain A4	5/10 shoots showing no response; 5/10 with slight necrosis at wound sites	transfer to cef. medium	all shoots forming callus at wound sites; 3/10 with roots at basal internodes	transfer to fresh cef. medium	as for week 4

2.4.6 Excised root cultures

The use of non-transformed excised root cultures for cocultivations of phylloxera with primary roots would provide useful parallels to existing techniques of excised secondary root cocultivations. This technique would also have the advantage of readily allowing observation of the settling and feeding behaviour of the phylloxera.

Excised root culture trial one

The purpose of this trial was to test the survival and growth of excised roots in culture, and determine whether they would be suitable for use in cocultivations with phylloxera. Grapevine varieties tested for excised root culture were *V. vinifera* cv. Shiraz accession PT 23/A.N.61.0020, Schwarzmann accession WA/A.S.74.2257, *V. riparia*, Ramsey accession A11V2/I.V.63.2065, Börner, and *V. rotundifolia.*

The root culture medium (RGM) was poured into sterile 90×14mm petri dishes (Sarstedt), and when set, half the plates were overlaid with sterile filter paper (Whatman No. 1). The presence of filter paper was tested here for adverse effects on root growth, as it was planned to use it to provide a protective layer between the phylloxera and the culture medium in cocultivations.

Roots 4-5cm in length, 1-2mm diameter, with a healthy growing tip, were excised from micropropagated vines. The cut end was inserted into the medium, through a hole in the filter paper where necessary. Three roots were placed into each petri dish, and the dishes sealed with Glad Wrap™ (figure 2.3c). Three dishes of each variety were established for each treatment (a total of nine roots, in three replicates, per treatment). Cultures were incubated at 26°C in the dark for six weeks. Root length was visually assessed weekly for three weeks, and survival of the whole root was assessed after six weeks (table 2.6).

While the excised roots of most varieties had stopped growing by six weeks, most were still alive at week three and around 50% of roots of some varieties still remained alive along the length of the root (although the root cap was often brown) at week six.

An analysis of variance (Zar 1984) showed no significant difference between survival on media with or without filter paper. There were, however, significant differences in survival between varieties, shown in table 2.6.

Excised root culture trial two - cocultivation trial

Following trial one, another trial was carried out to test the practicality of this method for cocultivation. This was done in order to determine survival rates of phylloxera, establishment of feeding sites and the feasibility of observing phylloxera behaviour.

Roots were cultured as for excised root trial one, with four roots per petri dish, three petri dishes per variety. Because filter paper did not adversely affect survival of the roots, it was used in all dishes. VWL-1 phylloxera, originally isolated from Brown Brother's Whitlands vineyard, King Valley and bulked up on excised secondary roots of *V. vinifera,* was used as inoculum. Immediately after initiating excised root cultures, ten phylloxera eggs (surface-

sterilised using egg sterilisation method two) were placed on each root. Over a 25 day period phylloxera behaviour and root response to phylloxera feeding were observed, and infestation

Table 2.6 Growth and survival of excised roots in culture.

	without filter paper						with filter paper				
	mean no. growing per petri dish[1]			mean no. alive[2]			mean no. growing per petri dish[1]			mean no. alive[2]	
	week 1	week 2	week 3	week 0	week 6		week 1	week 2	week 3	week 0	week 6
V. vinifera[a]	3.00	1.33	0.33	3.00	2.00		2.66	1.66	2.66	3.00	2.33
Ramsey[c]	2.00	1.33	0.66	3.00	0.66		2.66	2.66	1.66	3.00	1.66
Schwarzmann[a]	2.33	1.33	0.00	3.00	2.00		1.33	2.00	0.33	3.00	1.33
V. riparia[c]	2.00	0.00	0.00	3.00	0.00		3.00	0.00	0.00	3.00	1.66
Börner[a]	2.00	0.00	0.66	3.00	3.00		2.66	1.00	0.33	3.00	1.33
V. rotundifolia[b]	0.80	0.33	0.33	3.00	1.33		2.00	1.33	1.00	3.00	2.00

[1] Continued growth was determined by visual assessment of root length over time.
[2] Roots which had become dark brown for most or all of their length were no longer considered alive.
[a, b, c] Indicates groups of significantly similar root survival rates (p=0.03). a: >50%; b: 50%; c: <50%.

parameters determined. Data for *V. vinifera* cv. Shiraz are presented in table 2.7, in order to illustrate the maximum potential survival and gall initiation rates with this method. See Chapter 5, table 5.2 and Appendix four for discussion of this trial in terms of assessing the relative resistance of these varieties and phylloxera behaviour on each.

The survival of phylloxera on excised roots was low (9.2%). This was due in part to the low hatch rate and in part to the tendency of phylloxera crawlers to move off the roots and become trapped in condensation on the lid of the petri dish, or in a film of water between the root and the filter paper. Crawlers trapped in condensation were not counted in the assay. The number of eggs hatched could only be determined by counting hatched crawlers on or near roots as unhatched eggs were difficult to locate. Egg hatch data presented for excised root cocultivations are therefore not accurate.

Once hatched, crawlers were observed moving along the length of the roots. Typical behavioural observations included the initial preference of phylloxera for root tips, with a subsequent tendency to settle behind the tip; apparently aimless wandering without probing on some varieties. See Chapter 5, section 5.2.2 for illustration and discussion of these observations.

Table 2.7 Survival of VWL-1 phylloxera life stages and nodosity formation on *V. vinifera* excised root cultures. Twelve roots were each inoculated with ten surface-sterilised eggs.

		mean no. of live phylloxera at each developmental stage after 25 days cocultivation					
eggs hatched (±SD)	**nodosities formed (±SD)**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults[1]**	**total no. survivors (±SD)**
2.83 ± 2.29	0.25 ± 0.45	0.00	0.25	0.00	0.25	0.33	0.83 ± 1.64

[1] Egg laying adults were counted separately from other fourth instar insects.

Discussion of excised root culture trials

Excised root cultures have the major advantage of enabling easy observation of phylloxera behaviour and feeding and, given a high enough survival rate, the assessment of fecundity. Behavioural studies can give important clues as to the nature of the interaction and possibly also the mechanisms of resistance (e.g. deterrence to feeding, necrotic response to feeding, lack of gall initiation).

The assumption was made that survival of most of the length of the root for the period of a bioassay (usually 21-25 days) would be sufficient for this system to be suitable for cocultivations with phylloxera. This should enable phylloxera to proceed through one generation, since on excised secondary roots, phylloxera may develop to adulthood within 16 to 28 days (Granett *et al.* 1983). In trial two, the survival rate was actually too low to determine if this was the case. This appeared to be mainly due to problems with excess moisture; similar problems were reported by Askani and Beiderbeck (1991).

Because of the relatively low survival of phylloxera, these cultures were not considered an appropriate method for assessment of rootstock resistance or phylloxera biotype. They were, however, very useful for observations of feeding behaviour, and as such provided insight into possible resistance mechanisms. In this respect, excised root cultures can be used to complement the data obtained from perlite-based medium cocultivations.

2.4.7 Callus cultures

Phylloxera have been observed to feed on callus produced on secondary root pieces of some grapevine varieties in excised root bioassays (Granett 1990, Corrie *et al.* unpublished). This callus forms in response to root excision, and not in response to phylloxera feeding. The development of a system in which phylloxera could feed on cultured callus would make it

possible to assay the resistance of non-differentiated tissue. Any observed differences in phylloxera feeding preference or fecundity could therefore be ascribed to differences in chemical composition. If the same, or similar responses to phylloxera feeding were observed on cultured callus as on roots of the same variety, it could be deduced that biochemical, rather than physical characteristics are most important in determining the nature of the interaction of phylloxera with roots.

Callus cultures have been derived from a number of tissues of grapevine, including leaf, stem, petiole and berry (Hawker *et al.* 1973, Benson and Roubelakis-Angelakis 1994, Perl *et al.* 1995, Feucht *et al.* 1996, Harding *et al.* 1996, Sefc *et al.* 1997), but not from root. Root-derived callus was assumed to be most suitable for cocultivation trials here. Callus growing on excised secondary roots is not sterile and therefore not suitable for continuous culture.

The aim of this trial was to establish a method for generating callus from root tissue of a range of vine varieties, and test cocultivation of phylloxera with callus cultures. This involved two successive trials using various media types. Callus generated from trial two was used in a third trial for cocultivation with phylloxera.

Callus culture trial one

Four tissue types (leaf, stem, petiole and root) from four vine varieties of tissue cultured grapevine (*V. vinifera* cv. Shiraz accession PT23, Schwarzmann accession WA-AS.74.2257, Börner and *V. rotundifolia*) were used in an attempt to generate callus cultures.

Callus culture initiating medium consisted of MS nutrients (Murashige and Skoog 1962), 2% (w/v) sucrose, 0.2% (w/v) Phytagel®, supplemented with auxin (NAA or NOA) and/or cytokinin (BAP) as listed in table 2.8, based on recommendations from other researchers.

Table 2.8 Callus culture media types used in callus culture trial one.

callus culture medium type	final hormone concentrations	recommendation
medium 1	5.4µM NAA	Dai *et al.* 1995c
medium 2	10µM NAA, 2µM BAP	Modification of medium 3
medium 3	5µM NAA, 2µM BAP	Benson and Roubelakis-Angelakis 1994
medium 4	20µM NOA, 40µM BAP	T. Franks, CSIRO Plant Industry, pers. comm.
medium 5	5µM NOA, 1µM BAP	T. Franks, CSIRO Plant Industry, pers. comm.

Five to seven tissue pieces approximately 0.5cm long (0.5cm^2 for leaf tissue) were placed on media in petri dishes which were sealed with Glad Wrap™, and incubated at 26°C in the dark. After three weeks, any adventitious roots (short roots with a proliferation of root hairs) were removed and healthy callus was subcultured onto similar media types, and incubated under the same conditions. Callus growth was assessed over seven weeks (table 2.9).

Summary of results:

- *Callus culture medium 1*: Adventitious roots were produced on all grapevine varieties. There was little development of callus, so tissue on this medium was not subcultured at week three.
- *Callus culture medium 2*: All grapevine varieties produced healthy callus from leaves, stems and petioles on this medium except *V. rotundifolia*. However, there was only a moderate amount of callus generated from root tissue of any variety, and none from *V. rotundifolia*.
- *Callus culture medium 3:* Healthy callus developed from most tissues of most grapevine varieties. There was development of some adventitious roots on *V. vinifera*, but these were removed at week three and did not re-develop.
- *Callus culture medium 4:* Very little callus developed on the tissue from any grapevine varieties so tissue on this medium was not subcultured at week three.
- *Callus culture medium 5*: Callus developed on all grapevine varieties except *V. rotundifolia*. This callus was slower growing, and often more brown than on medium 2 or 3, and there was also development of adventitious roots on most varieties.

Callus culture medium 3 was optimal for production of callus from most tissue types, especially roots. Root tissue proved the most difficult from which to establish callus, with the highest occurrence of adventitious roots. A higher concentration of BAP than NOA or NAA prevented callus formation (medium 4), while a lower concentration of BAP relative to NAA (media 1 and 2 compared with medium 3) encouraged development of adventitious roots. Most published methods for callus development use stem or petiole as explant (Benson and Roubelakis-Angelakis 1994, Dai *et al.* 1995c), and the ease of establishing callus from these tissues was reflected in the results here. In the case of these tissues there was little difference in this trial between callus culture media 1, 2 or 3.

Table 2.9 Initiation and growth of callus from four tissue types, on five media, over seven weeks.

A: *V. vinifera* cv. Shiraz

		medium 1		medium 2		medium 3		medium 4		medium 5	
	week	a	b	a	b	a	b	a	b	a	b
Root	3[1]	0	ar	++	ar	++		++		++	
	5	n/a[2]		++		+		n/a		-	
	7	n/a		++		+		n/a		-	br/ ar
Leaf	3	0	ar	++		++	ar	++		++	
	5	n/a		++		++		n/a		-	
	7	n/a		++	ar	++	br	n/a		-	br
Stem	3	++	ar	+++		+++		+		++	ar
	5	n/a		+++		+++		n/a		+	br
				+		+					
	7	n/a		+++		+++		n/a		+	br
				+		+					
Petiole	3	0	ar	+++		+++		-		++	
	5	n/a		+++		+++		n/a		+	
				+		+					
	7	n/a		+++		+++		n/a		+	br
				+		+					

[1]After three weeks callus was subcultured. Replicates of medium three callus were subbed onto medium where the auxin napthoxy-acetic acid (NOA) was substituted for napthalene acetic acid (NAA) and replicates from medium five vice versa to determine which auxin was optimal. There was no significant difference between auxin types, so these results have not been included.

[2]Media one and four were not subcultured at week three, due to lack of healthy callus growth.

B: Schwarzmann

		medium 1		medium 2		medium 3		medium 4		medium 5	
	week	a	b	a	b	a	b	a	b	a	b
Root	3	+	ar	-		+		-		+	ar
	5	n/a		+	an	++		n/a		+	
	7	n/a		+++	an	++	ar	n/a		++	ar
Leaf	3	+	ne	+	ne	+		0	ne	-	
	5	n/a		++		++		n/a		n/a	
	7	n/a		+++		++	ar	n/a		n/a	
Stem	3	+	ar	++		++		-		+	
	5	n/a		+++		++		n/a		+	br
	7	n/a		+++		+++		n/a		++	
Petiole	3	-		++		++		0		++	
	5	n/a		+++		++		n/a		+	
	7	n/a		++		++		n/a		++	

Legend:

column a - callus growth:

0	none
-	negligible
+	little
++	moderate
+++	good
++++	very rapid

column b - visual characteristics:

ar	adventitious roots
ne	necrotic
br	browning
ye	yellowing
an	anthocyanins

Table 2.9 Initiation and growth of callus from four tissue types, on five media, over seven weeks - continued.

C: Börner

		medium 1		medium 2		medium 3		medium 4		medium 5	
	week	a	b	a	b	a	b	a	b	a	b
Root	3	+	ar	+	ar	++		+		+	ar
	5	n/a		++		++		n/a		++	
	7	n/a		++	an	++		n/a		++	
Leaf	3	+	ar	+++		++		+	ye	++	
	5	n/a		++		++		n/a		+++	
	7	n/a		+++	an	+++		n/a		++	ar
Stem	3	++		++		+++		-		+++	
	5	n/a		++	an	++		n/a		+++	
	7	n/a		+++		++		n/a		+++	
Petiole	3	++		++		+++		+		+++	
	5	n/a		++		++		n/a		+++	
	7	n/a		+++		++		n/a		+++	

D: *V. rotundifolia*

		medium 1		medium 2		medium 3		medium 4		medium 5	
	week	a	b	a	b	a	b	a	b	a	b
Root	3	+	ar	+		+		-	ne	-	ne
	5	n/a		-	br	-	br	n/a		n/a	
	7	n/a		-	br	-	br	n/a		n/a	
Leaf	3	-	br	+	br	+	br	-	br	-	br
	5	n/a		-	br	-	br	n/a		n/a	
	7	n/a		-	br	+	br	n/a		n/a	
Stem	3	-	br	+		+		-		+	br
	5	n/a		-	br	+		n/a		+	br
	7	n/a		+	br	++	br	n/a		++	br
Petiole	3	+	br	+		+		+		+	br
	5	n/a		-	br	+		n/a		+	
	7	n/a		+	br	++		n/a		++	

Legend:

column a - callus growth:

-	negligible
+	little
++	moderate
+++	good
++++	very rapid

column b - visual characteristics:

ar	adventitious roots
ne	necrotic
br	browning
ye	yellowing
an	anthocyanins

Callus culture trial two

Based on the results of trial one, the use of callus culture medium 3 was further investigated for development of callus from roots. These trials included some modifications of this medium. Casein hydrolysate was added in to optimise the growth rate. 1% (w/v) agar was used as gelling agent to produce more solid callus. 2,4-D and activated charcoal were added in to reduce the browning seen with some varieties, particularly *V. rotundifolia*. Root

segments 0.5cm long of the four micropropagated grapevine varieties were placed on medium consisting of MS salts, 2% (w/v) sucrose, pH 5.6, together with supplements listed in table 2.10.

Table 2.10 Callus culture media used in callus culture trial two.

callus culture medium type	hormone concentrations	other additives	recommendation/ modification
medium 3	5µM NAA, 2µM BAP	0.2% (w/v)Phytagel®	Benson and Roubelakis-Angelakis 1994
medium 6	5µM NAA, 2µM BAP	1% (w/v) agar	Modification of medium 3
medium 7	5µM NAA, 2µM BAP	1% (w/v) agar, 0.25% (w/v) activated charcoal	Modification of medium 3
medium 8	5µM NAA, 2µM BAP	1% (w/v) agar, 0.5% (w/v) casein hydrolysate	Modification of medium 3
medium 9	5µM 2,4-D, 2µM BAP	1% (w/v) agar	Nakano *et al.* 1994
medium 10	10µM 2,4-D, 2µM BAP	1% (w/v) agar	Choi *et al.* 1995, Perl *et al.* 1995

Root callus growth on the five media types was assessed over five weeks (table 2.11) with the best callus obtained using callus culture medium 3. The callus generated was relatively wet and friable, but healthy with rapid growth except that from *V. rotundifolia* which had a very slow growth rate. Phytagel® was the most suitable solidifying agent.

Callus culture trial three - cocultivation trial

A cocultivation trial was set up to determine the potential for phylloxera to survive on root-callus cultures. Four healthy clumps of callus (approximately 7mm diameter) from each of three varieties (*V. vinifera* cv. Shiraz, Schwarzmann and Börner) were cultured in petri dishes, then inoculated with ten phylloxera eggs (surface-sterilised using sterilisation method two, section 2.4.8) per clump. All eggs hatched after three days (figure 2.3d), but crawlers died without reaching the second instar. Phylloxera did not move off the callus, but it was not possible to determine whether they attempted to feed.

Table 2.11 Initiation and growth of callus from root tissue of four vine varieties, on six media types, over five weeks.

		medium 3		medium 6		medium 7		medium 8		medium 9		medium 10	
	Week	a	b	a	b	a	b	a	b	a	b	a	b
V. vinifera cv. Shiraz	2	++		+		0		++		+		+	
	3	++		+		0		+	br	+		+	
	4	++ +		+		0		+	br	+		+	
	5	++ +		+	ar	0		-	br	+	ye	-	
Schwarz.	2	++		++		0		+		++		++	
	3	++		++		0		++		++		+	
	4	++	ar	++		0		++		++		+	
	5	++	ar	+		0		+		-	ye	-	ye
Börner	2	+		++		0		++		+		+	
	3	++		++		0		++		+		+	
	4	++		++		0		++		+	ye	+	ye
	5	++		+		0		+		-	ye	-	ye
V. rotund.	2	+		+		0		++		-	br	-	br
	3	+		+	br	0		+	br	-	br	-	br
	4	+		-	br	0		-	br	-	br	-	br
	5	-		-	br	0		-	br	-	br	-	br

Legend:

column a - callus growth:

0	none
-	negligible
+	little
++	moderate
+++	good
++++	very rapid

column b - visual characteristics:

ar	adventitious roots
ne	necrotic
br	browning
ye	yellowing
an	anthocyanins

Discussion of callus culture trials

Phylloxera were not able to survive on root-derived callus under the conditions described here. This is in contrast to the observation that phylloxera are able to maintain high reproductive rates on callus produced on excised secondary roots in bioassays (Granett *et al.*1987, Corrie *et al.* unpublished). In the latter case, firmer callus may enable the phylloxera to establish a feeding site. Excised secondary root pieces would also have a store of nutrients which could be actively mobilised into the feeding site upon establishment of a sink by the phylloxera. On cultured callus, however, phylloxera may be dependent upon passive diffusion of nutrients from the growth medium which may not be sufficient for their survival. It is also likely that the relatively wet state of the callus contributed to a high death rate, as phylloxera appeared to become trapped in pools of moisture on the callus surface. This method of cocultivation might be improved if firmer, drier callus could be generated. Pelet *et al.* (1960) successfully used callus cultures derived from leaf gall tissue for temporary cocultivations with phylloxera. Unfortunately this would not be useful for screening root resistance.

2.4.8 Phylloxera egg sterilisation

Considerable numbers of surface-sterilised eggs are required for tissue culture cocultivations when comparing several varieties of grapevine. 'Egg sterilisation method one' was routinely used at Agriculture Victoria, Rutherglen, but a more efficient method was required, leading to the development of 'egg sterilisation method two' described below.

Egg sterilisation method one

Working in a laminar flow cabinet, eggs were counted and placed into a sterile metal sieve, 10cm diameter, 60µm pore size. The eggs were rinsed several times with 70% (v/v) ethanol, then several times with sterile distilled water. Individual eggs were then collected under a dissecting microscope with a fine artist's brush, and placed directly onto the roots or stem of the vine to be infested. Percent hatch and degree of contamination were not routinely determined.

Egg sterilisation method two

One hundred eggs were counted and placed into a petri dish. These eggs were then rinsed into a Millipore filtration apparatus (Glass microanalysis system with stainless steel support (Millipore catalogue no. XX10 025 30), fitted with a 25mm diameter nylon net filter, 60µm pore size (Millipore catalogue no. NY60 025 00)) using 70% (v/v) ethanol from a wash bottle. The ethanol was drawn through the filter using a hand vacuum pump (Millipore catalogue no. XKEM 001 07). The eggs were rinsed several times with 70% (v/v) ethanol, ensuring gentle agitation with each rinse (around one minute total exposure to ethanol), and the eggs were then rinsed again several times with sterile water. On the final rinse all eggs were rinsed from the walls of the filtering apparatus onto the net filter, and surface dried using the vacuum pump. The nylon net filter was then removed from the apparatus, and gently placed into the culture vessel adjacent to the micropropagated vine.

From the collection of eggs onwards the entire procedure was carried out in a laminar flow cabinet to prevent contamination. At the end of the period of cocultivation, the filter was carefully removed from the culture vessel, and the hatch rate determined by counting the number of unhatched eggs. In a trial with six treatments, each containing ten replicates, the mean percentage hatch rate was 85.77 ± 3.01.

Discussion of egg sterilisation methods

Egg sterilisation method one was a very labour intensive process, and a cocultivation consisting of more than a few replicates in each treatment would take several days for one person to set up. Published sterilisation methods (Askani and Beiderbeck 1991,

Grzegorczyk and Walker 1997), provide more effective means of collecting eggs, but still require the use of a brush to transfer the eggs one or a few at a time onto the roots of the plant.

In phytatray culture trial two (section 2.4.3), egg sterilisation method two was used, and replicates nine and ten were inoculated by placing the sterilisation filter into the culture vessel. Thirty cocultivation replicates with 100 eggs each were set up in one day, demonstrating the practicality of this method. 70% (v/v) aqueous ethanol is suitable for surface sterilisation, as it effectively eliminates microbes in a relatively short time, while not causing significant mortality of eggs.

Sterilisation method two provides a quick and efficient means of infesting tissue culture plants with a known number of eggs, and has been published as a technical note (Kellow *et al.* 1999).

2.5 Conclusions

In the experiments described in this chapter, a number of different cocultivation systems have been developed and/or assessed.

Potted-vine cocultivations provide a useful method for generating large quantities of infested root material, provided environmental conditions can be maintained which are suitable for phylloxera.

The phytatray cocultivation method is probably not worth pursuing further because of problems with condensation and insect escape, and callus cultures only if a more solid callus can be generated. Perlite-based medium cultures, on the other hand, are ideal for generating infested tissue cultured root material. Phylloxera survival and infestation rates are relatively high and unwanted variables are eliminated. These cultures also provide a suitable system for assessment of the resistance of rootstocks. The cocultivation period required for phylloxera to complete one generation is less than thirty days, and the assay is season independent. The assay should be a relatively sensitive indicator of resistance (but not tolerance). If phylloxera cannot grow on the primary roots of tissue cultured plants, they are less likely to be able to grow on primary or secondary roots of field-grown vines. During the course of this work, a study was published by other researchers in which a similar *in vitro* system to that developed by Forneck *et al.* (1996) was used to screen a number of grapevine varieties for resistance to a mixture of phylloxera biotypes (Grzegorczyk and Walker 1998). This study also confirms the amenability of such a system for assessing rootstock resistance to phylloxera.

Cocultivation of phylloxera with excised root cultures potentially provides all the information which could be generated from an excised secondary root bioassay such as fecundity and establishment of feeding sites, without interference from contaminating secondary pathogens. Survival of phylloxera is, however, relatively low due to the high moisture content in these cultures, which means statistically significant tests are currently not possible. Also, in contrast to the perlite-based medium whole plantlet cocultivations, eggs still need to be collected following sterilisation and manually placed on the excised roots to ensure that phylloxera come into contact with the root (they have great difficulty moving across the moist filter paper). At present, excised root culture cocultivations are most valuable as a system for observing phylloxera behaviour and root response to feeding. Further development removing excess surface moisture would be valuable.

The results of cocultivation trials described in this chapter, and infested root material generated, form the basis of chapters 4,5 and 6.

3. DEVELOPMENT OF METHODS FOR ANALYSIS OF ROOT SAMPLES

3.1 Introduction

Little information is available about grapevine roots and their response to phylloxera feeding. Most published studies of the interaction are old (1950s and 1960s), and therefore were unable to take advantage of recent developments in microscopy and analytical methods such as molecular biology and HPLC. In order to select techniques for this study it was necessary to look further afield, to studies of grapevines with other pathogens, and to studies of other root pest-root interactions.

There are numerous published studies on the interaction of nematodes with plant roots (Wyss *et al.* 1988, Wyss 1992, Goddijn *et al.* 1993, Opperman *et al.* 1994, Böckenhoff *et al.* 1996, Møller *et al.* 1998), however many of these utilise either transgenic plants, or direct observation of the live organisms on a model host such as *Arabidopsis*, neither of which were possible in this study. Standard techniques such as microscopy, histochemistry and northern blot hybridisation which have also been used, were considered suitable for this investigation.

Quarantine regulations limited the range of analytical techniques which could be used. Viable grapevine material or phylloxera could not be removed from the Vine Disease District (Rutherglen) in which cocultivations were carried out. Laboratory facilities suitable for most analytical techniques were not available in Rutherglen therefore, all plant material and phylloxera was rendered inviable either by freezing in liquid nitrogen, or by fixing for microscopy before being transported to a PC2 (physical containment level 2) laboratory at The University of Adelaide. Microscopic and histochemical techniques requiring fresh tissue (such as confocal microscopy, *in situ* enzyme activity assays) were therefore not possible. This chapter describes the development and/or testing of several analytical techniques which were applicable to frozen or fixed tissue.

3.2 Analysis of free phenolic compounds in grapevine roots using HPLC

High Pressure Liquid Chromatography (HPLC) is a powerful tool for separating compounds such as free phenolics, and when coupled with a full-diode-array detector, can be used for their identification. Mass spectrometry can be used in combination with HPLC to help identify unknown compounds.

Grapevine resistance to pathogens has been correlated with rapid accumulation of *trans*-resveratrol and its derivatives, and HPLC has been used to detect and identify these compounds (Stein and Blaich 1985, Dercks and Creasy 1989, Calderón *et al.* 1994, Dai *et al.*

1995a, Feucht *et al.* 1996). HPLC is also suitable for detecting phenolic compounds such as those proposed to be present in galls and associated with resistance of some grapevine varieties to phylloxera (Denisova 1965).

A range of techniques for extracting free phenolics from plant tissues was tested. Previously published methods for extraction of phenolics from grapevine roots (Korhammer *et al.* 1995, Mattivi *et al.* 1996) were not suitable due to the very limited amounts of root or nodosity tissue available (particularly from tissue culture-based cocultivations). HPLC chromatography conditions (solvent gradients etc.) were developed to give the optimal separation and resolution of individual compounds present in the grapevine root extracts using the system available. This section outlines this developmental process and the results of various methods used.

HPLC was subsequently used to compare the free phenolic profile of roots and nodosities of a range of grapevine varieties. Results are presented in sections 4.3.5, 5.3.3, 5.4.2 and 6.4.2.

3.2.1 Trials of methods for extraction of free phenolics from grapevine roots

Different procedures for extraction of free phenolics from grapevine roots were compared in duplicate, using sub-samples of the same tissue (powdered frozen roots from potted *V. vinifera* cv. Shiraz). Extraction was optimised in order to be able to detect the full spectrum of free phenolics present in grapevine roots. Chromatography method four, as described in section 3.2.2 was used for separation of compounds in extracts.

Free phenolic extraction method one

Hot ethanol extraction adapted from Cole (1984).

100mg frozen powdered root tissue was extracted in 1ml 95% (v/v) ethanol at 85°C for 15 min, chilled on ice, and centrifuged for 10 min. The supernatant was filtered through a 0.45µm filter. 200µl extract was diluted to 500µl with solvent A (section 3.2.2) and 300µl of this loaded on the HPLC column.

Free phenolic extraction method two

Hot ethanol extraction followed by Sep Pak® purification, adapted from Cole (1984).

Extraction was as for method one, but the final extract was purified through a Sep Pak® Plus C18 cartridge (Waters) to remove polysaccharides. The Sep Pak® column was primed with 7ml methanol, rinsed with 7ml H_2O, and the sample loaded. Carbohydrates were eluted with H_2O, followed by elution of the sample with 1ml methanol (according to manufacturer's instructions). Sample was diluted and loaded as above.

Free phenolic extraction method three

Methanol extraction of soluble phenolics, adapted from Münzenberger *et al.* (1990).

100mg frozen powdered root tissue was extracted for 1 hr in 4ml 80% (v/v) methanol at RT, and centrifuged. The pellet was re-extracted twice with 2ml 80% (v/v) methanol at RT and the three supernatants pooled. The supernatant was dried under vacuum and resuspended in 500µl 50% (v/v) methanol. The extract was centrifuged, and 100µl diluted to 500µl with solvent A then 300µl of this loaded onto the HPLC column.

Free phenolic extraction method four

Cold methanol extraction and phase partitioning, adapted from Calderón *et al.* (1993).

100mg frozen powdered root tissue was extracted in 4ml 70% (v/v) methanol for 24 hr, at 4°C. The extract was filtered through Whatman filter paper (No. 1) and dried under vacuum. The residue was extracted by phase partitioning for 10 min with ethyl acetate and 3% (w/v) $NaHCO_3$. The organic phase was washed with water, and the ethyl acetate evaporated under vacuum at 45°C. The residue was resuspended in 100µl MeOH. 20µl was diluted to 500µl with solvent A and 300µl loaded onto the column.

Results of extraction method trials

Areas of the seven major peaks obtained using the four extraction methods are given in table 3.1. Extraction method one yielded five major peaks eluting between 25 and 40 min (numbers 3, 4, 5 and 7); also several minor peaks with earlier elution times. Further purification with the Sep Pak® column (method two) reduced the overall yield, resulting in almost no peaks before 25 min, and eliminating peak six. The yield ratio between extraction methods one and two ranges from 5-50% for most peaks, with only peak three being equivalent in area. Extraction method three yielded the same four major peaks as method one, but with only 5-20% of the yield for most of these, again with the exception of peak three. No minor peaks were visible at all. Method four yielded several minor peaks eluting before 25 min, but all except one of the major peaks (number three) shown by method one were greatly reduced. The duplicates of all extraction methods gave quantitatively reproducible yields (data not shown).

Table 3.1 Mean peak area $\times 10^{-5}$ AU (from duplicate root extracts) of the seven major peaks at A_{280nm} or A_{330nm} for four extraction methods.

peak #	extraction method 1	extraction method 2	extraction method 3	extraction method 4
1	8.75	4.81	n	4.03
2	10.75	3.57	1.82	5.42
3	33.31	33.54	29.37	32.34
4	36.47	6.86	2.19	n
5	15.83	2.56	1.59	1.45
6	5.62	n	1.27	n
7	168.42	9.21	6.97	4.03

n: Peak was either not present, or present in negligible quantity.

Based on the number, size and resolution of peaks obtained with these extracts, extraction method one was judged to give the best yield of free phenolic compounds. This was therefore adopted as the method of choice for subsequent HPLC analyses. Extraction of wall-bound phenolics was attempted using the method published by Münzenberger *et al.* (1990). This method proved unsuccessful and was not pursued further.

3.2.2 Trials of HPLC chromatography conditions

Chromatography conditions (solvent gradients) were optimised in order to obtain maximum sensitivity and resolution of the HPLC chromatogram.

The HPLC system consisted of a Beckman System Gold HPLC apparatus, running Beckman System Gold Nouveau software version 1.6. Peak areas were quantified using the 'Custom Report' feature of this software; System Gold 168 diode array detector; System Gold 26 pumps; Beckman 503e autosampler; Vydac C18 reversed phase column 250mm×4.6mm internal diameter or ×2.1mm internal diameter, 5µm pore size and guard columns of the same diameter and matrix. HPLC solvents were: solvent A: 0.1% (v/v) trifluoroacetic acid (TFA) in water; solvent B: 0.085% (v/v) TFA in 80% (v/v) acetonitrile.

Chromatography conditions - trial one

Chromatography conditions were optimised by running sub-samples from the same root extract. Solvent gradients were sequentially adjusted such that the gradient was progressively made more shallow where clusters of peaks were occurring in order to improve their separation. *V. vinifera* cv. Shiraz root extracts were prepared by extraction method one (section 3.2.1). 100µl extract was diluted to 500µl with solvent A in an autosampler vial, and 300µl of this sample was loaded into a 500µl loading loop using the autosampler.

Chromatography methods (solvent gradients) tested using the 4.6mm diameter column were as follows:

Chromatography method one: 0% solvent B (100% solvent A) for 5 min, 0-40 % solvent B over 30 min, 40-100% solvent B over 10 min, 100% solvent B for 5 min, 100%-0% solvent B over 10 min. Flow rate 0.6ml/min.

Chromatography method two: 0% solvent B for 5 minutes, 0-40 % solvent B over 30 min, 40-100% solvent B over 10 min, 100% solvent B for 5 min, 100-0% solvent B over 10 min. Flow rate 0.8ml/min.

Chromatography method three: 0% solvent B for 2 min, 0-15% solvent B over 3 min, 15-25% solvent B over 10 min, 25-35% solvent B over 2 min, 35-50% solvent B over 15 min, 50-100% solvent B over 5 min, 100% solvent B for 5 min, 100-0% solvent B over 10 min. Flow rate 0.8ml/min.

Chromatography method four: 0% solvent B for 2 min, 0-15% solvent B over 3 min, 15-25% solvent B over 10 min, 25-35% solvent B over 2 min, 35-40% solvent B over 10 min, 40% solvent B for 5 min, 40-50% solvent B over 5 min, 50-100% solvent B over 5 min, 100% solvent B for 5 min, 100-0% solvent B over 10 min. Flow rate 0.8ml/min.

Duplicate samples were run using each method to determine reproducibility of results.

The best separation of major peaks was achieved using chromatography method four which was therefore used for further optimisation of HPLC analyses using the 4.6mm diameter column (section 3.2.3).

During the development of these chromatography conditions, it became apparent that the high concentration of ethanol in the sample (around 38% v/v) affected the affinity of the phenolic compounds for the column matrix. When running commercially available standards this problem was alleviated by reducing the ethanol concentration. 100µl of root extract run previously were dried under vacuum at 35°C, dissolved in 500µl solvent A, and chromatographed as before (data not shown). It was clear that all compounds in solution were binding to the column, as the large initial peak, detected in previous runs (e.g. figure 3.2), with a retention time of about six minutes, representing the delay between loading and reaching the detector, was no longer evident. All peaks were also resolved more clearly than they had been previously, and elution times were changed slightly. Unfortunately, some of the major peaks previously detected, originally eluting towards the end of the run, were no longer detectable, suggesting that these compounds are insoluble in water. It was therefore necessary to find a means of minimising the ethanol concentration in the sample loaded on the column, while avoiding the need to dry down the extract.

Chromatography conditions - trial two

A column of the same matrix (C18 reversed phase), but with a narrower bore (2.1mm diameter) than that used previously was tested. 20µl root extract (compared to the 100-200µl used previously) was diluted into 480µl solvent A in a 500µl autosampler vial. The final ethanol concentration in the sample was approximately 4% (v/v). This was low enough to allow effective binding of compounds to the column, while not requiring further concentration of the sample by evaporation. Detection of peaks using the 2.1mm diameter column was approximately five times more sensitive (estimated by running a series of standard compounds - data not shown) than using the 4.6mm diameter column, and a number of peaks were detected in root extracts which had not previously been apparent.

A new series of solvent gradients was developed to optimise separation of all peaks now detectable using the narrow bore column. The first gradient used (method five) was chromatography method four, with a modified flow rate of 0.2ml/min. Each new gradient was adjusted as described previously. Chromatography methods were as follows:

Chromatography method five: 0% solvent B for 2 min, 0-15% solvent B over 3 min, 15-25% solvent B over 10 min, 25-35% solvent B over 2 min, 35-40% solvent B over 10 min, 40% solvent B for 5 min, 40-50% solvent B over 5 min, 50-100% solvent B over 5 min, 100% solvent B for 5 min, 100-0% solvent B over 10 min. Flow rate 0.2ml/min.

Chromatography method six: 0% solvent B for 2 min, 0-15% solvent B over 3 min, 15% solvent B for 5 min, 15-25% solvent B over 10 min, 25-35% solvent B over 2 min, 35-40% solvent B over 10 min, 40-50% solvent B over 5 min, 50-100% solvent B over 5 min, 100% solvent B for 5 min, 100-0% solvent B over 10 min. Flow rate 0.2ml/min.

Chromatography method seven: 0% solvent B for 2 min, 0-5% solvent B over 3 min, 5-25% solvent B over 35 min, 25-50% solvent B over 10 min, 50-100% solvent B over 10 min, 100% solvent B for 5 min, 100-0% solvent B over 10 min. Flow rate 0.2ml/min.

Chromatography method eight: 0% solvent B for 2 min, 0-5% solvent B over 3 min, 5-25% solvent B over 25 min, 25-50% solvent B over 5 min, 50-100% solvent B over 15 min, 100% solvent B for 5 min, 100-0% solvent B over 10 min. Flow rate 0.2ml/min.

Chromatography method nine: 0% solvent B for 2 min, 0-5% solvent B over 3 min, 5-25% solvent B over 20 min, 25-35% solvent B over 5 min, 35-40% solvent B over 15 min, 40-50% solvent B over 5 min, 50-100% solvent B over 5 min, 100% solvent B for 5 min, 100-0% solvent B over 10 min. Flow rate 0.2ml/min.

The separation of peaks using chromatography methods six, eight and nine is shown in figure 3.1. Chromatography method nine gave the best profile resolution and was used in all subsequent HPLC analyses with the 2.1mm diameter column.

3.2.3 Assessment of the reproducibility of subsampling

The reproducibility of subsampling from the same root system was tested. The roots of a potted *V. vinifera* cv. Shiraz vine were spread out and 5×100mg root tip (0-2cm from root apex) samples were taken from different places in the root system. Each sample was snap frozen in liquid nitrogen and extract prepared using free phenolic extraction method one (section 3.2.1). 200µl of each extract was diluted with 300µl solvent A, loaded by autosampler and run using chromatography method four. Extraction and chromatography of each sample was not duplicated, as the reproducibility of these operations was previously established during the optimisation of extraction and chromatography methods (data not shown).

Chromatograms were compared by overlaying traces. Three of the five chromatograms are shown in figure 3.2. Peak areas for four of the major peaks absorbing at 330nm in the five root tip samples were calculated (table 3.2). Whilst the profiles were qualitatively similar. there were significant quantitative differences between individual samples, despite the fact that the same amount and type of tissue was used, and the same amount of extract loaded.

Similar variation between samples was observed in comparisons of primary roots from potted vines, and between samples of roots from tissue cultured vines (data not shown). The identification of compounds present in root extracts is discussed in Chapter 5, section 5.3.3.

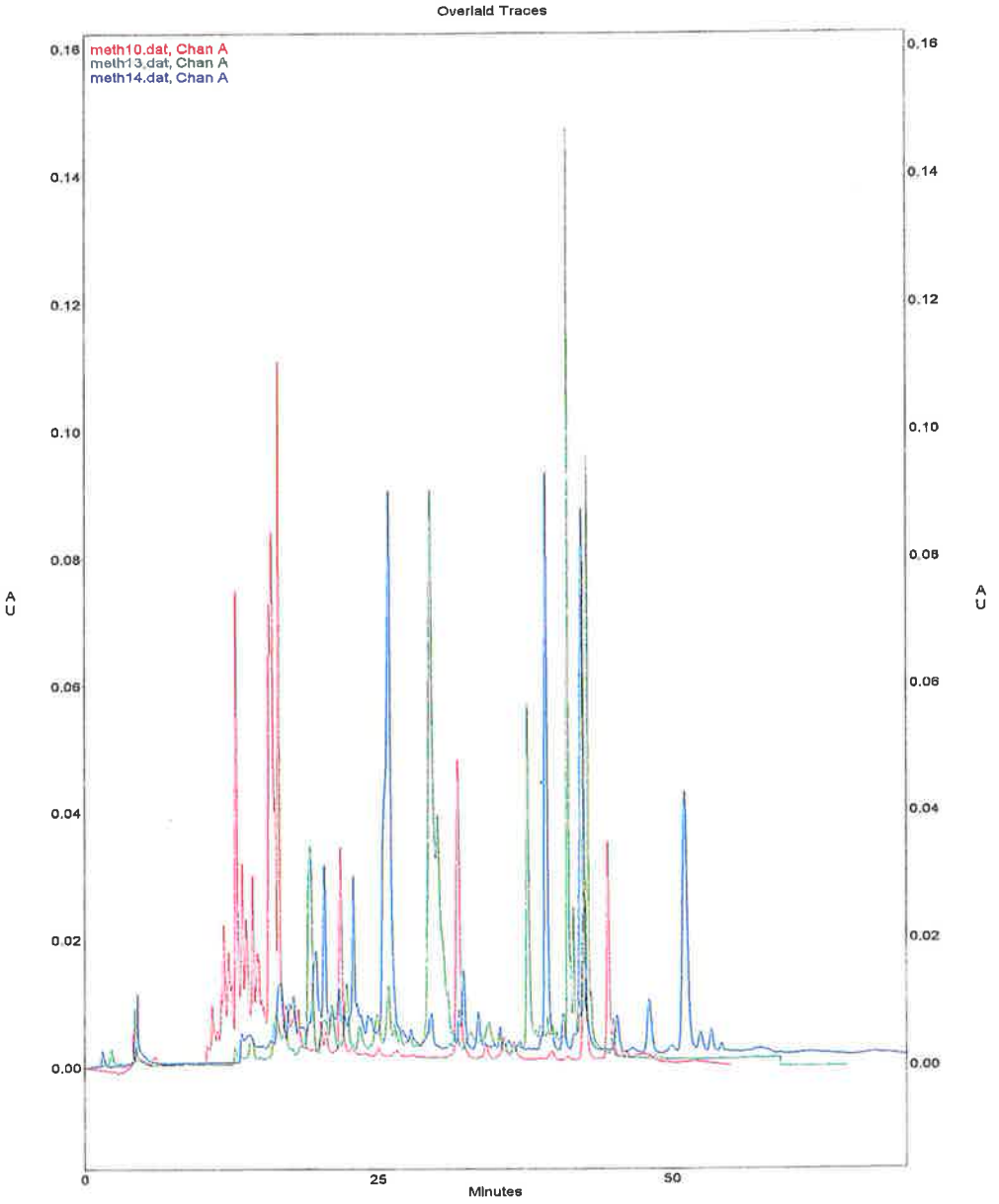


Figure 3.1 Overlayed HPLC chromatograms showing absorption profiles at $A_{330nm,}$ for comparison of chromatography methods. Chromatography method six, red; Chromatography method eight, green; Chromatography method nine, dark blue.

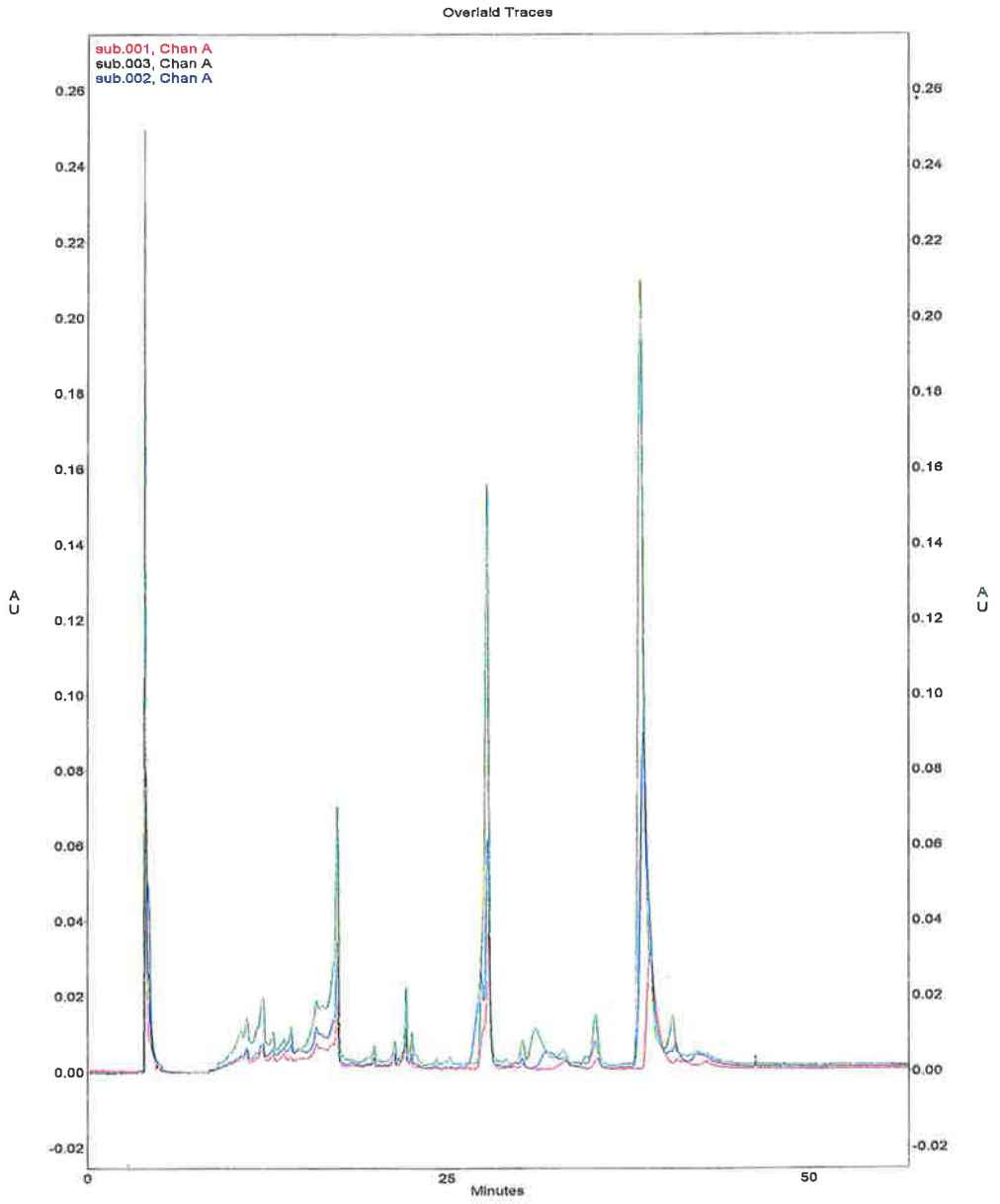


Figure 3.2 Overlayed HPLC chromatograms showing absorption profiles at $A_{330nm,}$ for comparison of root tip extracts. Extracts of free phenolics were compared from three (of five) different *V. vinifera* cv. Shiraz root tip samples. Extracts were prepared with extraction method one and separated using chromatography method four.

Table 3.2 Peak area ($\times 10^{-5}$ AU) of four major peaks at A_{330nm} in five root tip samples from the same root system.

peak no.	sample 1	sample 2	sample 3	sample 4	sample 5
1	2.28	4.58	10.71	1.57	1.61
2	0.89	0.46	1.23	0.42	0.96
3	8.96	10.45	32.13	5.48	7.28
4	13.61	37.41	62.56	11.72	12.71

3.2.4 Discussion of HPLC methods

Optimal conditions for extraction and separation of free phenolics from grapevine roots were determined as extraction method one, and chromatography method nine. Retention times and absorption spectra of a series of commercially available standard compounds run using chromatography method nine are given in Appendix five.

Comparison of the HPLC chromatograms (figure 3.2) and major peak areas (table 3.2) obtained for five different subsamples of the same root system demonstrate that there is a high level of quantitative variation between samples. The methods developed here were specifically designed for small samples of roots such as those from tissue culture plants. It is possible that improved quantitative reproducibility would be obtained if larger root samples were used. Despite this, qualitative differences in free phenolic content between varieties were still of interest, therefore the results of HPLC analyses in subsequent sections are presented as presence or absence of peaks.

3.3 Light microscopy and histochemistry

Microscopy and histochemistry are powerful techniques for studying the interaction between plants and pathogens or pests. Plant responses can be localised and characterised through the use of specific staining or fluorescence techniques, providing an important first step in characterising the defence mechanisms of plants, and a broader picture of an interaction compared with a more targeted method (e.g. molecular analysis).

Microscopy and histochemistry were used in this study for three purposes. To locate the feeding site of phylloxera within the root, to document primary root anatomy and histochemistry and investigate the possibility of their involvement in constitutive resistance mechanisms, and to investigate changes which take place in response to phylloxera feeding, including the activation of defence responses.

Histochemical techniques can be used to characterise the biochemical composition of particular tissues within an organ (e.g. root), and to detect and characterise localised responses to pest or pathogen attack. Such techniques have not previously been applied to the study of grapevine root-phylloxera interactions. For this reason it was necessary to test a range of techniques to determine which were most applicable to this study. Techniques were selected which would be suitable for use with the root material available, taking quarantine restrictions into consideration, and then tested to see which could detect compounds possibly involved in the response of roots to phylloxera attack.

The selections were based largely on published studies of grapevine and plant defence responses. For example, histochemistry has been used to characterise the response of *Vitis* leaves to downy mildew infection, demonstrating that accumulation of *trans*-resveratrol and its derivatives, flavonoids and lignin correlated with resistance to downy mildew (Dai *et al.* 1995a, b, Mondolot-Cosson *et al.* 1997) (see also Chapter 1, section 1.4.1 for further discussion). Several of the techniques used by Dai *et al.* (1995a, b) were tested in addition to those which could detect accumulation of callose and suberin, other typical plant defence responses.

A staining technique was also required for the study of basic root anatomy. A technique was selected which would also allow specific staining of starch, as starch accumulation has been recorded in nodosities (Niklowitz 1954, Hofmann 1957).

3.3.1 Sample preparation

Due to quarantine restrictions, it was necessary to either freeze or fix infested root material, therefore both cryosectioning and resin embedding were tested. Uninfested primary roots and nodosities were harvested from *V. vinifera* cv. Shiraz and Schwarzmann vines in pot trial four (section 2.3.4). For cryosectioning, root material was embedded in cryomounting medium (OCT compound, Tissue Tek) and snap frozen in liquid nitrogen. Cryosectioning trials failed to produce good quality sections, so this technique was not pursued further. For resin embedding, root material was fixed in FAA or 3% glutaraldehyde then embedded in glycolmethacrylate (GMA) or epoxy resin and sectioned. Details of fixatives, embedding protocols and sectioning are presented in Appendix three.

Samples of uninfested roots and nodosities harvested from *V. vinifera* cv. Shiraz vines were prepared for TEM as described in Appendix three.

3.3.2 *Trials of microscopy and staining methods*

Slides containing ten sections each were prepared from triplicate samples of each tissue type (*V. vinifera* cv. Shiraz and Schwarzmann uninfested root tissue and *V. vinifera* cv. Shiraz and Schwarzmann nodosities). All methods were tested on one slide of each sample. Details of stains, staining schedules and microscopy methods are presented in Appendix three.

An example of the result of each staining method on GMA sections is shown in figure 3.3.

Fluorescence microscopy

Autofluorescence: Unstained sections were observed under UV excitation (365nm excitation filter, 420 nm barrier filter)(figure 3.3a). Autofluorescent compounds detected include lignin and suberin, as well as polyphenolics (confirmed by TBO staining - data not shown). This method was simple and very useful for observation of the interaction, and was subsequently adopted for observation of both uninfested and infested roots.

Aniline Blue: Stained sections were observed under UV excitation (365nm excitation filter, 420 nm barrier filter) (figure 3.3b). Callose was not detected in any of the samples tested. This method was not used for subsequent histological studies.

Neu's Reagent: Stained sections were observed under UV excitation (365nm excitation filter, 420 nm barrier filter) (figure 3.3c). No staining of flavonoids was observed, therefore this method was not used for subsequent histological studies.

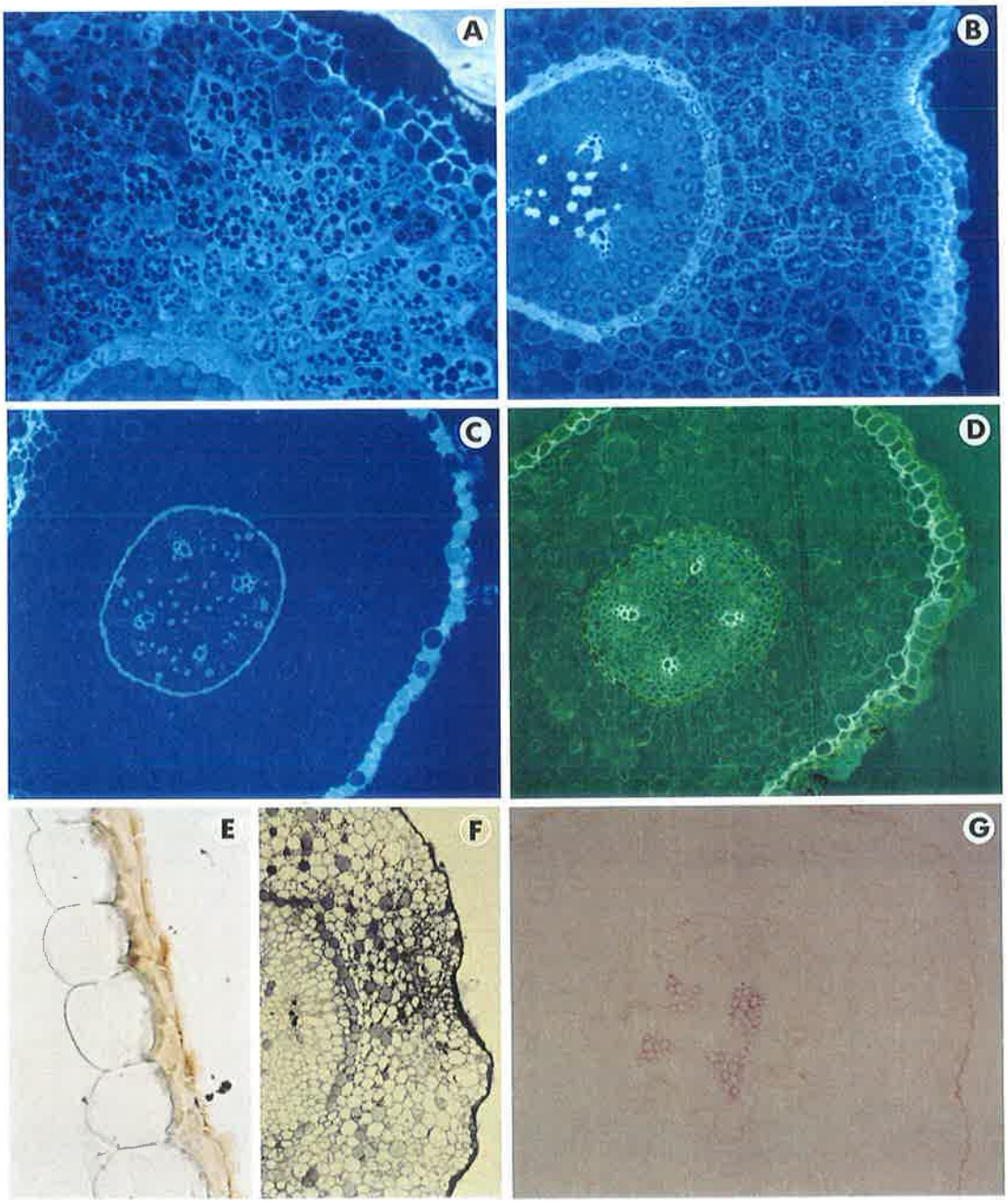
Auramine O: Stained sections were observed under blue light excitation (420nm excitation filter, 515-560nm barrier filter)(figure 3.3d). Auramine O staining caused the cell walls of the hypodermis and endodermis to fluoresce grass-green indicating the presence of suberin, while lignin fluoresced bright green. This stain was not specific for any compounds which could not be detected by autofluorescence, and was therefore not used for subsequent histological studies.

Bright field microscopy

Sudan Black: Stained sections were observed under bright field illumination (figure 3.3e). Suberin in the walls of the exodermis and endodermis was clearly stained blue-black. This method was time consuming, but useful for showing the suberisation of tissues without interference from other compounds as with, for example, autofluorescence. This method was therefore used in subsequent histological studies.

PAS/TBO: Stained sections were observed under bright field illumination (figure 3.3f). Anatomical features stained clearly, including cytoplasm, cell walls, polyphenolics and lignin, and starch. To differentiate starch from phenolic-containing globules, which may both

Figure 3.3 Transverse sections through *V. vinifera* cv. Shiraz uninfested roots or nodosities observed with a range of staining and microscopy methods. a) Nodosity under UV excitation. b) Nodosity stained with aniline blue. c) Uninfested root stained with Neu's reagent. d) Uninfested root stained with Auramine O. e) Epidermis of uninfested root stained with Sudan black. f) Nodosity stained with PAS/TBO. g) Uninfested root stained with phloroglucinol/HCl. a) - c) examined under UV excitation, d) examined under blue-light excitation, e) - g) examined under bright field illumination.


A
B
C
D
E
F
G

appear dark purple, PAS was also used without a counterstain so that polyphenolics were not stained at all (figure 4.3). PAS and PAS/TBO were used in subsequent anatomical and histological studies.

Phloroglucinol-HCl: Sections were observed under bright field illumination (figure 3.3g). Lignin stained pale pink. A delay in observation caused the colour to fade, and the section to wrinkle. While this stain is specific for lignin, PAS/TBO and autofluorescence also showed the presence of lignin clearly, therefore phloroglucinol-HCl was not used for subsequent histological studies.

3.3.3 Discussion of microscopy methods

While cryosectioning presents several advantages over resin embedding, including the ability to detect alcohol soluble compounds which may be washed out of fixed samples during dehydration, in this case the samples proved brittle and difficult to section, therefore a stain-permeable resin (GMA) was adopted for use. Two fixatives, FAA and 3% (v/v) glutaraldehyde, were compared. Similar material fixed with each was compared using a number of stains and as well as autofluorescence, but no difference was observed (results not shown). The two fixatives were subsequently used interchangeably.

Most stains tested were successful in that they allowed detection of the compounds for which they were recommended. The exceptions were aniline blue and Neu's reagent. As callose would be present in phloem tissue, it is possible that the aniline blue staining schedule may have been ineffective for unknown reasons. The failure of Neu's reagent may have been due to lack of flavonoids in the root tissue. It is also possible that dehydration of root samples in alcohol resulted in the loss of alcohol-soluble compounds such as flavonoids, making their detection by histochemistry impossible. However the chromatography of ethanol-soluble root extracts by HPLC did not detect the presence of flavonoids in similar tissue samples.

TEM was used for ultrastructural observation of uninfested root tissue (Chapter 4, figure 4.4), but unfortunately, the embedding protocol used for TEM samples was found to be incompatible with nodosity tissue. While good sections were obtained from uninfested roots, it was not possible to obtain suitable ultrathin sections of nodosities. This may have been due to a high content of hydrophobic compounds causing the tissue to become unstable in araldite resin. Alternative sample preparation methods (e.g. other resins, freeze substitution) were not tested, but this may be valuable in future.

Summary

Of the light microscopy and histochemical techniques tested, the following were adopted for use in this study: UV excitation for detection of lignin, suberin and autofluorescent phenolic

compounds; PAS/TBO for general anatomy, detection of polyphenolics and starch; Sudan black for detection of suberin. Together, these three techniques provide a good system for a histological and histochemical study of grapevine roots. To extend this work, conditions for cryosectioning could be improved, which would make other staining techniques possible. Alternatively, availability of a fluorescence microscope within a phylloxera quarantine zone would extend the range of techniques available by enabling the use of fresh tissue.

3.4 Extraction of RNA from primary roots

A number of plant-pathogen interactions are characterised by accumulation of defence-related compounds, and changes in associated gene expression patterns. This includes the grapevine defence response to fungal pathogens, where accumulation of PR proteins or products of the phenylpropanoid pathway (Langcake and Pryce 1976, Stein and Blaich 1985, Sbaghi *et al.* 1995, Renault *et al.* 1996, Derckel *et al.* 1999), or enhanced expression of genes encoding their synthesis (Melchior and Kindl 1991, Busam *et al.* 1997b, Jacobs *et al.* 1999) has been detected.

In order to investigate the grapevine-phylloxera interaction at the molecular level, the expression pattern of a range of grapevine genes for which cDNA clones were available was studied. Northern blot hybridisation, which requires large quantities of undegraded, pure RNA, was used. There was no published method for extraction of RNA from grapevine roots, which contain very high concentrations of carbohydrates and phenolic compounds that can interfere with the extraction of nucleic acids. It was therefore necessary to test the suitability of a number of RNA extraction methods. This section outlines the four methods tested and comments on the results of each. Details of the methods are presented in Appendix two.

All RNA extractions were performed over the same time period using a subsample of the same root material (primary roots of a potted *V. vinifera* cv. Shiraz vine). RNA yield and quality was measured by absorbence at 260nm and 280nm using a UV spectrophotometer. Absorption spectra for RNA obtained using the four methods are shown in figure 3.4. The integrity of the RNA was further assessed by electrophoresis in a denaturing formaldehyde agarose gel, followed by staining with ethidium bromide (figure 3.5).

3.4.1 RNA extraction method one

Modified from Levi *et al.* (1992).

This protocol was designed for extraction of RNA from Pecan fruits and leaves, and aims to suppress the interaction of polyphenols with RNA. The lithium dodecylsulphate-based

extraction buffer contained LiCl, Nonidet P-40 (Sigma), sodium deoxycholate, soluble PVP, insoluble PVP, DTT, and β-Mercaptoethanol. After homogenisation of the root tissue in extraction buffer, the mixture was extracted with chloroform and ethanol precipitated, followed by a LiCl precipitation, then two further ethanol precipitations. The entire procedure took two days to complete.

The estimated RNA yield obtained was 88.22μg/g fw *V. vinifera* root with an A_{260}:A_{280} of 1.47 (figure 3.4a).

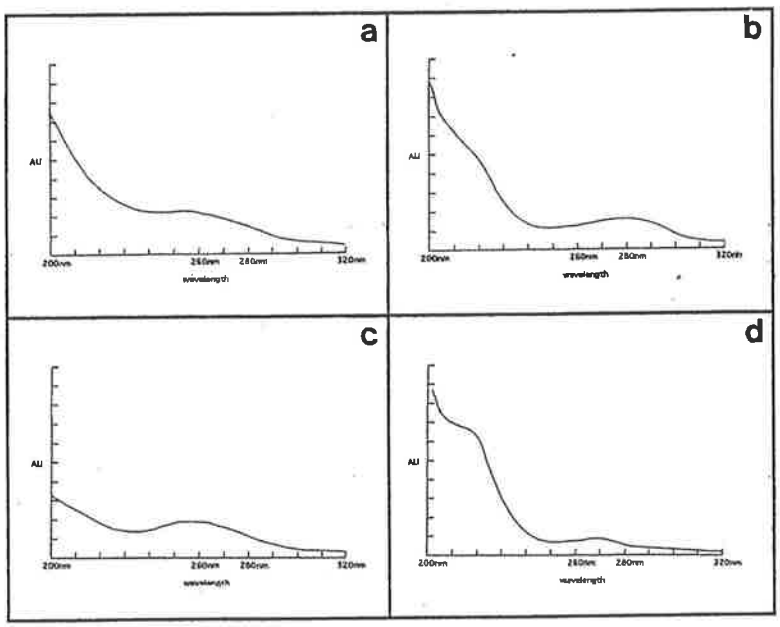


Figure 3.4 Absorption spectra of RNA isolated using four RNA extraction methods (section 3.4). a) Extraction method one. b) Extraction method two. c) Extraction method three. d) Extraction method four.

3.4.2 RNA extraction method two

From Pawlowski *et al.* (1994).

This sodium dodecylsulphate/sodium deoxycholate-based extraction buffer has been successfully used for isolation of RNA from *Casuarina* roots which contain high levels of polyphenolic compounds. The extraction buffer also contained LiCl, NP-40, polyclar AT (Serva), β-Mercaptoethanol and DTT. Following homogenisation of root tissue in extraction buffer, RNA was precipitated with *iso*-propanol, then extracted with phenol:chloroform

followed by chloroform. Finally, RNA was precipitated with ethanol. The entire procedure took one and a half days to complete.

The estimated RNA yield obtained was 5.3µg/g fw *V. vinifera* root with an A_{260}:A_{280} of 1.59 (figure 3.4b).

3.4.3 RNA extraction method three

From Davies and Robinson (1996).

This is a sodium perchlorate-based method designed for RNA extraction from grape berries. The extraction buffer was 5M Na perchlorate containing PEG 4000, SDS, and insoluble PVP. Following homogenisation of the root tissue in extraction buffer, RNA was precipitated with ethanol, then extracted with phenol:chloroform followed by chloroform, and finally precipitated with ethanol. The entire procedure took one day to complete.

The estimated RNA yield obtained was 38µg/g fw *V. vinifera* root with an A_{260}:A_{280} of 1.76.

The absorption spectrum showed this to be the purest RNA preparation, with an absorption maximum at 260nm (figure 3.4c).

3.4.4 RNA extraction method four

Trizol® is a commercial phenol/guanidine isothiocyanate-based RNA extraction reagent produced by Gibco BRL Life Technologies which was recommended for all tissues including plant tissues.

This method used Trizol® Reagent as recommended by the manufacturer, except that the root tissue was ground to a fine powder in liquid nitrogen in a mortar and pestle, before mixing with the extraction buffer. The mixture was extracted with chloroform, then precipitated with *iso*-propanol. The entire procedure took around one hour to complete.

The estimated RNA yield obtained was 134.8µg/g fw *V. vinifera* root with an A_{260}:A_{280} of 1.37.

While this appeared to be a high yield, the pellet was large, grey and largely insoluble. The absorption spectrum (figure 3.4d) of the supernatant showed that this preparation of RNA contained significant amounts of impurities, and no RNA was visible when electrophoresed (figure 3.5).

Figure 3.5 Denaturing formaldehyde-agarose gel electrophoresis of *V. vinifera* cv. Shiraz root RNA extracted using four extractions methods. RNA in lanes 1-4 was extracted using: **1**) Extraction method two (4.24µg total RNA). **2**) Extraction method four (6.4µg total RNA). **3**) Extraction method three (10µg total RNA). **4**) Extraction method one (10µg total RNA). The estimated amounts of RNA electrophoresed in each lane was based on the A_{260} of the RNA preparations.

3.4.5 Discussion of RNA extraction methods

Extraction method one appeared to give the highest yield of total RNA but the A_{260}:A_{280} ratio (1.47) and spectrum (figure 3.4a) showed that the RNA contained impurities. Ideally the A_{260}:A_{280} ratio should be at least 1.8 and as close to 2.0 as possible (Ausubel *et al.* 1999). Extraction method three gave the best quality RNA (A_{260}:A_{280} 1.76), and was relatively straight forward and reproducible. Figure 3.5 shows that the RNA extracted using method three was the least degraded, and that the yield, as estimated by ethidium bromide staining, was actually the highest of the four methods. This method was therefore adopted as the method of choice for extracting RNA from all grapevine root samples.

Subsequent use of extraction method three demonstrated that the yield could be improved to 103.5µg/g fw for uninfested root tips through careful selection of root tissue, targeting whitish 'healthy looking' roots and avoiding older, browner roots.

3.5 Supplementary methods

The following analytical methods were used without further adaptation. Details of each method are presented in Appendix two.

- Extraction of soluble proteins and SDS-PAGE gel electrophoresis.
- Extraction of free amino acids.
- Denaturing formaldehyde-agarose gel electrophoresis.
- Northern blot hybridisation.
- Enzymatic assay of starch content.

Mass Spectrometry analysis was carried out using an API-300 Mass Spectrometer at Australian Wine Research Institute (AWRI), Urrbrae, SA (Appendix six).

Free amino acid and amide analysis was carried out using a GBC Aminomate HPLC system at AWRI, Urrbrae, SA (Appendix two).

3.6 Conclusion

Analytical methods which can be used to investigate the grapevine-phylloxera interaction are currently limited by quarantine restrictions imposed on movement of phylloxera infested material. This chapter discussed a range of methods which comply with these restrictions and have successfully been developed and tested. The following chapters describe the use of these methods to investigate the interaction between phylloxera and a range of susceptible and resistant vine varieties.

4. THE INTERACTION BETWEEN *V. VINIFERA* CV. SHIRAZ AND A BIOTYPE B-LIKE STRAIN OF PHYLLOXERA

4.1 Introduction

Susceptible primary grapevine roots respond to phylloxera feeding by forming nodosities - metabolically active organs necessary for the nutritional requirements of the insect and able to support a high reproductive rate. Studies of this interaction, reviewed in section 1.2.1, have been carried out by a number of researchers. They reported anatomical changes during nodosity formation (Petri 1907, Niklowitz 1954, Hofmann 1957) and the biochemical (phenolic acids, sugars, proteases) composition of galls (Denisova 1965, Sobetskiy and Derzhavina 1973, Nedov *et al.* 1992). While such studies gave some clues as to responses that may be occurring in vine roots, they were unable to provide the detailed information that can be obtained using more recently developed techniques.

This chapter investigates the interaction between a phylloxera-susceptible grapevine, *V. vinifera* cv. Shiraz, and an aggressive strain of phylloxera, VWL-1 (Corrie *et al.* 1997a, b). This strain is known to survive and reproduce at high rates on *V. vinifera* and to exhibit similar biological characteristics to biotype B (Granett *et al.* 1985) when bioassayed on *V. vinifera* and ARG1 (Corrie *et al.* unpublished). An understanding of such a 'compatible' interaction is important if we are to determine the nutritional and other conditions required for a successful phylloxera infestation. This information also provides a backdrop for the study of incompatible interactions with resistant vines. It would not be possible to interpret defence responses of resistant varieties without being able to identify what makes them different from susceptible ones.

This study has taken a number of approaches to the investigation of the grapevine-phylloxera interaction. It describes the anatomy of 'typical' phylloxera-induced nodosities (primary root galls), locates the feeding site within nodosities, and investigates chemical and molecular changes which might be occurring in phylloxera-infested roots.

4.2 Materials and methods

For microscopy, uninfested and infested roots (nodosities) of *V. vinifera* cv. Shiraz were harvested from pot trial four (section 2.3.4), and from the perlite-based medium cocultivations (section 2.4.4) unless otherwise mentioned. Uninfested roots were harvested from within 2cm of the root apex, within the same region on which a nodosity would usually form. Root material was fixed with 3% glutaraldehyde fixative, embedded in GMA and observed for autofluorescence under UV excitation, or stained with TBO, PAS/TBO or Sudan

black. Details of methods are given in Appendix three. In all cases, triplicate samples were processed to ensure results were representative of tissue types. All figures illustrate 4μm GMA sections unless otherwise stated. TEM was carried out on root from pot trial four. Uninfested root material was harvested 0.5-1cm from the root apex, fixed in 4% (v/v) glutaraldehyde, 1.25% (w/v) paraformaldehyde and embedded in araldite resin as described in Appendix three. Material was harvested.

Insoluble starch content of uninfested roots and nodosities harvested from pot trial four (section 2.3.4) was determined using the enzyme-based assay described in Appendix two.

The ethanol soluble phenolic content of uninfested roots and nodosities was compared using methods described in sections 3.2.1 and 3.2.2. Roots and nodosities for analysis were harvested from perlite-based medium cocultivations (section 2.4.4).

Free amino acid and amide content, and total protein profile were determined for uninfested roots and nodosities harvested from pot trial four (section 2.3.4). Amino acid and protein extracts were prepared using methods described in Appendix two. Amino acid extracts were prepared from triplicate root samples. Proteins were separated using SDS-PAGE followed by staining with Coomassie Blue, also as described in Appendix two.

RNA was extracted from uninfested roots and nodosities harvested from pot trial four, using RNA extraction method three (section 3.4.3, Appendix two), or from leaves of pot grown *V. vinifera* cv. Shiraz (accession 12/BVRC-12-C12A) either uninfested or infested with VWL-1 strain phylloxera. Northern blot hybridisation was used to determine the expression of a wide range of genes using methods described in Appendix two.

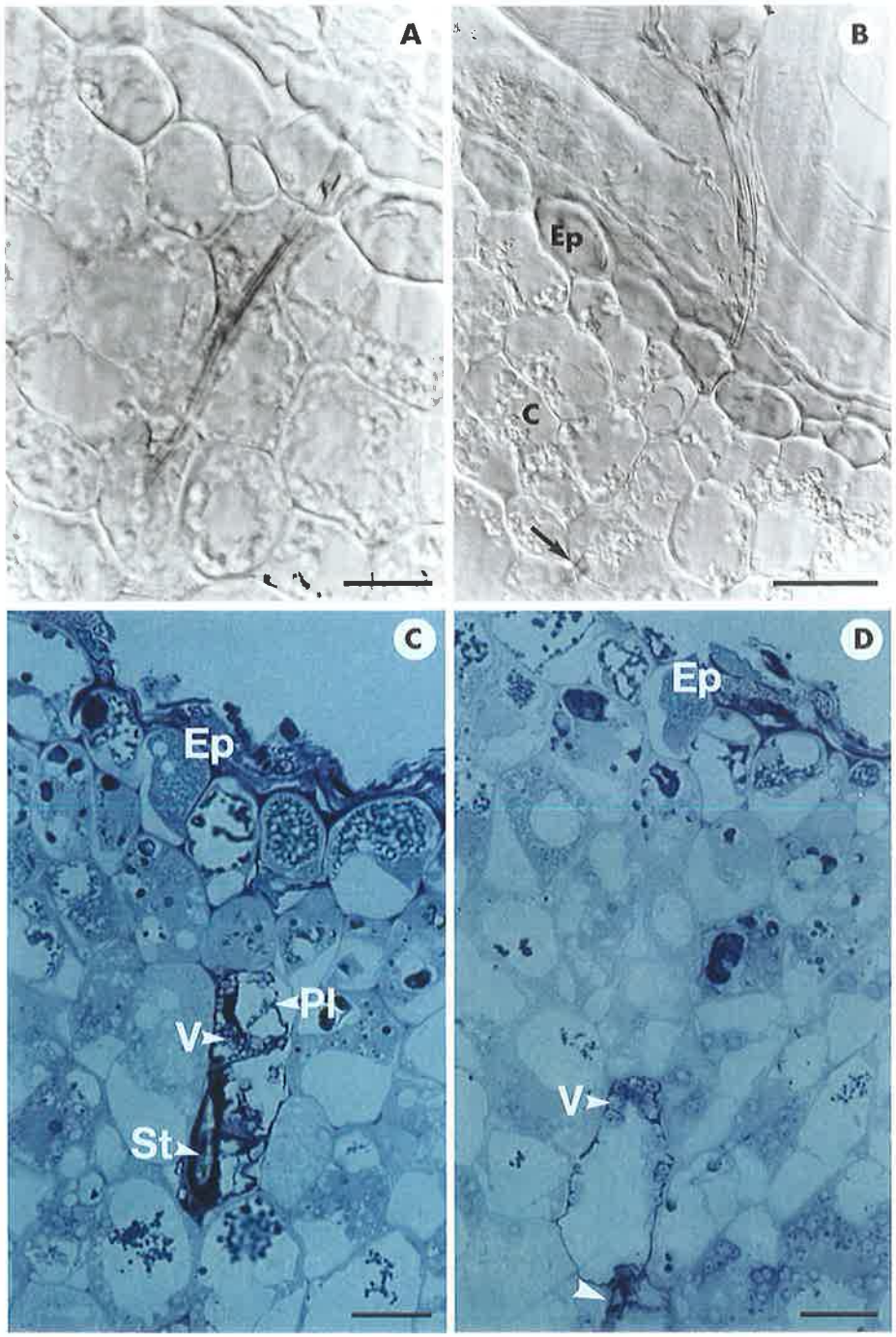
4.3 Results

4.3.1 Location of feeding site within primary roots

Transverse sections through a well developed nodosity at the site of phylloxera feeding demonstrate that the stylet apparently penetrated through the cortical cells with the stylet tip terminating within a single cell, four to five cell layers below the epidermis, from which the phylloxera was presumably feeding (figure 4.1a, b). It is unclear from this figure whether the tip of the stylet has penetrated the cytosol and/or the vacuole of the cell. Cells immediately surrounding those penetrated by the stylet appear unaffected by its presence.

Figures 4.1c and d illustrate the feeding site in another nodosity in which the phylloxera was apparently feeding at a depth of about six cell layers below the epidermis. The cells through which the stylet passed have become plasmolysed, possibly as a result of phylloxera feeding (figure 4.1c). In each, the cytosol appears dark and irregular, with the plasmalemma

Figure 4.1 Transverse sections through nodosities on *V. vinifera* cv. Cabernet Sauvignon at the point of stylet penetration. a) 4µm GMA section viewed with Nomarski differential interference contrast optics. Scale bar equals 10µm. b) As for (a), adjacent section showing stylet tip (arrow). The stylet penetrates through the epidermis (Ep) and several cell layers into the cortex (C) of the root, but does not approach the stele. Scale bar equals 20µm. c) 0.5µm araldite resin section, stained with TBO. Oblique section cut through stylet (St); epidermis (Ep); collapsed plasmalemma (Pl); vesicles (V). Scale bar equals 10µm. d) As for (c), adjacent section showing a further penetrated cell and stylet tip (arrow head); epidermis (Ep); vesicles (V). Scale bar equals 10µm.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C
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V
St
D
Ep
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collapsed inwards from the cell walls. Vesicles are apparent in what remains of the cytosol. The cytosol in unaffected cells is much more regular in appearance and lighter in colour, with one or more vacuoles, some of which contain darkly stained polyphenolics. Cells towards which the stylet was directed contain structures with a granular appearance. Penetration of a cell one to two cell layers deeper in the cortex resulted in even further reduction in the amount of cytosol (figure 4.1d). What remains of the cytosol here had a vesicular appearance, although similar vesicles were also observed in the cytosol of nearby, apparently unaffected cortical cells. A darkly stained layer, closely appressed to the cell wall may be either reduced cytosol or plasmalemma with associated high phenolic content. In all sections, the cells surrounding the immediate site of penetration appeared relatively unaffected.

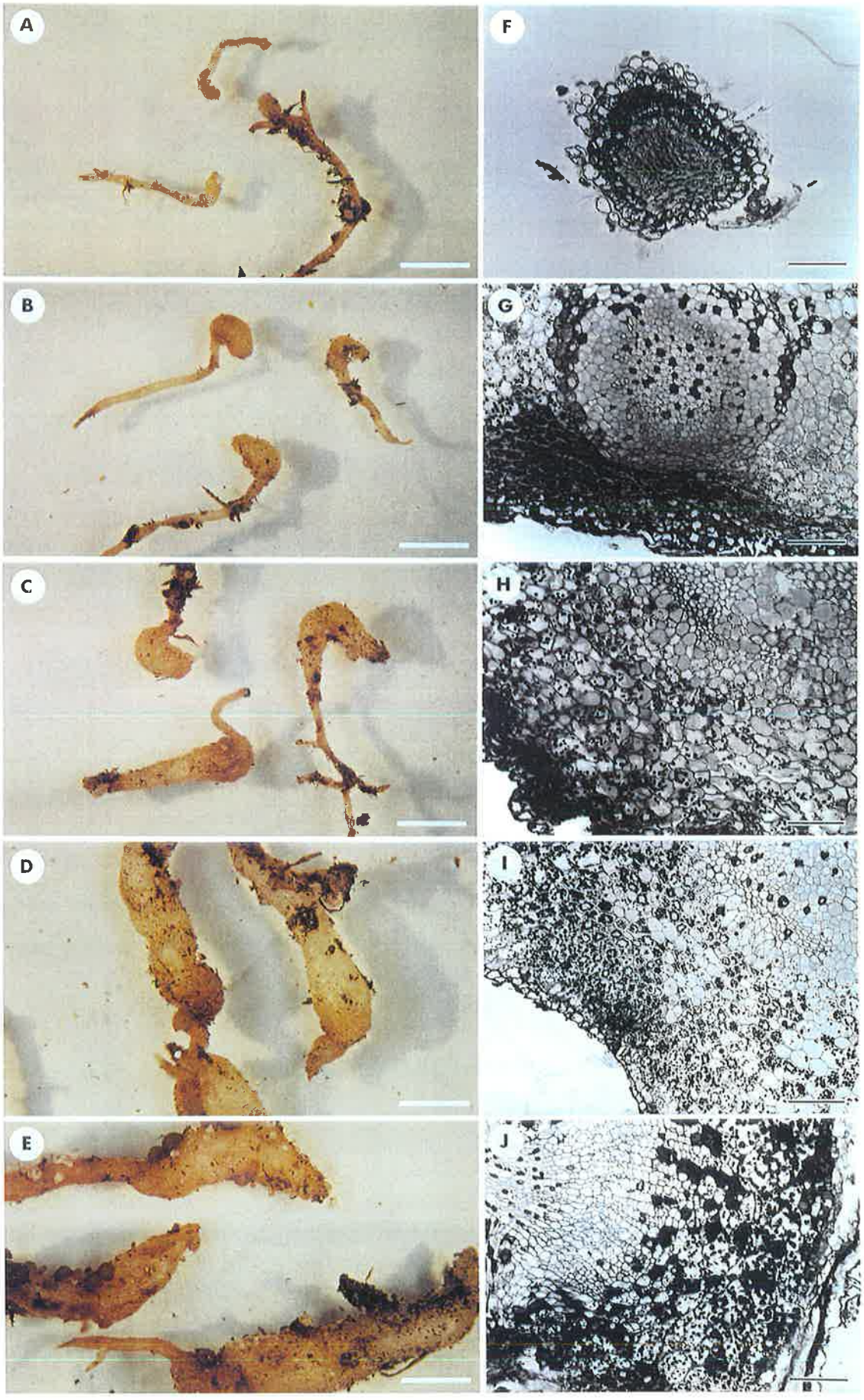
Feeding sites were dissected from nodosities and prepared for TEM, however, for unknown reasons, it was not possible to obtain suitable ultrathin sections for ultrastructural study. It is possible that the resin used was not compatible with the tissue type, as a similar problem was not encountered with uninfested roots treated the same way.

It was possible to detect feeding sites by observation of 'stylet tracks'. When the stylet of a feeding aphid is withdrawn, the stylet sheath, secreted from the stylet tip during plant penetration, remains behind leaving a 'stylet track' (Miles 1987). The stylet sheath is made up of a lipoprotein rich matrix, and possibly helps the aphid avoid recognition by its host, or seals the wound site to assist feeding (Miles 1999). Stylet tracks can be detected by autofluorescence under UV excitation. In all *V. vinifera* nodosities examined, there did not appear to be more than one stylet track or other evidence of multiple penetration points at any one feeding site (figure 5.12a), implying that once a feeding site was established, phylloxera remained sedentary. Observations of phylloxera feeding on primary roots of other grapevine varieties showed that while the site of feeding was the same, i.e. all stylet tracks terminated within the upper region of the cortex of primary roots, the presence of multiple stylet tracks was observed (figures 5.12c and 6.5c) suggesting that phylloxera were repeatedly probing the root cortex.

4.3.2 Nodosity anatomy and development

Nodosities were arranged in a series according to the developmental stage (instar) of the phylloxera present (figure 4.2). Four stages were selected representing first, second, third and fourth instar, and finally a fifth, which represents nodosities supporting mixed populations containing fourth instar insects together with other life stages. Typical transverse sections of each of the stages of nodosity development are shown in figure 4.2. The nodosity was often initiated very close behind the tip of the root (figure 4.2a) where the internal anatomy was essentially undifferentiated (figure 4.2f). Although some swelling and early signs of the

Figure 4.2 Development of nodosities on *V. vinifera* cv. Shiraz. Figures a) - e) Nodosities representing a)-d) 1st to 4th instar phylloxera respectively and e) a population of mixed instars. Scale bar equals approximately 2mm. f) - j) Transverse sections though nodosities of the same stages as a) - e) respectively, stained with PAS/TBO. Scale bars equal 100µm.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J

typical 'hook' form were present at this stage, there was little evidence that the phylloxera had affected the internal anatomy of the root. By the second stage, however tissues in the root had differentiated, and the phylloxera had already induced significant accumulation of starch (also see section 4.3.4 and figure 4.3) in the cortex adjacent to its feeding site (figure 4.2b, g). The epidermis and hypodermis appear to have collapsed, possibly due to poor penetration of fixative. The cortical cells proximal to the feeding site were flattened in comparison with those distal to the feeding site. This appears to be due to lack of expansion in the radial direction, as the cells appeared, in cross-section, to be elongated in the tangential direction. The stele had also become asymmetric, with the cells proximal to the feeding site flattened in a radial direction in comparison with the rounded cells distal to the feeding site. There was little evidence of hypertrophy of cortical cells distal to the feeding site, with swelling probably being accounted for by increased cell division. In other respects this section showed many features typical of nodosities as described by other authors (Petri 1907, Niklowitz 1954). The 'hook' shape observed here probably results from a combination of reduced cell expansion proximal to the feeding site combined with increased cell division distal to the feeding site.

The state of differentiation of tissues within the nodosity and extent of starch accumulation were variable throughout development. While the size of the nodosity increased steadily with age (figure 4.2 a-e), the corresponding sections (f-j) did not demonstrate a steady increase in starch accumulation. In the sections presented here, the endodermis was poorly defined, indicating that it may have been at least partly undifferentiated. The most well-developed nodosity (figure 4.2j) had more tissues in the stele demonstrating secondary differentiation (e.g. early development of vascular rays) than did other nodosities, indicating that nodosity vascular tissue may be capable of normal differentiation and function despite the presence of phylloxera.

4.3.3 Root responses to phylloxera feeding observed by histochemistry

Examination of sections of nodosities stained with PAS/TBO (figure 4.2) suggested that starch accumulation was taking place during nodosity formation. PAS stains amyloplasts, while TBO stains polyphenolics, which when present as cytoplasmic globules can appear similar to amyloplasts (section 3.3.2). Sections of nodosities were therefore stained with PAS only (no TBO counterstain), confirming the presence of starch in the form of amyloplasts in the cytoplasm of the cortical cells (figure 4.3).

Ultrastructural observation of uninfested roots revealed abundant plasmodesmatal connections between the stele and root cortex via plasmodesmata (figure 4.4), confirming the potential for symplastic transport of solutes into the root cortex from vascular tissue.

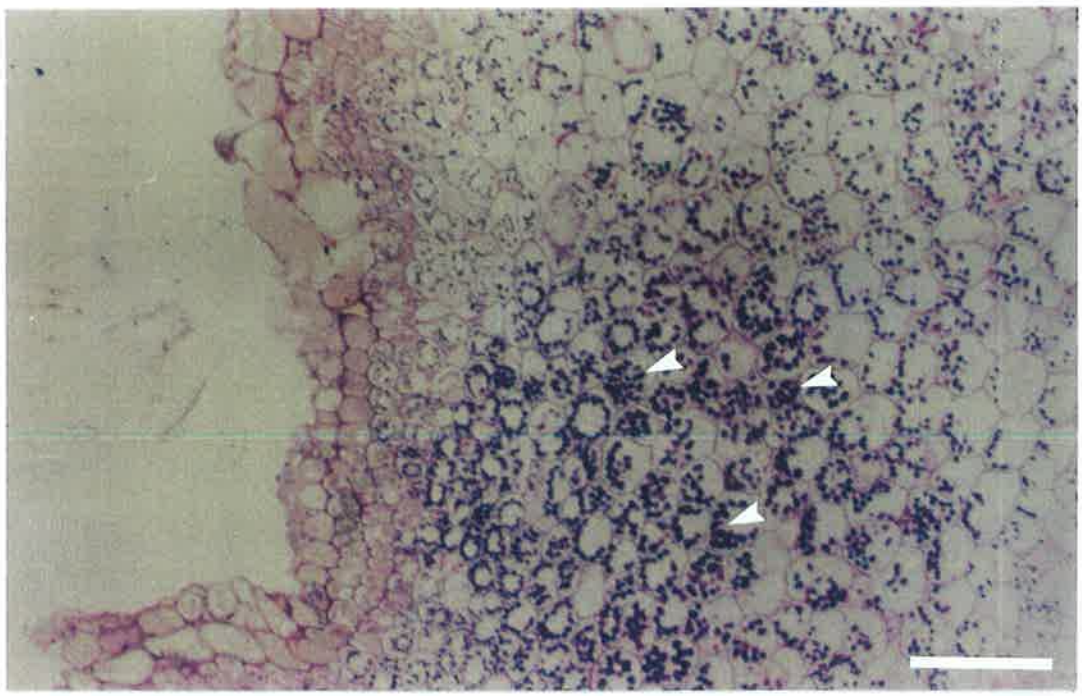


Figure 4.3 Transverse section through nodosity on *V. vinifera* root stained with PAS to visualise amyloplasts (arrow heads). Scale bar equals 100μm.

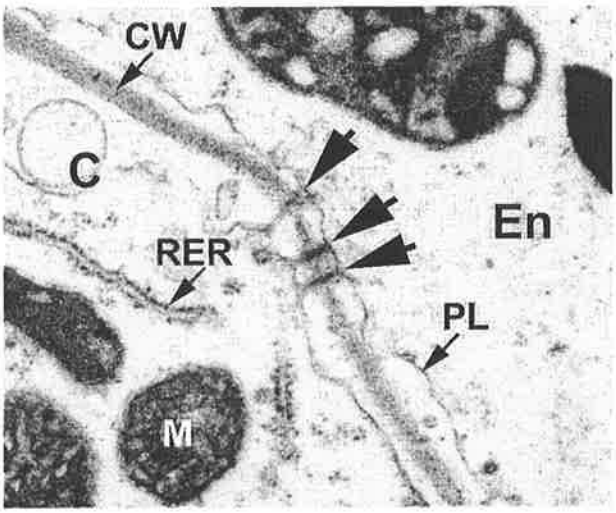


Figure 4.4 TEM photograph of transverse section through primary root of *V. vinifera* cv. Shiraz approximately 3mm from root tip (elongation zone) showing plasmodesmata (arrows) connecting cortical cell (C) with endodermal cell (En). Also shown are cell wall (CW), mitochondria (M), rough endoplasmic reticulum (RER), plasmalemma (Pl). Magnification 30,000×.

An uninfested root section stained with Sudan black showed the presence of suberin lamellae in all walls of endodermal cells (figure 4.5a), while there was no detectable suberin in the otherwise differentiated endodermis of a nodosity (figure 4.5b). Incomplete suberisation was consistently observed in all sections of *V. vinifera* nodosities stained with Sudan black. Suberisation of epidermal or cortical cells, which could indicate the activation of a defence response, was never observed at active feeding sites. Suberin lamellae had, however, been deposited in the walls of cells immediately surrounding a necrotic region on an older abandoned nodosity (figure 4.5c). (Note: the nodosity illustrated in figure 4.5c was formed in response to SRU-1 phylloxera, and harvested from pot trial three. See Chapter 6 for further discussion of the interaction of vines with this strain of phylloxera.) Suberisation of cell walls immediately adjacent to a necrotic area may function to protect the rest of the root from further necrosis.

No fluorescent compounds were detected in nodosities under UV excitation other than those also present in uninfested roots. A transverse section through the uninfested root of a tissue cultured vine (figure 4.6a) showed fluorescence indicating the presence of suberin in the endodermis and epidermis, as well as lignification of the xylem. The major difference between the sections shown in figures 4.6a and b (uninfested root and nodosity respectively)

Figure 4.5 Transverse sections through *V. vinifera* cv. Shiraz roots stained with Sudan black. Suberin is stained blue-black. a) Uninfested root with suberised endodermis (arrow). Scale bar equals 20µm. b) Nodosity with no suberisation in endodermis. Scale bar equals 20µm. c) Necrotic region of older nodosity with suberisation of healthy tissue (Su) surrounding necrotic region (Ne). Scale bar equals 50µm.

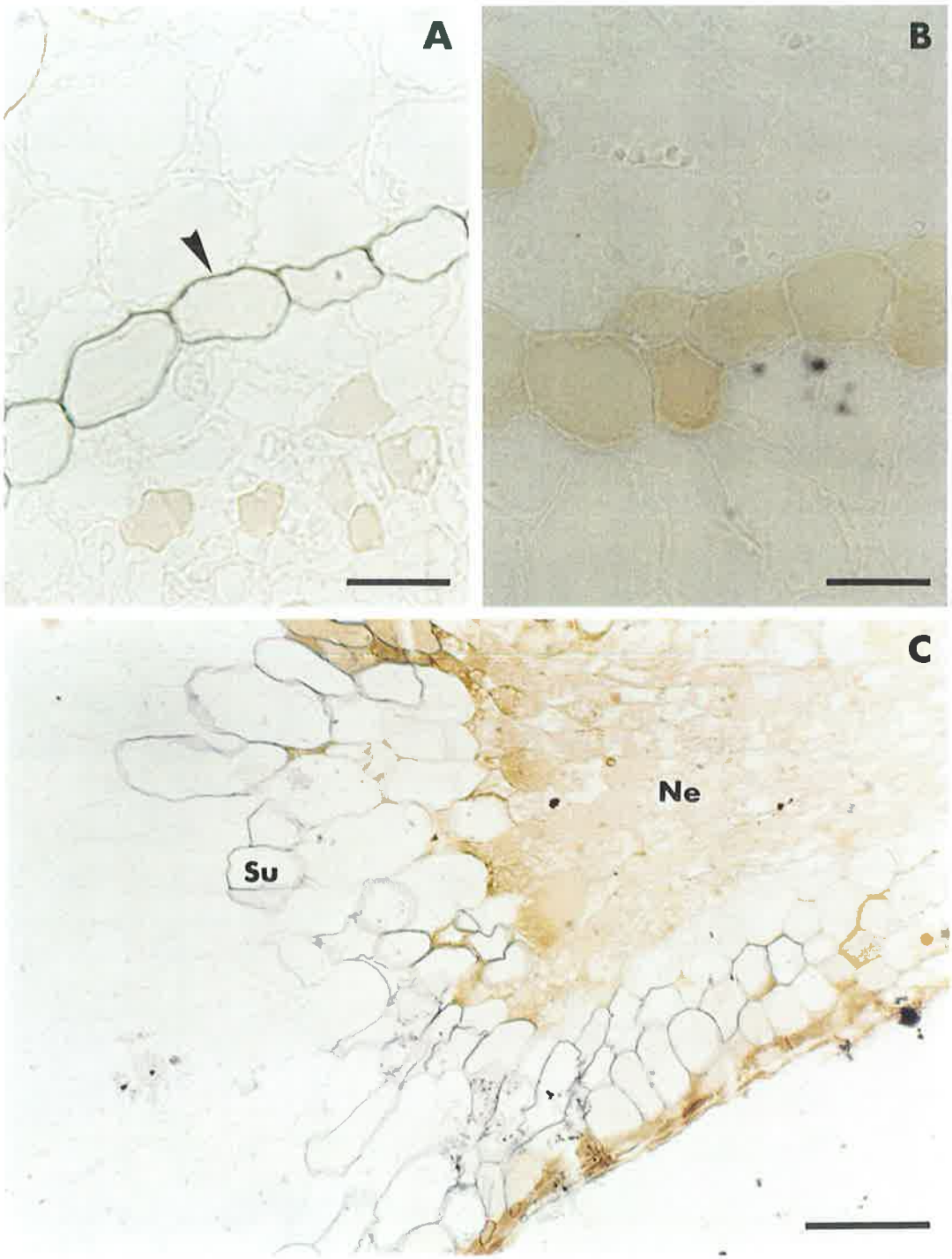
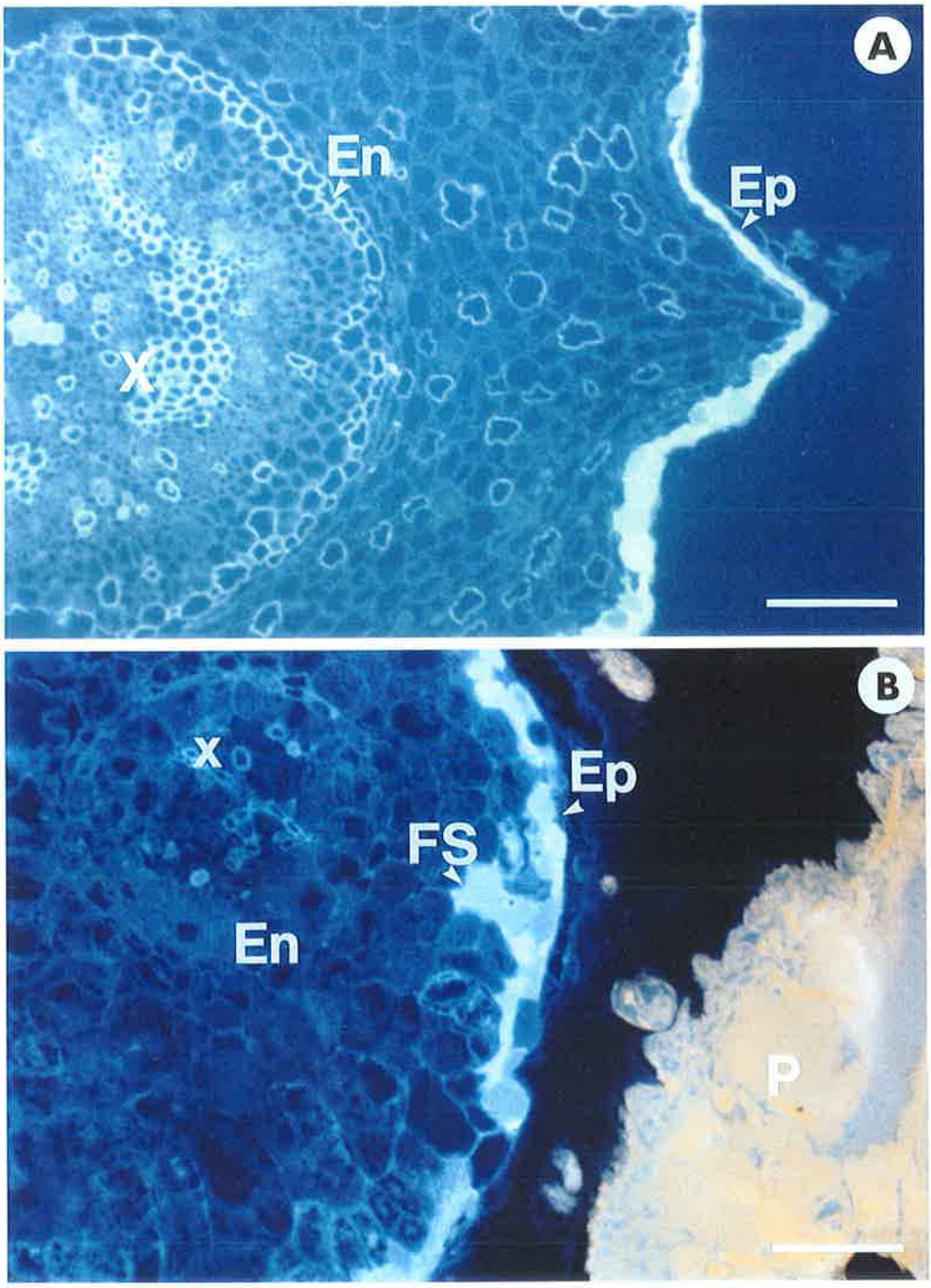

A
B
C
Ne
Su

Figure 4.6 Transverse sections through *V. vinifera* cv. Shiraz roots under UV excitation. a) Uninfested root: suberised epidermis (Ep); endodermis (En); lignified xylem vessels (X). b) Nodosity: epidermis (Ep); non-differentiated endodermis (En); lignified xylem (X); phylloxera (P); possible feeding site (FS). Scale bars equal 100µm.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was the state of suberisation of the endodermis. Although the nodosity shown in figure 4.6b was relatively young (indicated by the relative size of the phylloxera), the endodermis was only suberised on the side distal to the feeding site (the phylloxera is shown 'head down'). The stele of the nodosity was also generally less differentiated than that of the uninfested root, and xylem vessels were less clearly defined. The epidermis of the nodosity appeared to have been affected little by the presence of the phylloxera, although the cortical cells lacked the primary vacuolation normally seen in uninfested roots, possibly due to the presence of phenolic compounds and/or amyloplasts. Observation of a stylet track in a nodosity under UV excitation (figure 5.12a) further demonstrated the lack of even any very localised accumulation of autofluorescent compounds at the feeding site.

4.3.4 Starch accumulation in nodosities

Following the observation of starch accumulation in the cortex of nodosities (figure 4.3), samples of uninfested roots and nodosities at two different stages of development were assayed for starch content, in order to estimate the timing of starch accumulation. The approximate age of nodosities was estimated by size. Because only frozen nodosities were available, it was not possible to determine their stage of development in terms of the instar of the phylloxera as described in nodosity development, section 4.3.2. Similarly, it was not possible to establish the exact age of nodosities on the basis of the time of inoculation; phylloxera crawlers may not settle for several days. Based on an estimation of the lifecycle of VWL-1 phylloxera on *V. vinifera*, nodosities used in this assay were estimated to be either less than a week old ('small nodosities', e.g. figure 4.2a) or greater than four weeks old ('large nodosities', e.g. figure 4.2 i, j).

The concentration of starch in nodosities ranged between approximately 10 and 17 times that of uninfested root tips (table 4.1). The concentration in small nodosities was slightly higher than that of older nodosities, indicating that the onset of starch accumulation was very rapid, and preceded any significant expansion of the root cortex.

Table 4.1 Concentration of starch in uninfested roots or nodosities of *V. vinifera* cv. Shiraz.

tissue sample	mean starch concentration µg/mg fw[1]	
	rep. 1	rep. 2
uninfested root tips	1.80	2.89
small nodosities	30.52	31.55
large nodosities	19.72	26.63

[1] Each replicate represents individual tissue samples, each result being the mean of duplicate assays on these samples.

4.3.5 Free phenolic content of uninfested roots and nodosities

Phenolic compounds make up a high proportion of the dry weight of grapevine roots (Mattivi *et al.* 1998), and it is likely that they may be toxic to phylloxera, as they are to other insects (Todd *et al.* 1971, Cole 1984, Son *et al.* 1991). A change in concentration or composition of phenolic compounds (phenolic profile) could occur during the development of nodosities in a compatible (susceptible) interaction. Such changes might be induced by phylloxera to make the phenolic-rich tissue a more suitable food source. Alternatively, the phenolics may play a role in grapevine defence against phylloxera, and therefore a change in the phenolic profile may occur as part of an induced defence response.

The free phenolic content of samples of both uninfested roots and nodosities from tissue cultured vine roots was compared (figure 4.7, table 4.2). The putative identification of compounds detected as HPLC peaks is discussed in more detail in Chapter 5, section 5.3.3, table 5.4. There did not appear to be any significant qualitative changes in the profiles between uninfested root and nodosity tissue. One interesting difference detected, however, was a change in the absorption spectrum of peak number three (e.g. figure 5.14). A possible explanation for this is discussed briefly later in this chapter, and in more detail in Chapter 5, section 5.4.2.

It was not possible to make quantitative comparisons with these data because of the variation between replicate extracts. This resulted from both the small sample size (two to three nodosities make up a 100mg sample), and the naturally high variation in the distribution of phenolics in root tissues (F. Mattivi, Instituto Agrario, San Michele all'Adige, Italy, pers. comm.). Similar quantitative variation was discussed in section 3.2.3. It is thus important when interpreting results to be aware of the natural variation present.

The area of peak number three in the replicate samples of uninfested roots is indicative of localised differences in phenolic profiles between samples. In an earlier comparison of the phenolic profile of root tips compared with older well developed roots, (using chromatography method four, section 3.2.2), a significant difference was found in the area of peak 3 (data not shown). In older roots it was a major constituent of the free phenolic profile, while in root tips it was only a minor constituent, possibly because of the naturally high β-glucosidase activity in root tips (Rumpenhorst and Weischer 1978). Replicate one of the uninfested roots in table 4.2 may therefore represent tissue sampled closer to the root tip than replicate two.

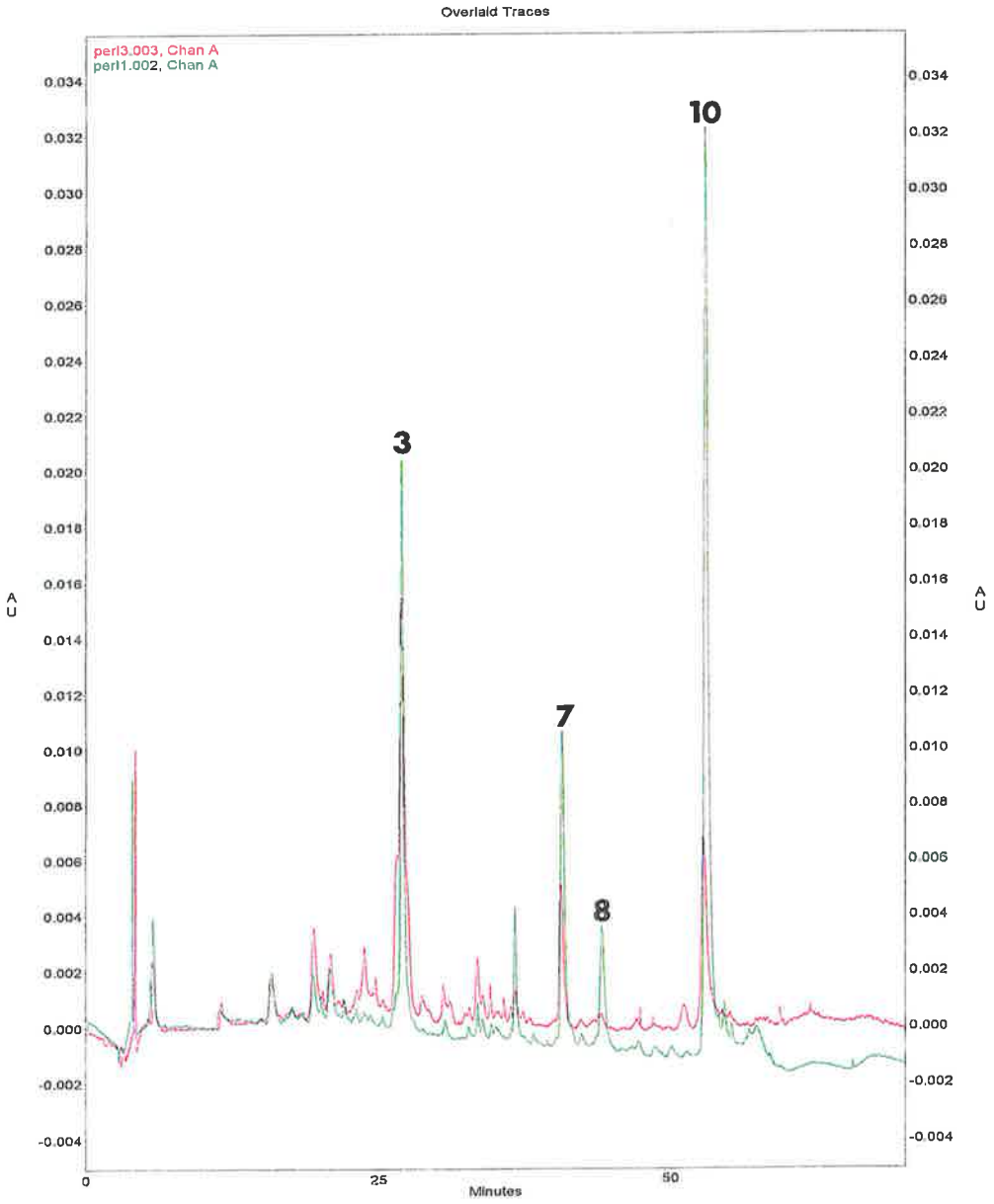

Figure 4.7 HPLC traces showing absorption profiles at 330nm for extracts from uninfested *V. vinifera* root (green) and nodosity (red). Numbers indicate peaks as discussed in section 5.3.3 and table 5.3.

Table 4.2 Peak areas for four major HPLC peaks detected at 330nm in extracts from nodosities and uninfested roots of tissue cultured *V. vinifera* cv. Shiraz. Peak areas ($AU \times 10^{-5}$) are given for extracts prepared from replicate samples from the same plant.

	uninfested root		nodosity		
peak no.[1]	rep. 1	rep. 2	rep. 1	rep. 2	putative identity[2]
3	3.66	43.43	3.74	5.54	*trans*-resveratrol 3-β-glucoside (piceid) plus unknown
7	2.31	8.32	2.12	0.92	ε viniferin
8	n	1.22	n	n	r-2-viniferin
10	7.95	10.52	2.87	1.77	r-viniferin

n: not detectable.
[1] Peak numbers refer to figure 4.6 and table 5.3.
[2] Putative identification of peaks is discussed in section 5.3.3.

From these results it appears that there was no significant reduction in the total amount of soluble phenolics in nodosities, nor was there a consistent accumulation of any novel soluble phenolic compounds. Extracts of root samples from infested and uninfested potted *V. vinifera* vines were also analysed by HPLC (data not shown), but did not show any significant difference from the results presented here.

Commercially available preparations of a number of free phenolic acids were run under the same HPLC conditions (Appendix five, table A5.1), allowing them to be compared to every peak in the profiles of these extracts. No peaks corresponding to any of these compounds were detected in the *V. vinifera* uninfested root or nodosity extracts.

4.3.6 Free amino acid and amide content of uninfested roots and nodosities

The total concentration of free amino acids and amides in nodosities (table 4.3) was more than double that in uninfested roots. There were also changes in the relative amounts of amino acids and amides. The nodosity:root ratio of their concentration varied from 0.89 (aspartic acid) to 26.66 (histidine). Aspartic acid was almost the only compound to decrease in concentration relative to the total amino pool (from 49% of the total pool in roots to 17% of the total pool in nodosities), while all other amino acids except ornithine were increased, relative to the total pool. Glutamine, in particular, increased from 13.76% to 30.78% of the total pool, to become the predominant form of free amino nitrogen.

Table 4.3 Mean measurements of free amino acids and amides in uninfested roots and nodosities from potted *V. vinifera* cv. Shiraz vines.

	mean conc. (μg/g fw) in uninfested roots	% total amino acid pool[1]	mean conc. (μg/g fw) in nodosities	% total amino acid pool	ratio nodosity:root[2]
aspartic acid	653.64	49.75	581.39	17.60	0.89
glutamic acid	209.15	15.92	448.68	13.58	2.15
hydroxyproline	6.66	0.51	20.08	0.61	3.02
asparagine	36.07	2.75	212.89	6.44	5.90
glutamine	180.83	13.76	1016.88	30.78	5.62
serine	22.76	1.73	84.35	2.55	3.71
histidine	1.28	0.10	34.13	1.03	26.66
glycine	7.09	0.54	14.80	0.45	2.09
threonine	12.15	0.92	81.42	2.46	6.70
alanine	47.32	3.60	143.33	4.34	3.03
GABA	50.57	3.85	87.30	2.64	1.73
proline	25.18	1.92	154.51	4.68	6.14
tyrosine	8.42	0.64	32.49	0.98	3.86
arginine	9.72	0.74	139.28	4.22	14.33
isoleucine	10.07	0.77	60.48	1.83	6.01
leucine	2.45	0.19	8.21	0.25	3.35
valine	7.40	0.56	32.77	0.99	4.43
methionine	7.35	0.56	42.76	1.29	5.82
phenylalanine	6.28	0.48	87.51	2.65	13.93
ornithine	3.54	0.27	2.47	0.07	0.70
lysine	6.00	0.46	18.39	0.56	3.07
Total	**1313.90**	**100.00**	**3304.13**	**100.00**	**2.51**

[1] Amount of each as % of total amino acid/amide pool.
[2] Ratio between amino acid concentration in nodosities and uninfested roots.

4.3.7 Gene expression in uninfested roots and nodosities

Soluble protein profiles

Changes in gene expression in response to phylloxera feeding might be expected in the light of predicted increased metabolic activity and starch accumulation in nodosities. These changes could be reflected in the accumulation of novel proteins, or down-regulation of constitutively expressed proteins. Analysis of root extracts by SDS-PAGE was used as a preliminary assay for detecting differences in protein profiles of uninfested roots and nodosities (figure 4.8).

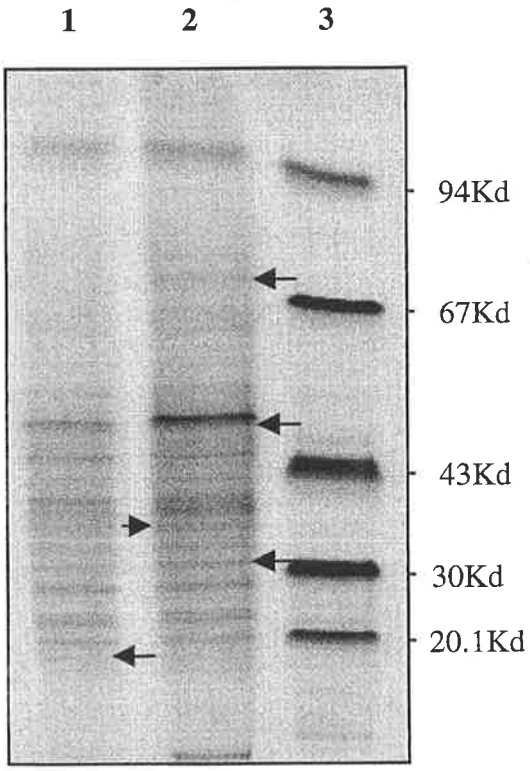

Figure 4.8 SDS-PAGE separation of total protein extracts of *V. vinifera* cv Shiraz roots. lane **1**) uninfested roots; lane **2**) nodosities; lane **3**) molecular weight marker. Arrows mark bands potentially altered in concentration between uninfested root and nodosity tissues. Extracts were prepared from the same amount of tissue (g fw) but twice the amount of extract was loaded in lane one than in lane two.

No major differences were detected, but indications of minor differences included enhanced accumulation of proteins of approximately 31.5, 34, 53 and 70kD in nodosities, and reduced accumulation of a protein of approximately 20kD. The failure of this method to detect major differences suggested that a more sensitive method, such as targeting the expression of specific genes by northern blot hybridisation, might be necessary to detect changes in gene

expression. The concentration of total soluble protein (per g fw), as estimated by SDS-PAGE, in extract from nodosities was approximately double that in uninfested roots.

Northern blot hybridisation analysis of gene expression

Northern blots containing total RNA isolated from both uninfested roots and nodosities were hybridised with a range of grapevine cDNA probes, obtained from various sources, as listed in table 4.4. The steady state levels of the hybridising transcripts in uninfested roots and nodosities from *V. vinifera* vines are illustrated in figures 4.9-4.12.

Stilbene synthase (StSy) transcripts were present at low to moderate levels in both roots and nodosities (figure 4.9b). Chalcone synthase (CHS) transcripts were present at higher levels in both roots and nodosities (figure 4.9c). PAL transcripts were present at moderately high levels in both roots and nodosities (figure 4.9d), with possibly slightly higher levels in nodosities. Hybridisation of PPO was at a low level and the signal was indistinct in both uninfested roots and nodosities (figure 4.9e).

VvTL1 (figure 4.10b), VvPR4a (figure 4.10d) did not appear to be expressed in roots or nodosities. VvTL2 (figure 4.10c) transcripts were present at low to moderate levels in both roots and nodosities.

The hybridisation signal for Grip 13 (figure 4.11b) was strong, but of the wrong transcript size (1.4 and 1.8kb instead of the expected 1.2kb). Grip 15 (figure 4.11c), Grip 28 (figure 4.11d) and Grip 68 (figure 4.11f) transcripts were present at moderate levels in both roots and nodosities. Grip 31 (figure 4.11e) did not appear to be expressed in either tissue.

Sucrose transporter 12 showed little to no expression in roots or nodosities (figure 4.12b); sucrose transporter 27 (figure 4.12c) and Grip 21 (figure 4.12d)showed moderate expression in both.

Northern blots containing total RNA isolated from leaves of both infested and uninfested vines were hybridised with probes for phenylalanine ammonia-lyase, stilbene synthase, VvTL1 and VvTL2. Steady state transcript levels were similar in both tissue types for all probes (data not shown).

Figure 4.9 Northern hybridisation of *V. vinifera* cv. Shiraz root and nodosity total RNA with defence-related probes. For each hybridisation: left lane 10µg uninfested root RNA, right lane 10µg nodosity RNA. a) Ethidium bromide stained lanes showing relatively even loading of RNA. b) Stilbene synthase probe cDNA clone pSV696. c) Chalcone synthase probe cDNA clone pBS305. d) Phenylalanine ammonia-lyase probe cDNA clone pBS204. e) Polyphenol oxidase probe cDNA clone pID96.

Figure 4.10 Northern hybridisation of *V. vinifera* cv. Shiraz root and nodosity total RNA with defence-related probes. For each hybridisation: left lane 10µg uninfested root RNA, right lane 10µg nodosity RNA. a) Ethidium bromide stained lanes showing relatively even loading of RNA. b) Thaumatin-like protein probe cDNA clone VvTL1. c) Thaumatin-like protein probe cDNA clone VvTL2. d) PR-4 type protein probe cDNA clone VvPR4a.

Figure 4.11 Northern hybridisation of *V. vinifera* cv. Shiraz root and nodosity total RNA with nodule-related probes. For each hybridisation: left lane 10µg uninfested root RNA, right lane 10µg nodosity RNA. a) Ethidium bromide stained lanes showing relatively even loading of RNA. b) Nodule cell wall-related protein cDNA clone Grip 13. c) Nodule cell wall-related protein cDNA clone Grip 15. d) Nodule cell wall-related protein cDNA clone Grip 28. e) Nodule pericycle-related protein cDNA clone Grip 31. f) Nodule pericycle-related protein cDNA clone Grip 68.

Figure 4.12 Northern hybridisation of *V. vinifera* cv. Shiraz root and nodosity total RNA with sugar-related probes. For each hybridisation: left lane 10µg uninfested root RNA, right lane 10µg nodosity RNA. a) Ethidium bromide stained lanes showing relatively even loading of RNA. b) Sucrose transporter cDNA clone VvSUC12. c) Sucrose transporter cDNA clone VvSUC27. d) Sucrose starvation-related protein cDNA clone Grip 21.

Figure 4.9

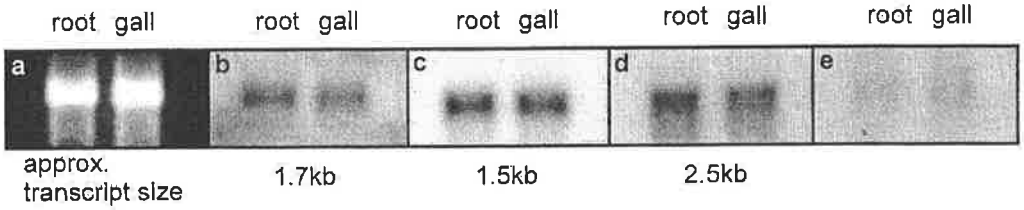

Figure 4.10

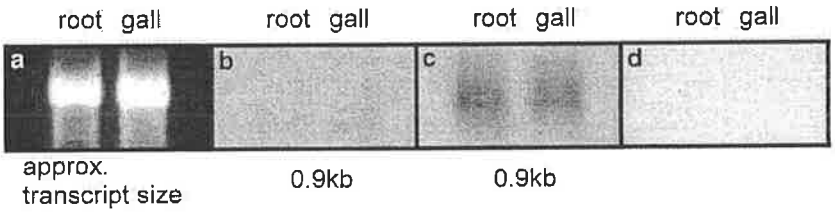

Figure 4.11

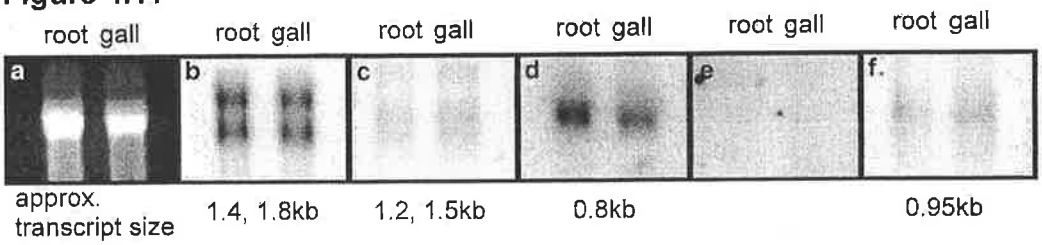

Figure 4.12

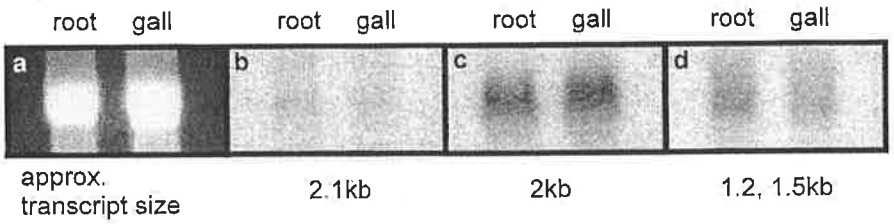

Table 4.4 cDNA clones used as probes in northern blot hybridisations. All clones are from *Vitis spp.* Reported expression patterns refer to those seen in *V. vinifera* by other researchers as listed in references.

Gene/ cDNA	**Clone/ Genbank acc. no.**	• **Reported function or closest homologous gene** • **Previous reports of expression pattern in grapevine roots**	**Source/ reference.**
Phenylalanine ammonia-lyase VvPAL	pBS204 X75967	• Key regulatory enzyme in phenylpropanoid pathway; upregulated in *V. vinifera* on fungal elicitation. • Expression in grapevine roots unknown, but constitutive in some other tissues.	F. Sparvoli, Universita degli Studi di Milano, Milan, Italy. (Boss *et al.* 1996, Sparvoli *et al.* 1994)
Stilbene synthase VvStSy	pSV696 X76892	• Catalyses synthesis of resveratrol and derivatives; induced in cell cultures by fungal elicitation. • Expression in grapevine roots unknown, but constitutive in some other tissues.	H. Kindl, University of Marburg, Germany. (Melchior and Kindl 1991)
Chalcone synthase VvCHS	pBS305 X75969	• First dedicated enzyme in flavonoid synthesis pathway. • Expression in grapevine roots unknown.	F. Sparvoli, Universita degli Studi di Milano, Milan, Italy. (Boss *et al.* 1996, Sparvoli *et al.* 1994)
Polyphenol oxidase GPO1	pID96 A27657	• Polyphenol oxidase from grape berries. • Expressed at high levels in young grapevine roots, but not mature tissues.	I. Dry, CSIRO, Adelaide, South Australia. (Dry and Robinson 1994)
Thaumatin-like protein VvTL1	pTL3 AF003007	• Thaumatin-like PR protein; expression not induced in leaves. • Not expressed in grapevine roots.	D. Tattersall, University of Adelaide, SA. (Tattersall *et al.* 1997)
Thaumatin-like protein VvTL2	Y10992	• Thaumatin-like PR protein; expression induced in leaves on pathogen attack. Homologous to osmotin-like protein from *V. vinifera.* • Expression in grapevine roots unknown.	Dry, CSIRO, Adelaide, SA. (Jacobs *et al.* 1999)
PR-4 protein VvPR4a	pWIN52 AF061329	• PR4-like protein. • Not expressed in grapevine roots.	D. Tattersall, University of Adelaide, SA. (D. Tattersall, pers. comm.)

Table 4.4 continued

Gene/ cDNA	Genbank acc. no.	• **Reported function or closest homologous gene** • **Previous reports of expression pattern in grapevine roots**	**Source/ reference.**
Grip 13	AJ237983	• Homology to nodule cell wall protein from yellow lupin. • Not expressed in grapevine roots.	C. Davies, CSIRO, Adelaide, SA. (Davies and Robinson 2000)
Grip 15	AJ237984	• Homology to nodule cell wall protein from *Maackia amurensis*. • Not expressed in grapevine roots.	C. Davies, CSIRO, Adelaide, SA. (Davies and Robinson 2000)
Grip 28	AJ237985	• Some similarity to pectin methylesterases, may be involved in carbohydrate binding; homology to protein from alfalfa nodules. • Not expressed in grapevine roots.	C. Davies, CSIRO, Adelaide, SA. (Davies and Robinson 2000)
Grip 31	AJ237986	• Homology to protein in young nodule pericycle and in older senescent nodule cells in *Alnus glutinosa*. • Expressed in grapevine root at a low level.	C. Davies, CSIRO, Adelaide, SA. (Davies and Robinson 2000)
Grip 68	AJ237987	• Homology to Grip 31, homology to protein from potato stolon tip. • Expressed in grapevine root at a low level.	C. Davies, CSIRO, Adelaide, SA. (Davies and Robinson 2000)
Grip 21	AJ237988	• Related to proteins responding to glucose starvation in maize roots. • Expressed in grapevine root at a low level.	C. Davies, CSIRO, Adelaide, SA. (Davies and Robinson 2000)
Sucrose transporter 12 VvSUC12	AF021809	• Sucrose transporter from grape berries; homology to maize sucrose transporter. • Expression in roots unknown.	C. Davies, CSIRO, Adelaide, SA. (Davies *et al.* 1999)
Sucrose transporter 27 VvSUC27	AF021810	• Sucrose transporter from grape berries; homology to maize sucrose transporter. • Expression in roots unknown.	C. Davies, CSIRO, Adelaide, SA. (Davies *et al.* 1999)

4.4 Discussion

The formation of nodosities appears to be a complex process. A gradient of some sort, presumably hormonal, is established across the root by unknown means, causing swelling on one side of the root (possibly including hypertrophy of cortical cells (Niklowitz 1954), although there was little evidence of this noted here), and inhibition of cell expansion on the other as well as preventing differentiation (or possibly causing dedifferentiation) of the endodermis and in some cases, the stele. The accumulation of starch and amino nitrogen in the cortex suggest the initiation of a strong sink for photoassimilates.

There is little published information available as to the mechanisms by which cecidogenic insects induce gall formation (for reviews see Forrest 1987, Hori 1992). There have been suggestions that IAA, either in saliva or synthesised *in planta* from tryptophan, is the main inducing agent for aphid galls (reviewed by Miles 1990). Miles (1990) further proposed that while IAA levels in aphid saliva may not be high enough to entirely account for gall induction, inhibition of IAA oxidase activity in the plant, brought about by competing oxidative reactions induced by aphid saliva, may allow endogenous IAA levels to increase to the extent that they cause gall formation.

Mechanisms proposed (although not demonstrated) for the induction of phylloxera galls are reviewed by Hori (1992). Chemicals demonstrated to be gall-inducing agents on grapevine roots under experimental conditions range from amino acids (e.g. tryptophan, with the effect intensified by added lysine and valine) or IAA, or a combination of both, to a simple phosphate buffer (the disruption of meristematic tissues being cited as the causative agent in this case (Miles 1968)). Cecidogenesis remains poorly understood, and mechanisms for the induction of gall formation by phylloxera remain to be elucidated.

4.4.1 Location of the feeding site and localised root response

The feeding site of phylloxera within primary roots was clearly shown to be in the upper regions of the cortex. Most aphids feed from phloem tissue, however there are a number of aphid groups (e.g. Adelgids) which feed from parenchymatous tissue (Pollard 1973). The stylet tracks observed in this study showed that the stylet penetrates intracellularly, and the insect feeds from one, or possibly from a column of adjacent cells, progressing successively towards the stele. Without the use of transmission electron microscopy (TEM), it is not possible to determine if phylloxera penetrates both the cell wall and the plasmalemma. For example, the ring nematode *Criconomella xenoplax* feeds from cortical root cells in a manner which has parallels with the feeding of phylloxera (Hussey *et al.* 1992). The stylet of *C. xenoplax* appears to penetrate the cortical cell from which it is feeding, however

ultrastructural study has shown that the plasmalemma of the cell is not pierced. The aperture of the stylet is closely appressed to the plasmalemma, and the nematode feeds through a small (c. 150nm) hole.

The implications of the location of phylloxera's feeding site are two-fold. First, by feeding from cortical cells, rather than phloem, phylloxera are potentially exposed to the various cellular chemicals stored in the vacuoles, cytosol, periplasmic and apoplastic spaces. Second, phylloxera apparently feed from a localised region of one or only a few cells. They are predominantly sedentary feeders, and more than one stylet track was not seen per feeding site in roots of susceptible *V. vinifera* vines. Also, few cells appeared damaged within the feeding site. From such feeding sites phylloxera are able to gain sufficient nutrition to support the production of a significant amount of biomass; they have been shown to be able to lay, on average, three to six eggs per day for around 30 days when feeding on a susceptible vine variety (Granett *et al.* 1983, de Benedictis *et al.* 1996). These facts suggest that phylloxera are able to induce enhanced mobilisation of nutrients to the feeding site. This might occur via an apoplastic or symplastic route, or both.

The changes seen in cortical cells in the immediate path of the stylet, or adjacent to it, were similar to those reported by Niklowitz (1954), in particular, the collapsed plasmalemma, 'granular' looking contents of nearby cells, accumulation of tannins (polyphenolics) and 'bubble filled looking structures' (noted here as 'vesicles') in penetrated cells. These observations are difficult to interpret at the level of magnification which can be achieved using light microscopy. The use of TEM to better interpret the ultrastructure of the food cells and the cells immediately surrounding them would be valuable in this respect.

Deposition of suberin lamellae was observed in the walls of cells surrounding a necrotic region in a nodosity undergoing the early stages of decay. There is a parallel here with the response to phylloxera seen in secondary roots of resistant vine varieties, where the development of a suberised wound periderm isolates and protects the root from extensive phylloxera damage (Boubals 1966a, King and Rilling 1991). It is possible that here we have observed a related response in the *V. vinifera* nodosity. Clearly, however, the response in *V. vinifera* roots was not strong enough, or not rapid enough to protect the root from either phylloxera attack or subsequent nodosity decay.

4.4.2 Changes in the endodermis and possible implications for photosynthate unloading

One possible explanation for the increased levels of starch and amino acids in nodosities is that phylloxera feeding might result from enhanced unloading of solutes from the phloem

and into the root cortex. The main carbohydrate imbibed by aphids is sucrose (Klingauf 1987), which is also the main solute transported in the phloem (Salisbury and Ross 1992, Gholami 1996). Presumably, therefore, enhanced phloem unloading would specifically contribute to the high levels of nutrients which must be required to support the continued production of biomass throughout phylloxera development. This study has shown that nodosities possess structural features which might play a role in phloem unloading.

Firstly, the lack of radial cell expansion in both the cortex and stele has the effect of bringing the feeding site of the phylloxera closer to the phloem inside the stele. Secondly, transport of nutrients to the feeding site may be facilitated by the normal development of vascular tissue. Finally, and importantly, the endodermis in nodosities lacks the detectable suberin lamellae seen in uninfested roots. Endodermal structure can influence both apoplastic and symplastic transport pathways and will be discussed further below.

Symplastic transport of solutes

Plasmodesmata probably play an important role in phloem unloading and transport of solutes to the feeding site. Wright and Oparka (1996, 1997) have demonstrated that the symplastic pathway (via plasmodesmata) of solute transport is the normal route for solute unloading into the root elongation zone. Despite this pathway being considered the 'norm' for roots (Fisher and Oparka 1996, Wright and Oparka 1996, 1997), both Patrick and Offler (1996) and Wright and Oparka (1997) suggest that the rate of transport through 'normal' plasmodesmata may not be sufficient to account for the magnitude of solute movement into strong sinks such as actively growing root tips. Presumably the same considerations would apply to movement of solutes into phylloxera galls, suggesting that either enhanced transport through plasmodesmata, or enhanced movement of solutes via an apoplastic route is required.

Plasmodesmata have been shown to be dynamic structures which normally remain constricted through an active ATP-dependant process, and their enlargement can effect enhanced phloem unloading (Wright and Oparka 1997, Patrick and Offler 1996). Plasmodesmatal widening accompanies increased unloading of solutes from phloem in root tips of pea under osmotic stress (Schulz 1995). It is possible that alteration in the size of plasmodesmata in sink tissues might also result from a change of the sink/source ratio or of the osmotic gradient for sucrose across the cortex (Wright and Oparka 1997).

The grapevine root pest dagger nematode, *Xiphinema index,* feeds near the root tip, and causes galling involving cellular hypertrophy (Rumpenhorst and Weischer 1978). The feeding site of *X. index* consists of both multinucleate giant cells and mononucleate

hypertrophied cells, both of which have enlarged pitfields, presumably resulting in the enhanced import of solutes. A study of the ultrastructure of feeding cells of the ectoparasitic nematode *Criconomella xenoplax*, which feeds from single cells in the root cortex in a similar manner to phylloxera, shows a striking enlargement of plasmodesmata connecting the 'food cell' with the adjoining cells (Hussey *et al.* 1992). This enlargement would greatly facilitate the symplastic transport of nutrients, and is proposed as the mechanism by which these relatively large nematodes obtain their nutrients over several days from a single cell. There is no evidence yet to support the widening of plasmodesmata or the occurrence of altered pit fields at the phylloxera feeding site, however parallels between feeding of phylloxera and these nematodes mean the possibility warrants investigation.

Apoplastic transport of solutes

The enhanced movement of solutes into strong sinks such as growing root tips and phylloxera galls could also occur via an apoplastic route. Wright and Oparka (1997) claim that there is no apoplastic movement of the phloem mobile dye carboxyfluorescein into normal growing root tips, but admit that this may not be indicative of the transport of sucrose. Böckenhoff *et al.* (1996) were able to demonstrate apoplastic transport of solutes into cyst nematode-induced syncytia developing within the stele of the root. They used carboxyfluorescein to show that the syncytium was symplastically isolated from surrounding root tissue. They also demonstrated that the phloem unloading which occurred at syncytia along the length of the root was highly specific for syncytia and did not occur in other tissues. Apoplastic unloading in this case appears to be a very specialised phenomenon, not simply the enhancement of normal functions of the root. One hypothesis as to how this apoplastic unloading was induced is that it resulted from the inactivation of proton pumps which act as sucrose transporters (Grundler and Böckenhoff 1997). These pumps normally function to retrieve sucrose which diffuses passively from the phloem, and the effect of their inactivation is the net 'leakage' of sucrose from the phloem into the apoplastic space. The sucrose might then move along a negative osmotic gradient through the apoplastic space into the sink region.

Role of the endodermis

The endodermis in primary roots of dicotyledons goes though two states of suberisation as it differentiates (Peterson and Enstone 1996). Figure 4.13 illustrates the positions of these states in a typical root relative to the root tip. State one endodermis, with only localised regions of suberin (the 'casparian strip') in the radial and transverse walls of the cells only restricts apoplastic movement of solutes (Peterson and Enstone 1996). Such cells occur very

early in endodermis differentiation in the zone of elongation behind the root tip. This zone has also been shown, by the use of fluorescent tracers and C14 labelling, to be the site of phloem unloading in a growing root (Oparka *et al.* 1994, 1995) suggesting that the relatively non-suberised endodermis allows unrestricted symplastic transport of solutes across the root. This would effect rapid movement of solutes away from the phloem, creating a sucrose gradient between sieve cells and transfer cells and enhancing the observed phloem unloading (Patrick 1990). In this study, plasmodesmata in the zone of elongation of an uninfested grapevine root have been shown to be abundant (figure 4.4), confirming the plausibility of a symplastic pathway for transport of solutes into growing grapevine root tips.

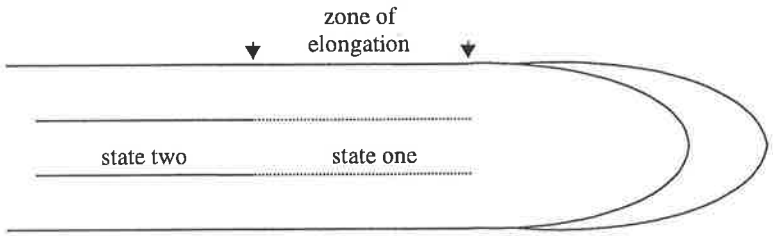

Figure 4.13 State of differentiation of endodermis in a longitudinal section of a dicotyledon root (from Peterson and Enstone 1996).

State two endodermis has all walls thickened with a suberin lamella which results in constriction of the plasmodesmata of endodermal cells, and consequently reduced symplastic transport of solutes to the root cortex (Warmbrodt 1985). This greatly restricts the flow of water and solutes across the endodermis. The endodermis in primary roots of *V. vinifera*, as seen in figure 4.5a quickly differentiated to state two in potted vines.

The phloem unloading which occurs in the zone of elongation may be the factor which attracts phylloxera to settle in this region (see section 4.3.2). Phylloxera feeding appeared to inhibit further differentiation, especially suberisation, of the endodermis. The development of nodosities described in this study showed that the endodermis was often not differentiated to the same extent as in uninfested roots, and that even when it was well defined it had either state one suberisation, or was not suberised at all. This probably resulted from an inhibition of endodermis differentiation, rather than dedifferentiation, since it is unlikely that phylloxera could cause the loss of secondary wall deposits such as suberin lamellae. Petri (1907, cited in Niklowitz 1954) also commented on prevention of differentiation of the endodermis by feeding phylloxera.

The non-suberisation, or in some cases non-differentiation, of the endodermis in nodosities possibly enables these organs to function as a strong nutrient sink through unrestricted apoplastic and/or symplastic (via more open plasmodesmata) transport throughout their development. The observation that phylloxera show no apparent preference for root tips in tissue culture (perlite-based medium) cocultivations, where it remained in state one for the entire length of the root (figure 5.6a), further supports this hypothesis.

The potential for enhanced symplastic movement of solutes across the endodermis in response to phylloxera feeding could be assessed through ultrastructural observations of plasmodesmata abundance and the use of symplastic tracers such as carboxyfluorescein during nodosity formation. If symplastic transport seems insufficient to account for the sugar (and starch) accumulation in nodosities, the hypothesis that non-suberisation of the endodermis also enhances apoplastic solute transport would gain support.

4.4.3 Starch accumulation in nodosities

The accumulation of starch in the cortex of galled roots is an indication that establishment of a phylloxera feeding site does indeed enhance the transport of sucrose into the nodosity. Starch is normally rapidly turned over in growing primary roots (Farrar 1991), and in this study amyloplasts in uninfested roots were small and scattered throughout the root cortex. In nodosities, however, abundant amyloplasts were observed in PAS-stained sections, and enzymatic assays show the starch content to be around 3% (w/fw), at least ten times that of uninfested roots. This level of starch accumulation can be compared with a starch storage organ such as a potato tuber, which contains 10-15% w/fw starch. (C. Davies, CSIRO Plant Industry, Adelaide, SA. pers. comm.). The accumulation of starch could result from a direct effect on the starch synthesis pathway caused by phylloxera feeding, or it may be an indirect effect of other physiological changes within the root cortex.

The presence of starch in nodosities as detected by PAS staining appeared to be relatively variable from section to section (figure 4.2). It is possible that accumulation of starch may be localised to the feeding site of the phylloxera within the nodosity. Its detection by microscopy would then be determined by the proximity of the section to the feeding site. Also, the number of phylloxera on any one nodosity might determine the total starch content of that nodosity, and the differences in phylloxera numbers might then explain the variation in starch levels detected enzymatically. If starch is a food source for phylloxera, more phylloxera on a single nodosity would result in less starch accumulation. If it is not a food source, however, the presence of more phylloxera might induce higher levels of accumulation. Serial sections through several entire nodosities would help to elucidate this.

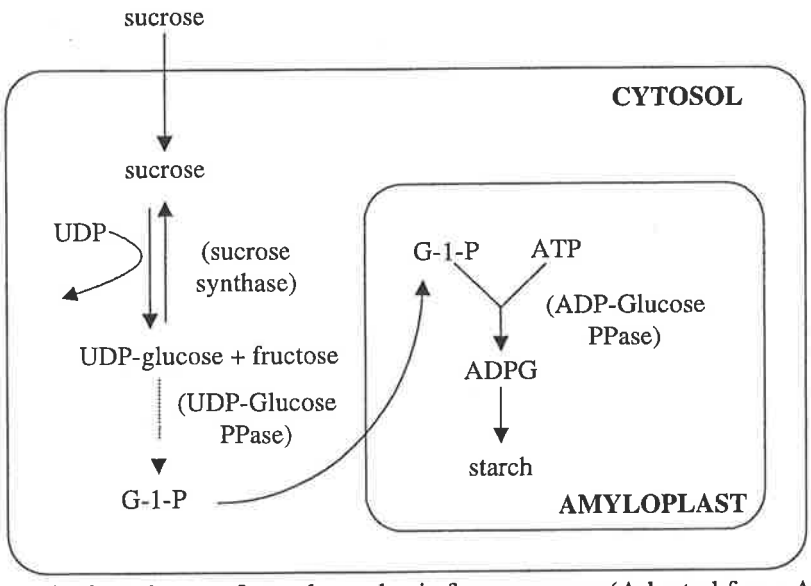

Figure 4.14 Pathway of starch synthesis from sucrose (Adapted from Atwell *et al.* 1999).

The metabolism of sucrose imported into sink tissues is summarised in figure 4.14. Sucrose is imported directly into sink tissues as the major component of phloem sap (Salisbury and Ross 1992). UDP-glucose is formed in the cytosol by sucrose synthase, then converted to G-1-P by UDP-glucose pyrophosphorylase. Following transport of G-1-P into the amyloplast, it is converted to ADP-glucose by ADP-glucose pyrophosphorylase. Starch is synthesised from ADP-glucose via a complex pathway involving polymerisation into chains by starch synthase and branching of the chains by a branching enzyme to form the polymer amylose (Mares *et al.* 1985, Ziegler 1991, Atwell *et al.* 1999). The exact pathway for starch synthesis remains unclear, but at least two enzymes (starch synthase and ADP-glucose pyrophosphorylase) appear to be involved.

Starch synthesis requires the supply of substrate (UDP-glucose), and sucrose import depends on a negative sucrose gradient (Sung *et al.* 1989, Patrick 1990, Wang *et al.* 1993), hence both the rate of starch synthesis and the strength of the photosynthate sink appear to be determined by the rate at which imported sucrose is metabolised. This metabolism may be catalysed by invertases or by sucrose synthase, however, in organs where sucrose or starch are the main storage compounds the key enzyme involved is sucrose synthase (Sung *et al.* 1989, Wang *et al.* 1993, Zrenner *et al.* 1995, Koch 1996). Wang *et al.* (1993) also suggest that in some organs other enzymes such as ADP-glucose pyrophosphorylase may be involved in regulating the rate of starch synthesis.

It is possible that phylloxera feeding causes upregulation of sucrose synthase (and possibly other enzymes responsible for starch polymerisation) thus increasing the sink strength of the tissue, as well as resulting in increased accumulation of starch. This could occur through

altered gene expression as a result of phytohormone activity induced by the phylloxera. In support of such a hypothesis, Blee and Anderson (1998) suggest that in the vesicular-arbuscular mycorrhiza-root interaction, plant growth regulators (compounds with auxin-, gibberellin- and cytokinin-like activities *in vitro*) produced by the mycorrhiza in arbusculated cells are responsible for upregulation of genes encoding sucrose metabolising enzymes, thus establishing a sink for sucrose.

The transcription of the sucrose-metabolising invertases and sucrose synthase is also sugar-responsive (Koch *et al.* 1992, Koch 1996). It appears that different isoforms of each enzyme class exist, the genes of which are either up- or down-regulated in response to variations in sugar concentration. Genes encoding enzymes involved in starch synthesis (ADP-glucose pyrophosphorylase and starch synthase) have also been shown to be positively responsive to sugar concentration in potato (Koch 1996). Clearly the regulation of these enzymes is under complex control. and it is possible that high starch accumulation in nodosities could also be precipitated, via gene upregulation, by a high level of sucrose import.

Analysis of the expression of sucrose synthase genes, and those encoding other enzymes/proteins involved in sugar transport and starch synthesis during nodosity formation may elucidate the mechanism(s) by which starch accumulation is effected. At present, the only cDNAs encoding these genes from grapevine are putative sugar transporters (Davies *et al.* 1999) and an invertase (Davies and Robinson 2000). Northern blot analysis using two of the sugar transporters and Grip 21 (a sugar starvation-related cDNA) showed only low to moderate accumulation of transcript in uninfested roots, and no change in transcript accumulation in nodosities. This might indicate that apoplastic transport of sucrose is not significant in roots and nodosities, or that transporters other than these are involved. Gene expression is discussed further in section 4.4.6.

The only suggested role for starch in nodosities comes from Cornu (1873, cited in Niklowitz 1954) who proposed that it provided an energy reserve for phylloxera. Root galls formed by the endoparasitic nematode *Nacobbus aberrans* (false root-knot nematode) also accumulate large amounts of starch, accompanied by the proliferation of abnormal phloem, which presumably enhances sucrose transport to the gall (Jones and Payne 1977). The quantity of starch in galls declined during the reproductive phase of the nematodes, which suggests its importance as an energy source for nematode feeding. Other gall-forming nematodes (e.g root-knot and cyst) induce only minor starch accumulation in plant roots, however these nematodes create much more elaborate and specialised feeding sites, which may obviate a need for photosynthate storage in this form.

4.4.4 Free phenolic content of uninfested roots and nodosities

The results of HPLC analyses of the free phenolic content of roots and nodosities showed that no significant qualitative changes in response to phylloxera feeding or gall formation could be detected by these methods. The simplest inference that can be drawn from these results is that *V. vinifera* cv. Shiraz does not have a phenylpropanoid-based defence response to phylloxera, which may not seem surprising as it is a susceptible variety. Other *V. vinifera* cultivars have also been noted to have little, or only very delayed accumulation of defence-related phenolics in response to fungal pathogen attack compared with resistant species (Dercks and Creasy 1989, Dai *et al.* 1995a, b).

The presence of potentially toxic compounds such as piceid (*trans*-resveratrol glucoside) and ε-viniferin as major chemical components of the roots clearly does not prevent establishment and survival of phylloxera; there was no consistent reduction in their concentration in nodosities compared with uninfested roots. The compound r-viniferin, which is probably the predominant phenolic component of *Vitis* roots (Mattivi and Reniero 1992), was found at reduced levels in nodosities, although the large degree of variation in the results from these HPLC analyses in general indicate that this apparent reduction may not be significant. The biological activity of r-viniferin has not been characterised. In general, the results of the HPLC analyses reported here do not support those of Sobetskiy and Derzhavina (1973) who had earlier reported a decrease in phenolic content in phylloxera leaf gall exudate (proposed to represent 'sap' ingested by the phylloxera), but an increase in total concentration of phenolics in leaf galls.

The change in absorption profile of HPLC peak three, which consists of *trans*-resveratrol glucoside plus at least one other co-eluting compound, is discussed in detail in Chapter 5, section 5.4.2. It appears to result from the de-glycosylation of the *trans*-resveratrol glucoside component of the peak. This may have the effect of removing a potentially toxic compound from storage in the root tissue and ultimately, since the aglycone is not accumulated to a significant level in *V. vinifera* cv. Shiraz, make the root more suitable as a food source. The aglycone could be further metabolised to form other stilbenes or stilbenoids, however, no novel compounds are detected in nodosities. If such novel compounds do occur in this variety, for example at very low concentrations, they do not appear to have any antimetabolic effect on phylloxera.

Denisova (1965) claimed that the free phenolics quercetin and chlorogenic acid were found in the nodosities of a susceptible grapevine (*V. vinifera* cv. Shasla) and that they act as 'gall enhancers'. In this study these compounds were not detected at all in either nodosities or uninfested roots of *V. vinifera* cv. Shiraz (section 4.3.5). As the HPLC method used was

adapted from one designed for investigation of chlorogenic acid content of (lettuce) roots (Cole 1984), and quercetin and chlorogenic acid were successfully tested as standard compounds (Appendix five), these compounds should have been detected if present.

In summary, it would appear that the concentrations of free phenolics found in *V. vinifera* roots in this study are not toxic to phylloxera. No other phenolic compounds were detected in concentrations that indicate that they have a direct role in gall formation.

4.4.5 Changes in free amino acid and amide profile

The profile of free amino acids and amides in uninfested roots was similar to that reported by Nassar and Kliewer (1966) with aspartic acid, glutamic acid and glutamine as predominant compounds. One difference was the content of arginine, reported by Nassar and Kliewer (1966) to comprise at least 55% of the total pool, while in this study it only comprised only 0.74%. This could be accounted for by the use of different cultivars of *V. vinifera* (Thomson Seedless compared with Shiraz) or root tissue type. Nassar and Kliewer do not indicate whether primary or secondary roots were used to generate the data presented, however since arginine is a nitrogen storage compound it may be assumed that they used secondary roots.

In this study, the concentrations of all amino acids and amides, except aspartic acid and ornithine, showed a marked increase in nodosities compared with uninfested roots. Glutamine increased in nodosities relative to uninfested roots to become the predominant form of amino nitrogen detected, constituting more than 30% of the total amino nitrogen pool. The content of asparagine also increased to become the fourth most predominant amino acid, although it still remains less than 7% of the total amino acid pool. The increases in the levels of almost all the amino acids are likely to be the result of increased phloem unloading and subsequent assimilation of amino nitrogen. Nitrogen is transported in the phloem in the form of nitrates and the amides asparagine and glutamine (Pate 1975, Sijmons *et al.* 1994), and amides are thought to become the prominent form of nitrogen transport into tissues during periods of nitrogen assimilation (Pate 1975). In *V. vinifera* cv Shiraz the concentration of amino acids and amides in the phloem has been show to vary throughout the season (Gholami 1996). As measured from sap exuded from severed peduncles, glutamine is the predominant from of amino nitrogen (48.4% of the total pool) although asparagine remains a minor component (0.7%). Glutamine can be transaminated to glutamate, the glutamate pathway being the major pathway for nitrogen assimilation in plants (Miflin and Lea 1982).

Changes in amino acid content in response to insect or pathogen attack have been reported for a number of plant species (Owens and Specht 1966, Stewart and Lahrer 1980, Dorschner 1990, Hedin and Creech 1998). Amino acid content and concentration have also been cited as being particularly important for aphid nutrition (McNeill and Southwood 1978, Dorschner 1990).

In phylloxera leaf galls the free amino nitrogen content was reported to be increased compared with uninfested leaves (Sobetskiy and Derzhavina 1973). Amino acid content was increased in the honeydew of the aphid *Aphis fabae* feeding from galls formed by the leaf rolling aphid *Dysaphis devecta* on apple (Forrest 1981) compared with those feeding on ungalled leaves. The honeydew of *D. devecta* itself also changed in amino acid and amide content over a period of two to nine days from the initiation of galling (Forrest 1987): phenylalanine dropped to undetectable levels, accompanied by a moderate rise in glycine, serine and lysine, and a dramatic rise in asparagine. In these examples the composition of honeydew is believed to be indicative of the composition of the food source. Asparagine in leaves also increases in response to rust infection, leaf hopper infestation and stress (reviewed by Stewart and Lahrer 1980).

McNeill and Southwood (1978) reviewed the role of nitrogen in insect-plant interactions. Requirements for particular amino acids vary, and may partly depend on the type of gut symbionts present, so that generalisations cannot be made about host suitability based on amino acid composition alone. Enhanced aphid survival and reproduction has, however, been correlated with levels of available amides. Both asparagine and glutamic acid play an important role in aphid nutrition, settling and development (Klingauf 1987). The presence of individual amino acids such as methionine, phenylalanine and leucine in artificial diets can enhance aphid uptake of sucrose solutions (Mittler 1988). The increase in amino acids in honeydew of *Aphis fabae* was correlated with their increased growth when feeding on galls of *Dysaphis devecta* (Forrest 1981). Some amino acids, on the other hand, may actually depress performance, for example phenylalanine with *Brevicoryne brassicae* (McNeill and Southwood 1978), although once again the authors point out that this is likely to be an effect specific to this interaction.

In plant-nematode interactions, the amino acid content of roots was increased by 27% in root-knot nematode-infested (susceptible) cotton (Hedin and Creech 1998), and by 304% in root-knot nematode-infested tomato roots (Owens and Specht 1966). Free amino acids were also increased in stem galls formed by the stem nematode *Ditylenchus dipsaci*, compared with uninfested stems on alfalfa and pea (Howell and Krusberg 1966). An increased glutamine concentration in roots of *Brassica napus* infested with the cyst nematode

Heterodera schachtii has been correlated with increased numbers of females in the population (Sijmons *et al.* 1994). It was postulated in this case that a high glutamine concentration ensures an adequate supply of free amino acids, which may be used either directly for nematode nutrition or to provide a pool of amino acids for protein synthesis in the highly metabolically active galls.

It is a common phenomenon then, that pest infestation, particularly involving gall formation, is associated with increased accumulation of amino nitrogen presumably for nutrition. Nitrogen availability is very important for aphid nutrition and can be closely correlated with reproductive status and population size (McNeill and Southwood 1978). Given the importance of glutamine in nitrogen transport and amino acid assimilation, the particular accumulation of glutamine in a metabolically active sink such as a phylloxera gall is perhaps not surprising.

4.4.6 Gene expression in uninfested roots and nodosities

Protein profile

The total protein profile in nodosities did not show any significant differences to that of uninfested roots, however the concentration of soluble protein, as estimated from amounts loaded on the gel, appeared to have increased approximately two fold. This is similar to what has been recorded for root-knot nematode-induced galls on tomato, where total protein content is increased by 80% over uninfested roots (Owens and Specht 1966). This is, if nothing else, an indication of the increased metabolic activity of gall tissue. The lack of accumulation of any major novel proteins suggests that major changes in gene expression might not be expected. It may be that differences exist which are expressed only transiently, or within only a few cells around the feeding site, making them difficult to detect using this fairly insensitive method.

Northern blot hybridisation analysis

Defence-related genes:

StSy, PAL, CHS and PPO are all defence-related proteins, the genes of which have been shown to be expressed at increased levels in response to pathogen attack in a number of plant species (McKhann *et al.* 1997, Melchior and Kindl 1991, Mayer 1987). StSy, PAL, and CHS are genes coding for enzymes of the phenylpropanoid pathway, the products of which have been implicated in grapevine defence against pathogen attack (Melchior and Kindl 1991, Calderón *et al.* 1992, Dai *et al.* 1995a, 1995b, 1995c). It has also been hypothesised that PPO may be involved in plant defence against insects and fungal

pathogens (Mayer 1987). Genes coding for these proteins were therefore expected to be induced in response to phylloxera feeding if the vine was capable of a phenylpropanoid- or PPO-based defence response to phylloxera. While PAL gene expression has been shown to be upregulated in incompatible nematode-host interactions (Goddijn *et al.* 1993), it may be down-regulated in susceptible interactions (Edens *et al.* 1995), suggesting that it could also be down-regulated in the susceptible interaction of *V. vinifera* with phylloxera. CHS gene expression has been shown to be upregulated in roots upon invasion by *Rhizobium* (McKhann *et al.* 1997) and root-knot nematodes (Hutangura *et al.* 1999), possibly associated with the proposed role of flavonoids in auxin regulation. CHS gene expression may also be induced by high sugar levels (Tsuyaka *et al.* 1991), suggested here in nodosities by starch accumulation. On these bases, induction of the gene coding for CHS might have been expected.

None of the transcripts of these genes was induced in nodosities above the level at which they are expressed in uninfested roots, with the possible exception of a slight upregulation of PAL. Expression of PPO genes in roots was not detected, although it is reportedly expressed at a high level in young roots (Dry and Robinson 1994). Hybridisations using this probe were repeated using fresh blots and freshly prepared RNA and the same result (no signal) was obtained each time. The reason for this discrepancy with published data is unknown. These results suggest that *V. vinifera* does not respond to the presence of phylloxera with a typical grapevine defence response unless it is a very localised or transient one. They also suggest that phylloxera do not cause suppression of the expression of these genes. The fact that StSy and PAL are expressed in uninfested roots is consistent with the roots' high phenolic content.

VvTL1 and VvTL2 are grapevine PR proteins of the PR5 (thaumatin-like) family. VvPR4a is a grapevine PR4-like protein. Neither VvTL1 nor VvPR4a are known to be induced in response to pathogens, nor have they been shown to be expressed constitutively in roots (Tattersall *et al.* 1997, D. Tattersall, Department of Horticulture, Viticulture and Oenology, The University of Adelaide, pers. comm.). These expression patterns were confirmed in this study. VvTL2 has been shown to be induced in leaves upon powdery mildew infection and ethephon treatment (Jacobs *et al.* 1999). In this study VvTL2 was moderately expressed in roots but was not up-regulated in nodosities, suggesting again that phylloxera do not elicit a PR protein-based defence response in *V. vinifera* roots.

Nodule-related genes:

Grip 13, 15, 28, 31 and 68 are grapevine cDNAs homologous to genes expressed during early nodule formation in other plant species (Davies and Robinson 2000). The superficial

similarity between nodules and nodosities, both consisting of metabolically active and swollen root tissue induced by exogenous factors, suggested that there may be some similarities at the molecular level as well. This is based on the assumption that the inducing organisms are causing the reactions in the roots by altering the expression of a discrete set of the plant's own genes.

Grip 13, and Grip 15 are homologous to genes expressed in the cell walls of nodules from yellow lupin and *Maackia amurensis* respectively (Davies and Robinson 2000), and are therefore thought to be involved in cell wall structure or expansion. Genes encoding extensins, which are proteins involved in cell wall expansion, are upregulated in root-knot nematode induced galls (Ohl *et al.* 1997). Neither Grip 13 nor Grip 15 were found to be induced in nodosities. Davies and Robinson (2000) reported that Grip 13 expression was not detected in *V. vinifera* roots. As the signal seen here is not of the expected transcript size, it is likely that it results from a cross hybridisation of labelled probe with a contaminant in RNA rather than with transcript in root RNA. A similar occurrence was noted in other northern hybridisation analyses (data not shown). This hybridisation was repeated, but the same results obtained. The reason for this is unknown. It is unlikely that Grip 13 was expressed in either tissue at a level which could be detected by this method.

Grip 31 is homologous to a gene expressed in the pericycle of *Alnus glutinosa* during early nodule formation, and Grip 68 is homologous to a group of Glu-rich proteins some of which are expressed during early potato tuber formation. Swelling of the root and accumulation of starch gives nodosities a superficial similarity to tubers, as well as their previously mentioned similarity to nodules. Both Grip 31 and Grip 68 are reportedly expressed at a low level in grapevine roots (Davies and Robinson 2000). Expression of Grip 31 could not be detected at all in this study, and Grip 68, while moderately expressed in roots, was not up-regulated in nodosities.

Sucrose transport-related genes:

The expression of genes encoding sucrose transporters might have been expected to show some differences between uninfested roots and nodosities, as the gall tissue appears to be a sink for sucrose, as indicated by starch accumulation. However, no transcript for sucrose transporter 12 could be detected in either tissue, while that for sucrose transporter 27 was moderately expressed at a similar level in both uninfested roots and nodosities. The lack of detectable induction in nodosities could be the result of a number of factors such as involvement of other sugar transporters, or the symplastic transport pathway in enhanced movement of sugars into the cortex.

Others:

Grip 28 is homologous to a group of proteins of unknown function, although they bear some similarity to pectin methylesterases, and therefore may have a function in carbohydrate binding. Its closest homology is to a gene expressed in alfalfa nodules (Davies and Robinson 2000). This gene showed moderate expression at roughly the same level in both uninfested roots and nodosities.

Grip 21 is reportedly expressed at a low level in roots and suggested to be related to a protein which might respond to sugar starvation in maize roots, although no definite function has been ascribed to it (Davies and Robinson 2000). A moderate level of transcript was detected in both roots and nodosities. There can be little doubt that the concentration of sugars changes during gall formation, but it is unlikely that 'starvation' would occur, so upregulation might not be expected.

Discussion of gene expression analyses

A phylloxera-induced nodosity is a highly metabolically active organ, as indicated by the accumulation of starch, rapid growth rate and reported increase in size of nuclei surrounding the feeding site (Niklowitz 1954). It could well be expected that the expression of at least some genes must be upregulated in response to phylloxera feeding. The results from this study, however, indicated little or no upregulation of a range of transcripts, including those of genes presumed involved in defence, cell wall structure and sucrose transport. There are several possible explanations for this. Induction may be limited to specific genes or members of gene families not effectively targeted here, or the expression of induced transcripts may occur in a very localised or transient manner.

Parallels for these scenarios exist in other plant root-parasite or symbiont interactions. The expression of many genes has been shown to be upregulated in roots of a number of plant species in response to infestation by both root-knot and cyst nematodes, including those involved in defence or stress responses, but this induction is most often transient and/or localised to the site of pest attack (reviewed by Bird and Wilson 1994 and Williamson and Hussey 1996). Gurr *et al.* (1991) used differential screening to determine expression of genes in potato roots infested with a compatible pathotype of potato cyst nematode *Globodera rostochiensis.* Only one upregulated transcript was cloned from infested roots, and its expression was localised at the feeding site. Several plant defence response genes were expressed in tomato roots in response to root-knot nematodes, including transcripts encoding a peroxidase, a chitinase and proteinase inhibitors, but the expression of all these genes was localised to the feeding site (Lambert 1995 in Williamson and Hussey 1996).

Møller (1998) used GUS fusion constructs to determine the pattern of expression in *Arabidopsis* of *atao*1, a gene involved in vascular differentiation, in response to both root-knot and cyst nematode attack. Expression was found to be transient and localised only to vascular tissue within the galls.

Expression of defence-related transcripts has also been found to occur in roots in response to mycorrhizal infection, but this has only been able to be detected by *in situ* hybridisation of arbusculated cells (Blee and Anderson 1996, Gianinazzi-Pearson *et al.* 1996); northern blot hybridisation is not sensitive enough (Blee and Anderson 1996). Blee and Anderson (1998) also describe experiments in which upregulation of genes encoding enzymes involved in sugar metabolism is detected, again only in arbusculated cells.

Pittock *et al.* (1997) investigated the expression of a basic chitinase in transgenic clover (*Trifolium pratense*) in response to *Rhizobium* and aphid (family Aphididae) attack. Chitinase was induced by both organisms, but its expression was very localised in response to aphids, and it was only transiently expressed in response to *Rhizobium*. Less targeted approaches, such as differential display (Goormachtig *et al.* 1995) or subtractive hybridisation (Gamas *et al.* 1996) have been used to successfully isolate several transcripts specifically induced in nodules (for example 29 novel nodulin transcripts were cloned by Gamas *et al.* 1996).

Nematode attack has also been shown to involve downregulation of many transcripts in roots, and this is often suggested to be an indication of the suppression of the host's defence response (Hammond-Kosack 1989, Rahimi *et al.* 1996, Williamson and Hussey 1996). Hammond-Kosack (1989) and Rahimi *et al.* (1996) both found accumulation of defence-related transcripts or proteins in leaves, but not roots of potato plants infested with potato-cyst (*Globodera* sp.) nematodes. They suggested this was due to the localised suppression at the site of pest attack of what would otherwise be a systemically induced defence response. Goddijn *et al.* (1993) made a wide range of GUS-fusion constructs to look at altered regulation of genes in nematode (cyst nematode *Heterodera schachtii* and root-knot nematode *Meloidogyne incognita*) feeding sites. It was found that while only a few transcripts were upregulated, many appeared to be downregulated, suggesting suppression of regulatory genes necessary for expression of many other transcripts. The downregulation of PAL was noted, and this was hypothesised to be necessary for the establishment of feeding sites. The results presented in this study (section 4.3.7) did not indicate any inhibition of expression of defence-related genes or other genes in phylloxera-induced nodosities, although this does not exclude the possibility of a localised suppression of a defence response immediately around the feeding site.

The above are only selected examples of changes in gene expression in roots in response to invasion by parasites or symbionts. From this it seems likely, however, that unless a systemic defence response is expected, for example in a resistant variety or otherwise incompatible interaction, any changes in gene expression which do occur may be only transient or localised to the site of attack, such that northern blot hybridisation analyses of RNA prepared from total gall tissue are not likely to detect them. For an approach where specific transcripts are targeted, *in situ* hybridisation would be the most appropriate technique to adopt. Similarly, immunohistochemistry would be useful for detection of localised changes in protein accumulation. Differential hybridisation or differential display may be sensitive enough to detect specifically expressed clones in a non-targeted manner. Promoter tagging or other use of GUS fusion constructs may be of use in the future when grapevine transformation techniques are more routine. As model plants such as *Arabidopsis* or tomato are not host plants for phylloxera, their use at present is not applicable to the study of this system.

4.4.7 Summary

The nodosity is an organ which provides nutrition for phylloxera in a specialised manner. It appears to be a strong nutrient sink, which possesses structural modifications that might enhance solute unloading and post-phloem transport into the root cortex. Its role as a sucrose sink may be confirmed by *in situ* hybridisation of tissue surrounding the feeding site with sugar metabolism-related transcripts (as used by Blee and Anderson 1998). The route of solute transport is most likely to be symplastic, possibly via enlarged or more numerous plasmodesmata. This might be elucidated by ultrastructural study of plasmodesmata in roots and nodosities. It is also possible that reduced suberisation of the endodermis allows enhanced apoplastic transport of solutes into the cortex.

Blee and Anderson (1998) hypothesise that production of phytohormones (e.g. IAA) may be responsible for establishment of a sucrose sink in arbusculated cells by upregulation of sucrose synthase and invertases. Phytohormones are also generally implicated as causative agents in insect gall formation (Forrest 1987). It is possible that production or accumulation of IAA in phylloxera galls may serve a dual purpose in both gall formation and inhibition of tissue differentiation, as well as in establishment of a sucrose sink. In this study, IAA was not detected in galls by HPLC analysis, so this theory remains unsupported.

The nodosity is also a sink for free amino nitrogen, and the elevated protein content indicates raised metabolic activity. Despite this, no significant induction of gene expression nor accumulation of novel proteins was detected by northern blot analyses or SDS-PAGE.

Together, these facts imply that the root is responding in a very selective manner to the presence of the phylloxera, possibly through localised or transient induction of genes, or alternatively induction of a relatively few novel transcripts.

The lack of defence response to phylloxera as detected by HPLC and molecular analysis in this study are in agreement. The microscopic study clearly demonstrated that some anatomical and biochemical changes occur, but there is no apparent recognition of the parasite attack in terms of a typical plant defence response. The results from the histochemical studies also indicated little, if any localised defence response. While phylloxera were actively feeding there was no indication of oxidation of any phenolic compounds in response to feeding, nor was there any accumulation of novel autofluorescent compounds. PPO was not expressed in nodosities, possibly indicating that *V. vinifera* has little endogenous capacity for a rapid oxidative response. Peroxidase activity assays on infested roots were not possible because they require fresh tissue which was not available due to quarantine restrictions.

V. vinifera is susceptible to many pathogens, and often shows little or no induced response to their attack (Langcake 1981, Calderón *et al.* 1992, Dai *et al.* 1995a, b, c). It has been suggested that the stylet sheath of aphids may act to disguise the presence of the aphid as an attacking pest and thus inhibit any defence response (Miles 1987). The lack of defence response may arise from this 'disguise', or from the intrinsic inability of *V. vinifera* to produce a strong defence response. If the former is the case, nodosity induction is a clear demonstration that *V. vinifera* still responds to the pest in other ways. If, as suggested previously, gall formation occurs primarily through induction of natural plant hormones, typical defence responses against an invading organism may not be elicited, or at least may be suppressed in the short to medium term.

5. COMPARISON OF THE INTERACTION BETWEEN SUSCEPTIBLE AND RESISTANT GRAPEVINE SPECIES OR ROOTSTOCKS AND A BIOTYPE B-LIKE STRAIN OF PHYLLOXERA

5.1 Introduction

Knowledge of the interaction between phylloxera and resistant *Vitis* species or rootstocks is integral to understanding the spectrum of grapevine-phylloxera interactions. Most of the grapevine rootstocks in use today were bred or selected in the late 1800s specifically to withstand infestation by phylloxera. Useful rootstocks were selected or developed from relatively few American *Vitis* species; Viala and Ravaz (1901) commented, even at that time, that most American *Vitis* species had already been relegated to collections or discarded from viticultural use. Rootstocks in common use in Australia today have been selected mainly from natural accessions of *V. riparia*, *V. rupestris* and *V. champini*, or interspecific hybrids of these species, including *V. vinifera* and *V. berlandieri* parentage (Whiting *et al.* 1987, May 1994). Other rootstocks developed more recently include the *V. rotundifolia* × *V. vinifera* (VR) hybrids (Davidis and Olmo 1964), bred to combine *V. rotundifolia's* natural resistance to a number of organisms with the graft compatibility of *V. vinifera*, and the newly released *V. cinerea* × *V. riparia* hybrid Börner (Becker 1988, 1989).

The interaction of rootstocks with phylloxera may be one of tolerance, resistance, or a combination of both. Tolerant rootstocks can support phylloxera populations on their primary roots but do not succumb to the infestation (i.e. secondary roots are not infested and grafted scions do not show signs of vine decline). Tolerance, therefore, is predominantly a function of secondary roots. In general, however, the survival and fecundity of phylloxera populations on primary roots of tolerant rootstocks is also reduced compared with susceptible vines such as *V. vinifera*, and most therefore also exhibit at least partial resistance.

Rootstocks supporting only very limited populations of phylloxera on their primary roots may be considered resistant, or in the extreme case, where phylloxera cannot even initiate nodosity formation, immune. In this study, focus was placed on mechanisms of resistance, since the interaction between phylloxera and primary roots was investigated.

The complexity of the relationship between various rootstocks and phylloxera implies that there may be several mechanisms involved in the tolerance or resistance of vines to phylloxera. The inheritance of these mechanisms remains essentially undefined. An

early report on this topic was published by Boubals (1966b). In a series of crosses of resistant species with *V. vinifera*, he attempted to determine the genetic nature of resistance. His findings were that susceptibility to phylloxera appears to be determined by a homozygous trait, while resistance can be heterozygous. The resistance of most species, including *V. riparia* and *V. rupestris,* showed partial dominance over the susceptibility of *V. vinifera*, while the resistance of a few species, namely *V. berlandieri*, *V. cinerea* and *V. rotundifolia,* was dominant. More recently, Bouquet (1983) conducted a series of crosses between *Vitis vinifera* and *V. rotundifolia*, from which he hypothesised that the resistance of *V. rotundifolia* may be conferred by a partially dominant gene which is heterozygous in this species, and under the regulation of three modifier genes. There have been no published reports of the identification of a particular gene or genes responsible for resistance to phylloxera.

In any plant-pest interaction, resistance may result from either non-preference of the pest for its potential host resulting from the presence of chemical deterrents (antixenosis) or toxins (antibiosis), or the capacity of the plant to activate an effective defence response upon recognition of pest attack. Finally, a lack of an induced response necessary for a compatible interaction, or of essential phagostimulants, may also result in tolerance and/or resistance. Which of these mechanisms are operating in grapevine-phylloxera interactions is still unknown.

There is relatively little published literature regarding the effects of phylloxera on resistant rootstocks. The few reports that do exist deal with disparate topics and do not together provide an integrated picture of the phylloxera-rootstock interaction (Denisova 1965, Boubals 1966a, Sobetskiy and Derzhavina 1973, King *et al.* 1982, Askani and Beiderbeck 1991, Nedov *et al.* 1992) (see Chapter 1, section 1.2.2 for more detail.). Many products of the phenylpropanoid pathway, especially stilbenes and stilbenoids, have also been implicated in the general defence response of grapevines to fungal pathogen attack and to wounding (Langcake and Pryce 1976, 1977, Langcake 1981, Dercks and Creasy 1989, Liswidowati *et al.* 1991, Calderón *et al.* 1993, Dai *et al.* 1995a, b, c).

In the light of this information, a range of techniques used to investigate root anatomy and histochemistry, and free phenolic content were adopted in this study. The main objectives were to determine the resistance status of a range of vines through the use of tissue culture-based bioassays, and then to determine the role, if any, of root anatomy and chemistry (in particular that of phenylpropanoid pathway products) in their resistance.

Techniques most suited for use with small amounts of infested root tissue were adopted since only limited numbers of nodosities are generated from cocultivations of resistant vines with phylloxera. Thus northern blot hybridisation analysis, as used in Chapter four, was not feasible.

5.2 Tissue culture-based bioassays of VWL-1 phylloxera cocultivated with and range of grapevine varieties

VWL-1 was chosen because of its aggressiveness in the field and, though its biotype had not been determined, preliminary observations indicated similarity to the well characterised biotype B (Granett *et al.* 1985). On this basis, the resistance of each grapevine variety could be reasonably predicted, enabling a suitable range of varieties to be selected for the study.

The grapevine varieties *V. vinifera* cv. Shiraz, Ramsey, Schwarzmann, *V. riparia*, Börner, and *V. rotundifolia* (table 2.1) were selected on the basis of previous reports to cover a range of resistance levels to phylloxera, from susceptible (*V. vinifera*) through resistant (Ramsey, Schwarzmann, *V. riparia*) to immune (Börner, *V. rotundifolia*), though none had been tested for resistance to VWL-1 strain.

Tissue culture-based bioassays were conducted using either micropropagated vines in perlite-based medium (Chapter 2, section 2.4.4), or excised root cultures (Chapter 2, section 2.4.6). As discussed in Chapter two, these methods provide different information regarding the interaction between phylloxera and grapevine roots. Observations were made of the gross morphology of feeding sites and of the behaviour of the phylloxera. Both uninfested and infested roots (nodosities) were harvested following the assays and analysed further using HPLC and/or histochemistry/microscopy.

5.2.1 Perlite-based medium bioassays

Materials and methods

'Perlite bioassays' were conducted according to section 2.4.4. Grapevine varieties used were *V. vinifera* cv. Shiraz accession PT23/A.S.80.5397, Schwarzmann accession WA/A.S.74.2257, Ramsey accession A11V2/I.V.63.2065, *V. riparia*, Börner, and *V. rotundifolia*. Each replicate consisted of one vine in a tissue culture vessel infested with 100 eggs. Bioassays were assessed after 30 days cocultivation for percent egg hatch, number of nodosities initiated, number and developmental stage of immature surviving phylloxera, and number of adults phylloxera producing eggs.

Results

Infestation parameters are presented in table 5.1. Figure 5.1 shows a typical example of the root response of each variety to phylloxera attack.

Populations of VWL-1 phylloxera established most effectively on *V. vinifera* cv. Shiraz roots, with phylloxera present at all developmental stages, and several adults reaching egg laying stage. Typical nodosities (figure 5.1a) were large, yellow and actively growing. There was no indication of a preference for feeding sites near root tips, with hook shaped nodosities formed along the length of roots (figure 5.1f). Phylloxera also formed large colonies on *V. vinifera* stems at the base of the cuttings (not shown) and along splits in the larger roots (figure 5.1b).

VWL-1 phylloxera were also able to form nodosities and reproduce on roots of Ramsey. As on *V. vinifera*, no tendency was observed to initiate nodosities at root tips. The nodosities (figure 5.1c) were smaller than on *V. vinifera*, with necrotic regions (possibly indicative of a hypersensitive-like response) which appeared while phylloxera were still feeding. When this occurred, phylloxera tended to move to an unaffected site on the same nodosity and continued to feed. However, after 30 days, most nodosities had been abandoned by phylloxera. It is possible that either the tissue browning made the nodosities unsuitable as a feeding site, or that the nodosities were unable to supply phylloxera with their nutritional requirements in the long term. On the few nodosities where phylloxera were able to feed long enough to reach reproductive age, generation time was relatively short (< 30 days), indicated by the presence of several second generation crawlers feeding on newly initiated lateral roots or on primary root tips (data not shown).

Figure 5.1 Response of roots to VWL-1 phylloxera in perlite cultures. a) Nodosity on *V. vinifera* cv. Shiraz supporting a colony of phylloxera b) Split root on *V. vinifera* cv. Shiraz supporting a very large phylloxera population c) Small nodosity on Ramsey supporting adult phylloxera. Arrow indicates regions where this nodosity is becoming necrotic d) Strong necrotic response of *V. riparia* root tip to phylloxera feeding. Arrow indicates dead crawler. e) Necrotic response of Börner to phylloxera attempting to feed. Arrow indicates dead crawler. Scale bars for a) -e) equal approximately 5mm. f) Root system of *V. vinifera* cv. Shiraz vine following 30 days cocultivation with phylloxera. Arrows indicate nodosities. g) Root system of *V. rotundifolia* after 30 days cocultivation with phylloxera.

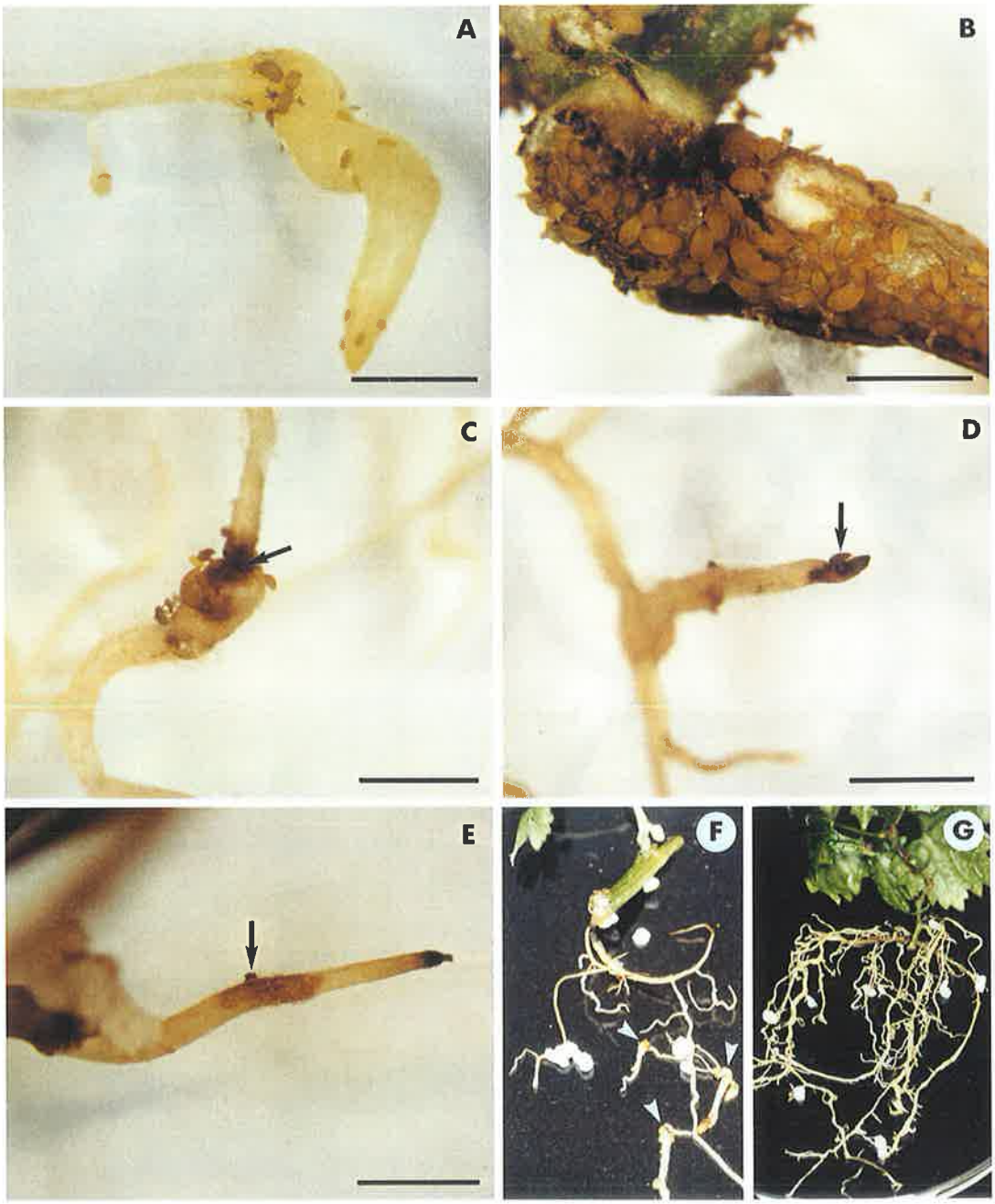
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Table 5.1 Survival of VWL-1 phylloxera life stages and nodosity formation in perlite-based medium bioassays (as described in Chapter 2, section 2.4.4) on six vine varieties. Ten replicates of each variety were each inoculated with 100 surface-sterilised phylloxera eggs.

			mean no. of live phylloxera at each developmental stage after 30 days cocultivation.						
vine variety	**eggs hatched (±SD)[1]**	**nodosities[2] formed (±SD)**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults**	**nymphs with wing buds**	**total no. survivors (±SD)**
***V. vinifera* cv. Shiraz**	86.63 ± 5.93	4.43 ± 3.87	2.29	2.29	3.14	0.71	6.43	2.28	17.14 ± 7.51
Ramsey	89.89 ± 5.99	4.66 ± 4.90	0.88	0.11	0.11	0.22	0.44	0	1.76 ± 2.59
Schwarzmann	83.75 ± 11.37	0	0	0	0	0	0	0	0
V. riparia	87.11 ± 9.31	2.56 ± 3.47	0	0	0	0	0	0	0
Börner	86.11 ± 9.36	0	0	0	0	0	0	0	0
V. rotundifolia	81.10 ± 11.40	0	0	0	0	0	0	0	0

[1]An analysis of variance at 5% significance (Zar 1984) showed no significant difference between the egg hatch rates on each grapevine variety. The high number of zeros in other data sets meant that meaningful statistical analysis was not possible.

[2]Nodosities were considered to be any feeding site causing swelling on a root. Other feeding sites were formed on the stem base and on larger roots which had split open; these have not been included in the nodosity numbers given here.

On *V. riparia*, nodosities were sometimes indicated by root swelling, but these aborted early, never reaching more than 2mm in diameter or length, and also rapidly became dark brown in colour. No surviving phylloxera were found at any developmental stage and phylloxera did not develop past the first instar on this variety. Figure 5.1d shows a site at which a crawler had attempted to feed (in this case no nodosity was initiated) resulting in strong and rapid necrosis of the root tip. It is interesting to note that the crawler died while attempting to feed, without searching for a new feeding site.

No nodosities were initiated on Börner, and phylloxera feeding resulted in localised necrosis of the root (figure 5.1e). Possibly the phylloxera did not settle long enough while attempting to feed, or root necrosis was rapid enough to prevent nodosity initiation from occurring. Alternatively, this variety may not be responsive to signals for nodosity initiation.

There were no signs of attempted nodosity initiation on Schwarzmann, and no necrotic regions were found. A large number of dead crawlers were found on the roots, indicating that the insects had been in contact with the roots, probably searching for feeding sites. Similarly, there was no evidence that phylloxera had made any attempt to feed on *V. rotundifolia*. The roots appeared healthy (figure 5.1g) and no necrotic lesions were seen, but there were no dead insects found on these roots, suggesting that phylloxera may not have attempted to find feeding sites on this variety.

5.2.2 Excised root culture bioassays

Materials and methods

The survival and behaviour of phylloxera on a number of different grapevine varieties was compared using excised root cultures as described in Chapter 2, section 2.4.6. The vine varieties and accessions were the same as for the perlite-based medium bioassays except that *V. riparia* was not included due to limited availability of root material. Each replicate consisted of a petri dish containing three roots with each root infested with 10 eggs. These bioassays were repeated three times.

Results

Survival data from one bioassay are presented in table 5.2 as a typical example. Data from further bioassays, showing similar trends, are presented in Appendix 4. In the trial reported in table 5.2, phylloxera were only able to develop to adulthood on *V. vinifera*, albeit in small numbers, although one small nodosity was also initiated on Ramsey. Survivors were found on Schwarzmann and Börner, although very few on the latter, and on neither variety had they developed past first instar. No survivors were found at all on *V. rotundifolia*.

Table 5.2 Survival of VWL-1 phylloxera life stages and nodosity formation on excised root cultures (as described in Chapter 2, section 2.4.6) of five vine varieties. Twelve roots of each variety were inoculated with ten surface sterilised phylloxera eggs.

			live phylloxera at each developmental stage after 25 days cocultivation					
vine variety	**eggs hatched per root (±SD)[1]**	**nodosities formed (±SD)**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults**	**total no. survivors (±SD)**
***V. vinifera* cv. Shiraz[3]**	2.83 ± 2.29	0.25 ± 0.45	0	0.25	0	0.25	0.33	0.83 ± 1.64
Ramsey	3.00 ± 2.04	0.08 ± 0.29	1.08[2]	0.17	0	0	0	1.25 ± 1.71
Schwarzmann	2.92 ± 1.93	0	1.50[2]	0	0	0	0	1.50 ± 2.32
Börner	2.17 ± 1.75	0	0.25[2]	0	0	0	0	0.25 ± 0.71
V. rotundifolia	2.14 ± 2.27	0	0	0	0	0	0	0

[1] Estimated by number of insects found dead or alive 21 days after inoculation.
[2] All had moved off roots onto filter paper.
[3] Data also presented in table 2.7.

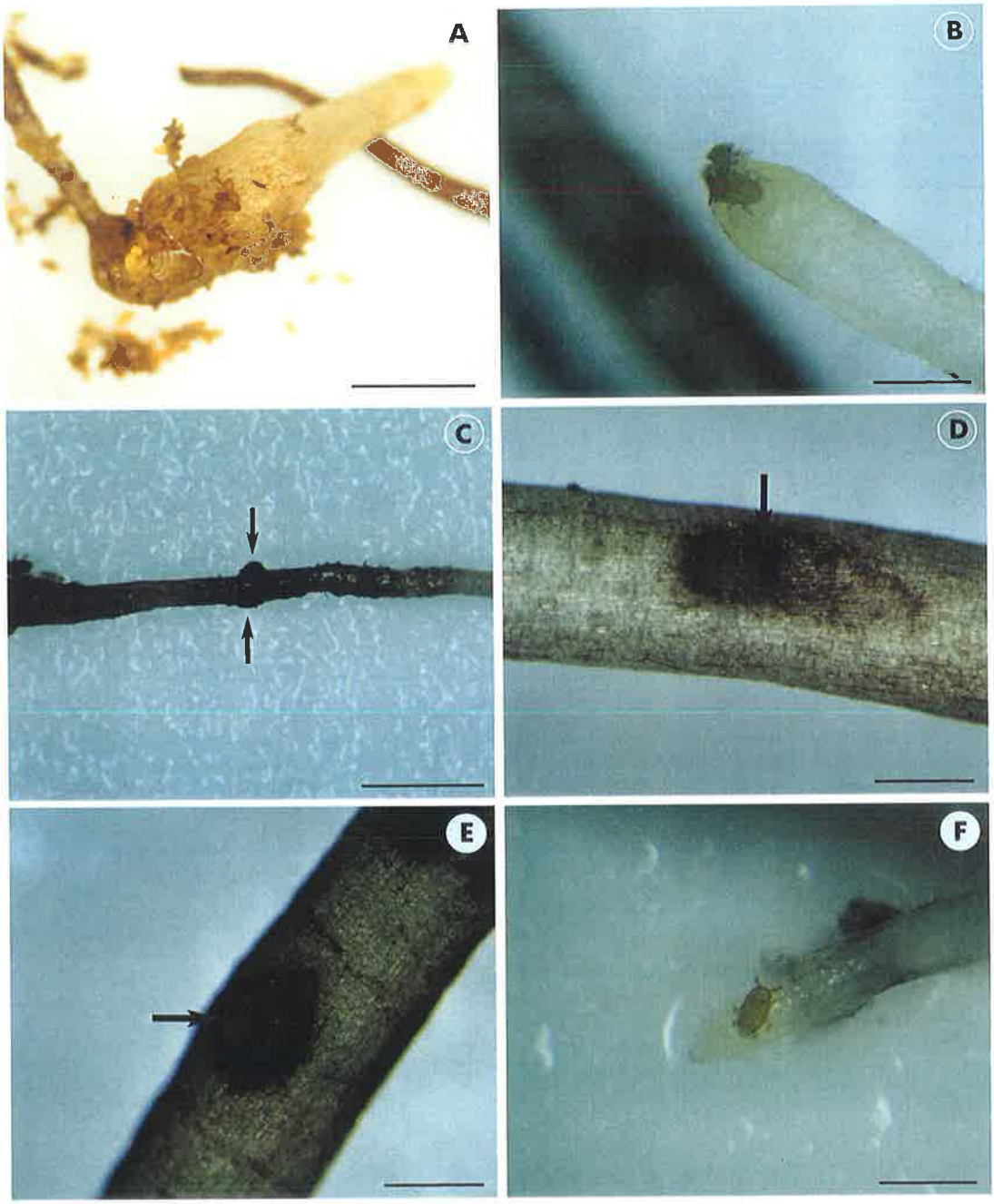
Despite the relatively low survival rates of phylloxera, presumably because culture conditions were not conducive to high survival, excised primary root cultures proved useful for observations of phylloxera behaviour and root response, a summary of which has been gathered from the results of all three excised root culture bioassays (table 5.3). Figure 5.2 shows typical examples of the root response of each grapevine variety.

Table 5.3 Summary of resistance ratings of various grapevine varieties to VWL-1 phylloxera as determined by infestation parameters from bioassays in perlite-based medium cultures and behavioural observations in excised root cultures.

Variety	Resistance rating	Comments
V. vinifera cv. Shiraz	Susceptible	Phylloxera established a range of feeding sites, and easily reached reproductive stage within the time of the assay. There was no visible necrosis on any of the nodosities formed.
Ramsey	Resistant (antixenosis)	Some phylloxera were able to feed, initiate nodosity formation and reproduce. Nodosities were small and had necrotic spots. Reproduction was at a much lower rate compared to that on *V. vinifera*.
Schwarzmann	highly resistant (antibiosis)	Phylloxera attempted to feed but were unable to become established or initiate nodosity formation. Feeding resulted in necrosis of surrounding root tissue and death of phylloxera.
V. riparia	highly resistant (antibiosis)	Phylloxera attempted to feed but were unable to become established. Nodosity formation was initiated, but nodosities quickly became necrotic, phylloxera died *in situ*.
Börner	highly resistant (antibiosis)	Phylloxera attempted to feed but were unable to become established or initiate nodosity formation. Feeding at sites with an intact epidermis induced necrosis of surrounding root tissue and resulted in death of phylloxera.
V. rotundifolia	immune (antibiosis and/or non-preference)	Phylloxera did not attempt to feed or initiate nodosities.

Newly hatched phylloxera crawlers tend to be extremely mobile, and may move constantly for several days before locating a suitable feeding site. This behaviour, while advantageous for dispersal of phylloxera, can be detrimental to their survival in excised root cultures. Most surviving phylloxera on all varieties did not develop past first instar, as they had moved off the roots and become trapped in moisture on the filter paper. (These were not counted in the assay.) Crawlers which had moved off the roots were occasionally observed probing the filter paper with their stylets. Aphids reportedly will probe any surface on which they are placed but only remain settled when that substrate provides them with a suitable food source (Mittler 1988).

Figure 5.2 Excised root cultures during cocultivation with phylloxera. a) Nodosity on *V. vinifera* cv. Shiraz supporting adult phylloxera. b) Crawlers on Ramsey showing a preference for the actively growing tip c) Schwarzmann root with necrotic region and dead crawlers (arrows) d) Necrotic lesion on Börner with dead crawlers (arrow). e) Crawler (arrow) on Börner feeding from the scar of a detached lateral root. f) Crawlers on *V. rotundifolia* on actively growing root tip. Scale bars for a), c) equal approximately 5mm; b), d) - f) equal approximately 2mm.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Crawlers on all varieties seemed to be initially attracted to the extreme tip of the root, i.e. within the zone of the root cap. Figure 5.2b shows phylloxera apparently settled (at least temporarily) within this region on Ramsey. After remaining sedentary for one or two days these crawlers moved off the tip without initiating a nodosity. In only one instance (on Ramsey) was a crawler observed to settle and initiate a nodosity at the very tip of a root. Nodosities were otherwise initiated behind the root cap (e.g. *V. vinifera,* figure 5.2a), as has been reported previously (Niklowitz 1954, King *et al.* 1982).

On Schwarzmann a few crawlers were observed feeding, usually at the tips of roots. In one case the root tip became slightly swollen in response. In all other cases the root or root tip became brown in response to attempted feeding and the crawlers died without moving off the roots (figure 5.2c).

Interestingly, crawlers were observed settled and apparently feeding on Börner, which has been reported to be immune (Becker 1988). Phylloxera seemed to show a preference for the scars left by detached lateral roots (figure 5.2e) or for preformed lesions made by handling with forceps during the establishment of the cultures. The phylloxera remained settled at these wounds or scars for about four days, then moved away. Where they penetrated the root epidermis directly, a rapid necrotic response was observed (figure 5.2d), and the crawlers died *in situ*. In one case a crawler was observed feeding from a root tip and there was slight swelling in response. The insect moved off after around six days, and the root tip subsequently became necrotic.

On one occasion a crawler was observed on the tip of a *V. rotundifolia* root (figure 5.2f), but this insect did not settle, moving away from the tip without making any observed attempt to probe the root. Crawlers appeared to move away from *V. rotundifolia* roots more quickly than from the roots of other varieties. No attempted feeding was observed directly, nor any necrosis which might suggest a response to attempted feeding. Dead crawlers were not observed on *V. rotundifolia* roots as they were on other varieties such as Schwarzmann, Börner and *V. riparia.*

Resistance ratings

The resistance ratings for grapevines to VWL-1 phylloxera, determined according to tissue culture based bioassays, were given in table 5.3.

The criteria used for the given resistance ratings were as follows: A susceptible vine supported phylloxera populations which were able to reproduce; no necrotic response to feeding was observed. *V. vinifera* cv. Shiraz was considered the susceptible standard in this study. Vines were considered resistant if phylloxera were able to feed and reproduce, but at reduced rates over that seen on *V. vinifera*; some necrosis was seen in response to feeding.

Highly resistant vines did not support phylloxera populations, although phylloxera attempted to feed and possibly initiate nodosities; a strong necrotic response to feeding was observed. On immune vines phylloxera were completely unable to feed or initiate nodosities.

5.2.3 Discussion of bioassays

The resistance ratings (table 5.3) determined in the perlite-based medium bioassays are, despite the use of different accessions and a very virulent Australian strain of phylloxera, consistent with the previously reported ratings of these vine varieties from potted or field grown vine trials (Viala and Ravaz 1901, Boubals 1966a, Whiting *et al.* 1987). The results are also consistent with those reported by Grzegorczyk and Walker (1998) who conducted a similar trial, published during the course of this study, where several accessions each of a range of vine varieties were assayed in tissue culture for resistance to phylloxera. The results reported by Grzegorczyk and Walker (1998) must be interpreted with care, however, as their assay was performed with a mixed phylloxera population, including different biotypes. Results presented later in this study (Chapter six) indicate that the tissue culture method is suitable for determining differences between biotypes, and it is therefore important that the phylloxera strain be taken into account. As an illustration of the amenability of tissue culture-based bioassays for screening rootstocks for phylloxera resistance, the resistance ratings of *V. champini*, *V. riparia* and *V. rotundifolia* are compared with published ratings in the following:

Ramsey is an accession of *V. champini;* it is rated here as resistant. Published ratings for this species vary from moderate (sufficient for use in sandy soils) (Viala and Ravaz 1901) to highly resistant (Boubals 1966a, Whiting *et al.* 1987). Grzegorczyk and Walker (1998) found it to have variable resistance depending on the accession, and to support low to moderate levels of phylloxera feeding in tissue culture.

V. riparia is generally rated as highly resistant (Viala and Ravaz 1901, Whiting *et al.* 1987), as it is rated in this study. Similar responses to those reported in this study were also observed by Forneck *et al.* (1996) who found that phylloxera attempted to feed but were unable to become established. A wide range of resistance levels have been reported between different accessions of this species, with Boubals (1966a) rating various accessions from resistant to immune. Similar variation was reported by Grzegorczyk and Walker (1998) for cocultivations in tissue culture; in this case phylloxera survived to adulthood on one out of four accessions, while the other accessions were highly resistant. The accession of *V. riparia* used in this study is unknown, however it was originally reported as 'Gloire'.

Subsequent attempts to confirm its identify through DNA typing proved unsuccessful due to limitations in available databases (data not shown).

Development of necrotic spots has been reported on *V. rotundifolia* roots in response to phylloxera feeding (Boubals 1966a). No evidence of attempted feeding was seen in this study, also reported by Grzegorczyk and Walker (1998). The accession used in the present study is unknown, and it is possible that it is more highly resistant (or immune) than accessions observed by Boubals (1966a).

Nodosity morphologies pictured in figure 5.1 were similar to those described by King *et al.* (1982) in a study of potted rootstock varieties. For example, necrotic spots similar to those seen here on Börner roots have been noted on 1202C; root tip swelling and necrosis similar to that observed here on *V. riparia* roots was noted on 1613C, 1202C, SO4 and 5BB; and small nodosities similar to those on Ramsey were noted on ARG-1. The biotype of phylloxera used by King *et al.* (1982) is unknown, but from their data, it appears to differ from VWL-1 phylloxera. It was able to produce nodosities on ARG-1, but unlike VWL-1 (Corrie *et al.* unpublished), its fecundity on this vine type was significantly lower than on *V. vinifera*. These biotype differences mean that direct comparisons between studies cannot be made.

The behaviour of phylloxera on excised root cultures was similar to that noted by Askani and Beiderbeck (1991), including initial feeding at the root apex. While the reason for this is not clear, it might be that the stylet readily penetrates the root cap and reaches the metabolically active meristem, which is possibly a rich source of nutrients. It is also plausible that phylloxera is attracted to root cap exudates, although the composition of these in grapevine roots is unknown. Phylloxera tend not to settle at these sites, however, and it is possible that the active growth of the root apex, and continuous elongation of cells, could make it difficult for a sedentary feeder to establish a suitable feeding site. Settling just behind the tip of the root, in the zone of elongation (figure 4.13), might avoid this problem. It might also allow more ready penetration of the root by avoiding the root cap. The relatively undifferentiated tissues of this region would presumably also be responsive to signals for the initiation of nodosity formation. This behaviour may also occur because the zone of elongation is the site of phloem unloading in a growing root (Oparka *et al.* 1994, 1995), providing a pre-established photoassimilate sink as a precursor to the feeding site. The dagger nematode, *Xiphinema index*, a common parasite of grapevine roots, also shows preference for the zone of elongation as a feeding site (Weischer and Wyss 1976).

Interestingly, and in contrast to the observations on excised roots and potted *V. vinifera* vine roots (Chapter 4, section 4.3.2), there was no obvious preference for root tips as a site for

feeding and nodosity initiation in perlite-based medium cultures. This may be a reflection of the tendency for roots of tissue cultured vines to retain a primary anatomy for the entire length of the root (section 5.3.1). Alternatively, there may be an increased level of sugars in the cortex of roots growing in tissue culture due to uptake from the medium, whereas in potted vines, free sucrose in the cortex would be restricted to the elongation zone.

The particular response of each vine type to attempted feeding can give some indication as to possible resistance mechanisms which may be operating. In general, rapidity and extent of root browning was proportional to resistance to phylloxera. On *V. riparia* the browning of the root and death of the feeding insect implies a rapid antimetabolic response by the root. A similar response is noted in Börner when the crawlers penetrate and feed through the root epidermis. In contrast, most of the insects feeding on Börner were settled at lateral root scars, and in these cases browning of the root was not seen and the insects moved away with no apparent antibiotic effect of the root. This implicates phenolics located in the epidermis in the browning and antibiotic effects seen. Feeding at lateral root scars may allow access to the root cortex while avoiding the need to penetrate the epidermis and hypodermis, which have a higher phenolic content than the underlying cortical cells (figure 5.5c). Alternatively these sites may provide more ready access to vascular tissue, facilitating access to solutes from the phloem.

No attempted feeding was observed on tissue cultured vines of *V. rotundifolia*, nor were dead crawlers found on the roots of this variety. This suggests that crawlers were not attracted to the roots and that *V. rotundifolia* may be a non-host plant. Alternatively, roots of this species may be either strongly resistant or immune to VWL-1 phylloxera.

The consistency of the results from this study with previously published resistance ratings indicates that a bioassay system based on the interaction between phylloxera and primary roots can predict the resistance of vines to phylloxera in the field.

The role of nodosities in field damage of vines is currently being reassessed due to reports of field decline of vines in Germany, where phylloxera is only producing nodosities, not tuberosities (Rühl 1997). Previously, tuberosities, not nodosities, have always been assumed to be the most significant cause of field damage of vines. Based on this assumption, Grzegorczyk and Walker (1998) suggested that use of tissue cultured vine bioassays might result in an oversensitive interpretation of a vine's susceptibility to damage by phylloxera in the field. This is because some phylloxera biotypes may produce many nodosities in tissue culture, but be unable to produce tuberosities. King and Rilling (1991) on the other hand, noted in their own work with potted vines, that particular combinations of phylloxera biotypes and vine genotypes can result in the production of tuberosities without

formation of nodosities. In such a case, tissue cultured vines would be unlikely to predict the potential field susceptibility of the vine genotype being tested.

5.3 Constitutive or preformed differences between resistant varieties

Analysis of constitutive differences between vine varieties involved microscopy, histochemistry and HPLC. Sufficient uninfested root material would have been available for molecular analysis, however there is evidence that grapevine resistance results not from constitutive expression of defence-related genes, but rather from gene induction, production of phytoalexins or a hypersensitive response (Stein and Blaich 1985, Calderón *et al.* 1994, Dai *et al.* 1995a, b, Feucht *et al.* 1996). For this reason, analysis of uninfested roots was limited to methods which were applicable to the limited amount of infested root material available, so that comparisons could later be made to determine any induced responses.

5.3.1 Anatomy of primary roots

While it is possible that primary root anatomy could play a role in resistance to phylloxera, there is little published information on comparative root anatomy of different grapevine varieties. The main purpose of this study was to determine which, if any, anatomical features of primary roots of several vine varieties might be involved in their resistance to phylloxera.

Materials and methods

Roots from both tissue cultured vines and potted vines were included for comparison. Tissue cultured vine roots were collected from perlite-based medium cocultivations (Chapter 2, section 2.4.4) and potted vine roots from pot trial four (Chapter 2, section 2.3.4) except for *V. rotundifolia* potted vine roots which were collected from potted vines grown in the glasshouse at The University of Adelaide. Root samples were harvested from approximately the same distance behind the root tip: around 5cm for tissue cultured vines and 0-1cm, 5-6cm and 10-11cm for potted vines. All samples were collected in triplicate (from three separate plants) and the figures shown are representative of the observations for each variety. Roots fixed in 3% (v/v) glutaraldehyde fixative for at least 48 hrs, then embedded in GMA, sectioned, stained with PAS/TBO and photographed with black and white film. Fixation and embedding methods are detailed in Appendix three.

Anatomy of tissue cultured vine roots

Primary roots of *V. vinifera* cv. Shiraz (figure 5.3a) had the typical anatomy of primary dicotyledon roots as illustrated in figure 1.4. The contents of the epidermal cells were

lightly stained throughout while the walls of the epi- and endodermis were heavily stained. The staining reaction, which appeared blue using TBO at pH 4.4, is indicative of phenolics. Contents of cortical cells other than the hypodermis were unstained and the cortex was approximately seven cell layers deep. Transmission electron microscopy (data not shown) has shown that these cortical cells generally contain one large vacuole which takes up most of the volume of the cell. Cytoplasm is limited to a thin envelope at the perimeter of the cell. This is not readily visible in these sections. The root shown in this figure had a triarch stele, but this was a variable characteristic in *V. vinifera* roots which may be either di-, tri- or tetrarch.

The primary roots of Ramsey (figure 5.3c) were very similar to *V. vinifera* except for the presence of heavily stained globules, probably consisting of polyphenolics, in epidermal cells (figure 5.3h), and a distinct hypodermis which was stained blue evenly throughout the protoplasm, implying that the vacuole contains phenolics. The walls or cytosol of the endodermis were also stained more heavily than in roots of *V. vinifera*.

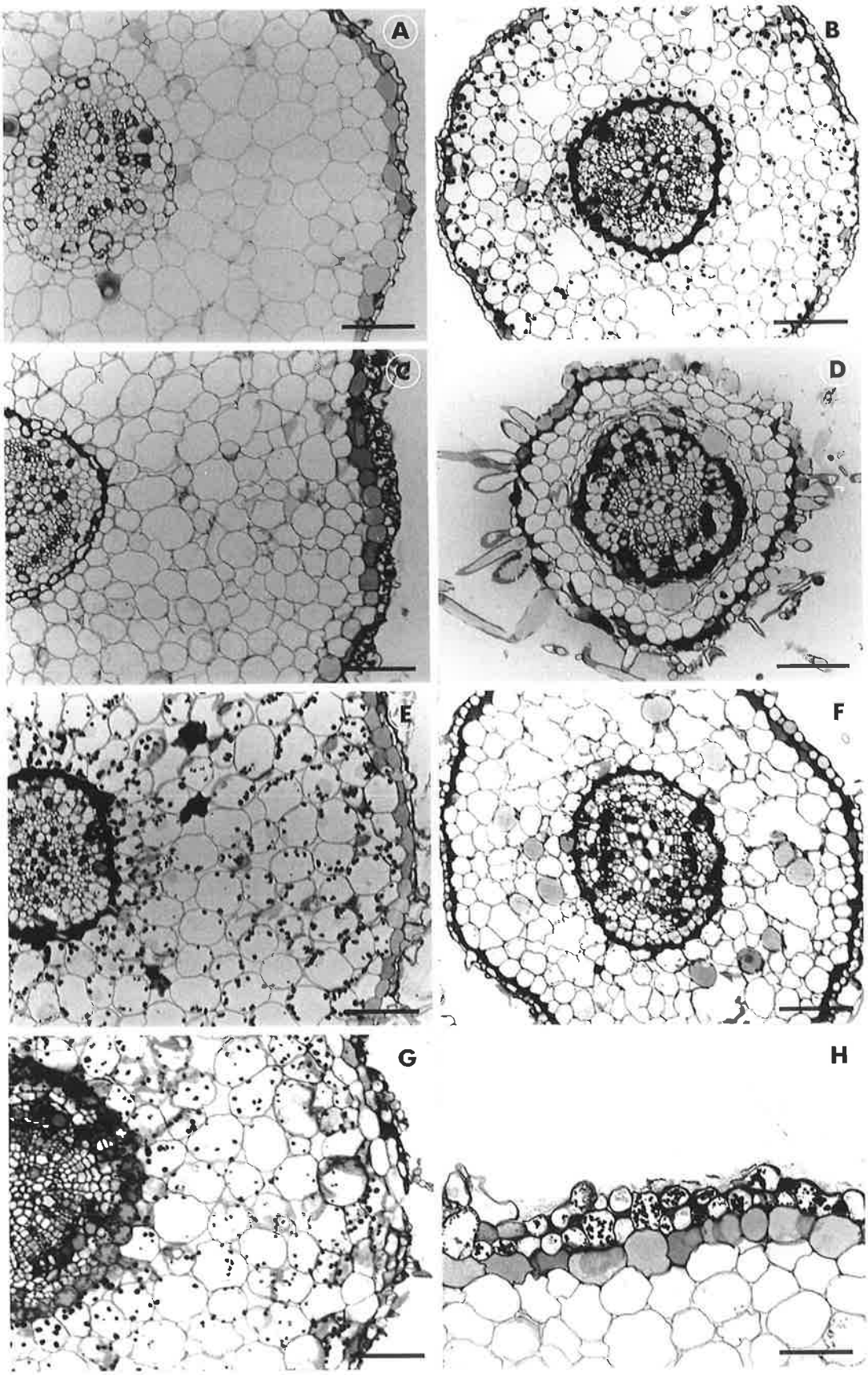
The primary roots of Schwarzmann (figure 5.3b), *V. cinerea* (figure 5.3e) and Börner (figure 5.3f) were very similar to each other, both having an epidermis with unstained contents and a distinct, evenly stained hypodermis. The cortical cells of Schwarzmann and Börner appeared loosely packed, with heavily stained granules scattered throughout. The endodermis was heavily stained, and the steles shown were triarch. Some cells scattered through the cortex in Börner had lightly and evenly stained contents.

The primary roots of *V. riparia* (figure 5.3d) had a much shallower cortex (two to three cells) than other varieties. The epidermis had lightly stained contents, and intercellular spaces below it were darkly stained. No hypodermis was apparent in this section, although present in others (figure 5.5d). The section shown in figure 5.3d was taken through the absorption zone (behind the zone of elongation), however similar sections cut either closer to or further from the root tip had a similar anatomy, often including large air spaces in the root cortex, which may be a product of growth in tissue culture medium. (This species naturally occurs along river banks and may be adapted to respond to waterlogging by the development of aerenchyma (Atwell *et al.* 1999).)

In the primary roots of *V. rotundifolia* (figure 5.3g) the epidermis was less continuous than other varieties observed and the stele showed formation of metaxylem. This species is more woody in tissue culture, and it is likely that this partly results from quicker differentiation to relative to distance from the root tip than other varieties.

The extent of staining of the endodermis appears to be a function of root development. Further from the root tip, the phenolic content, and hence staining, of the endodermis

Figure 5.3 Transverse sections through roots of tissue culture grown grapevines stained with PAS/TBO. a) *V. vinifera* cv. Shiraz b) Schwarzmann c) Ramsey d) *V. riparia* e) *V. cinerea* f) Börner g) *V. rotundifolia* h) Ramsey – epidermis and hypodermis. Scale bars for a) - g) equal 100µm. Scale bar for h) equals 50µm.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increased. In primary roots of tissue cultured vines of Schwarzmann, Börner, and *V. riparia* the endodermis was typically heavily stained This was less commonly observed in *V. vinifera* and Ramsey. There appears, therefore, to be some correlation between the resistance of a variety to phylloxera and the rate at which the primary roots mature relative to distance from the root tip.

Anatomy of potted vine roots

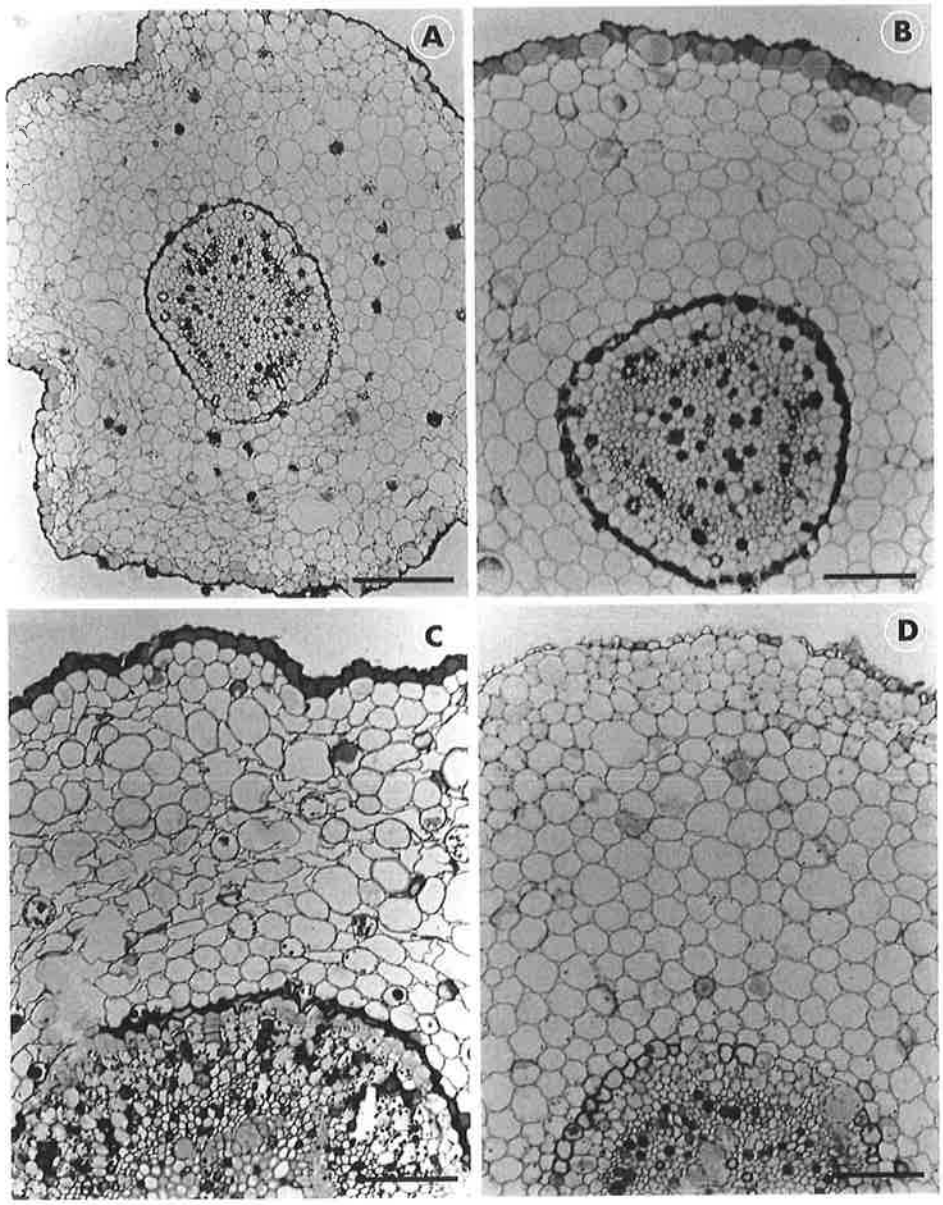
Transverse sections were cut through roots of the four potted varieties (*V. vinifera* cv. Shiraz, Ramsey, Schwarzmann and *V. rotundifolia*) at 5-6cm from the root tip. Initially, potted vine roots had been sectioned at 0-1cm, 5-6cm and 10-11cm from the root tip and the distance of 5-6cm was selected as giving the most consistent representation of primary root anatomy.

The primary roots of *V. vinifera* cv. Shiraz (figure 5.4a) had an evenly packed cortex approximately 12 cells deep. There were a few cells scattered throughout the cortex which were mostly filled with small, dark staining granules. The epidermis and walls of the endodermis were heavily stained, as were a few cells in the stele, and the hypodermis was faintly stained throughout. Other than these, contents of most cells were unstained. The stele was tetrarch, although, as in tissue cultured vines, this was variable from plant to plant. Further from the root tip (10-11cm) the contents of endodermal cells became heavily stained (data not shown).

At the same distance from the root tip, Ramsey roots (figure 5.4c) had developed more metaxylem than *V. vinifera* roots. The cortical cells were larger than those in *V. vinifera* roots, and also large in comparison with those in the stele. They were more loosely packed and the cortex showed some signs of degradation (characteristic of the transition of roots from primary to secondary anatomy). Contents of hypodermal cells were heavily stained. The stele was larger and more developed than in *V. vinifera* roots, with a heavily stained endodermis, two to three cells deep pericycle and far more cells either containing heavily stained granules, or with heavily stained contents. Further from the root tip (10-11cm), the phloem parenchyma and phloem tissue became filled with a mass of heavily stained contents. This was never observed at the same distance from the root tip in *V. vinifera* roots (data not shown).

The primary roots of Schwarzmann (figure 5.4b) were at a similar stage of development as *V. vinifera*. The epidermis had collapsed and the exodermis (suberised hypodermis, usually forming the outer layer of the roots after the epidermis has been sloughed off) had become the outer layer of the cortex, and had evenly stained contents. The underlying cortex was

Figure 5.4 Transverse sections 5-6cm from root tip through roots of pot grown vines stained with PAS/TBO. a) *V. vinifera* cv. Shiraz b) Schwarzmann c) Ramsey d) *V. rotundifolia*. Scale bars equal 100µm.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approximately nine cells deep with unstained cell contents. The endodermis and some cells in the stele parenchyma had heavily stained contents.

The primary roots of *V. rotundifolia* (figure 5.4d) also resembled *V. vinifera* in their state of development at 5-6cm from the root tip. The epidermis was intact and was made up of particularly small cells; contents of the hypodermis were not stained. The cortical cells were small and densely packed with unstained contents, and the cortex was ~15 cell layers deep. The endodermis cell walls or cytosol were stained but not the cell contents. There were some dark stained cells in the stele. In the same root further (10-11cm) from the tip the endodermis cell contents became heavily stained as in the other three varieties (data not shown).

Discussion of primary root anatomy

The differentiation of dicotyledon primary roots, such as those on potted grapevines, which have a primary anatomy, to woody roots with secondary anatomy involves degradation and sloughing off of the cortex, production of a corky layer, which becomes the outer layer of the root, from the tissues below the endodermis, and development of secondary vascular tissues including a woody pith. Unlike potted vine roots, the roots of tissue cultured vines tend not to undergo such differentiation as distance from the root tip increases. While sections of tissue cultured vine roots shown were taken close to the root tip, others (not shown) taken further from the tip, were not significantly more differentiated.

While secondary tissue were not present in roots shown here, the accumulation of phenolics in the endodermis and stele, shown by staining with TBO, gives an indication of root development. Based on this, primary roots of Schwarzmann, Börner, and *V. riparia* appeared to mature more quickly (assuming proximity to the root tip indicates age) than those of *V. vinifera* or Ramsey. This rapid maturation coincided with the poor performance of phylloxera as determined by tissue culture-based bioassays (section 5.2.1). Pratt (1974) mentioned a similar relationship between rate of root maturation and phylloxera resistance. An exception to this potential correlation is the immune *V. rotundifolia,* whose state of differentiation is similar to that of the susceptible *V. vinifera*. *V. rotundifolia*, however, differed from other varieties described in this study in that phylloxera did not appear to attempt to feed, therefore root anatomy or differentiation would have no role to play in its resistance status.

Apart from the state of differentiation of the endodermis, no major anatomical differences were seen between the primary roots of the varieties investigated. No correlation was found between cortical cell size and resistance, as had been suggested by Pratt (1974). It is possible that varieties on which phylloxera attempt to initiate nodosities may rely at least

partly on relatively early maturation to prevent extensive nodosity formation, as more differentiated tissues are less likely to respond to stimulation by hormones (Hori 1992).

5.3.2 Histochemistry of primary roots

The use of specific stains and other microscopy techniques can indicate the chemical composition of tissues examined. There is no published histochemical study of grapevine roots, nor a histochemical comparison between *Vitis* species or hybrids. The main objective of this study was to determine what correlation, if any, might exist between the presence of particular compound(s) in a range of vine varieties and their resistance to phylloxera.

Materials and methods

Trials of various microscopy techniques (Chapter 3, section 3.3.2) indicated that the most useful results for this study would be obtained by using autofluorescence under UV excitation, PAS/TBO staining, including PAS staining only as a control (results not shown) and Sudan black staining. These were used for examination of root samples taken 5-6cm from the tip of roots from vines used in pot trial four (Chapter 2, section. 2.3.4) and 5cm from the tip or roots from tissue cultured vines used in perlite-based medium cocultivations (Chapter 2, section 2.4.4). Potted vine roots of *V. rotundifolia* were collected as above from vines grown in the glasshouse at The University of Adelaide. Triplicate root samples from each variety and from each source (potted or micropropagated vines) were stained, and selected results, representative of each variety, are presented in this section.

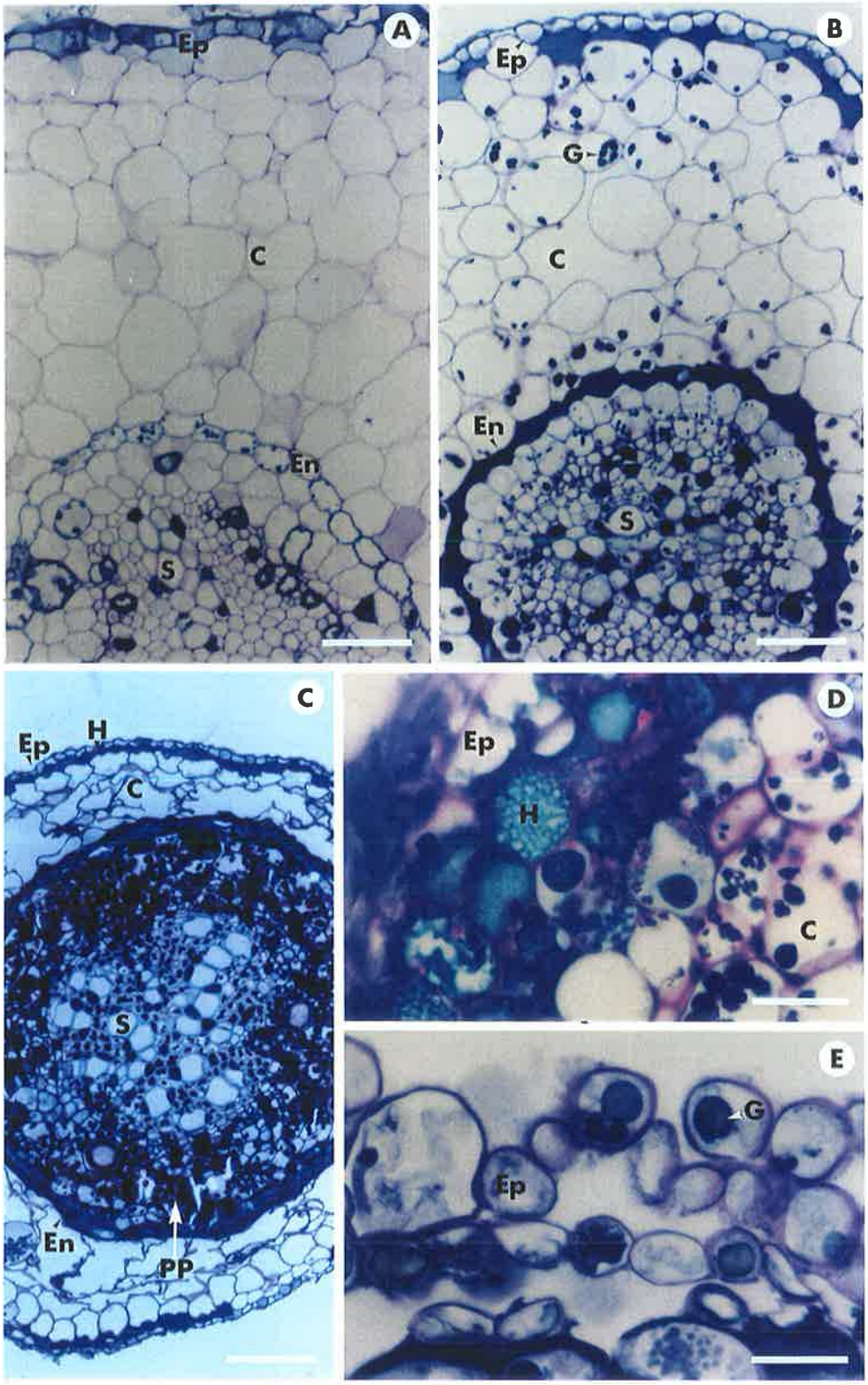
Polyphenolics and starch

Details observed using PAS/TBO stain (some of which are illustrated in figure 5.5) are described below.

V. vinifera cv. Shiraz: In roots from tissue cultured vines (figure 5.5a) a small quantity of starch was present in the cortical cells (although none was present in this section). In one root (not shown) dark-blue staining indicated the presence of phenolics in the vacuoles of some cells of the pericycle. The roots from potted vines were similar to those from tissue cultured vines in many respects, although they had a higher phenolic content in the outer cell layers, and in the endodermis, as demonstrated by dark blue staining.

Ramsey: Roots from tissue cultured vines (not shown) an endodermis with no evidence of phenolics in the endodermis nor of starch in the cortex. There were distinctive, dark-blue stained globules in the epidermis. Potted vine roots differed in that they had a high phenolic content (stained dark blue) in the endodermis, and a small amount of starch was present within the stele parenchyma. The epidermis of sections observed had been sloughed off.

Figure 5.5 Transverse sections through roots of tissue cultured vines stained with PAS/TBO a) *V. vinifera* cv. Shiraz Endodermis (En); epidermis (Ep); cortex (C); stele (S). Scale bar equals 50µm b) Schwarzmann. Endodermis (En); epidermis (Ep); cortex (C); stele (S); phenolic globules (G). Scale bar equals 50µm c) Börner. Endodermis (En); epidermis (Ep); hypodermis (H); cortex (C); stele (S); phloem parenchyma (PP). Scale bar equals 100µm d) *V. riparia*. Epidermis (Ep), hypodermis (H); cortex (C). Scale bar equals 20µm e) *V. rotundifolia*. Epidermis (Ep), globules (G). Scale bar equals 20µm.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Schwarzmann: Roots from tissue cultured vines (figure 5.5b) appeared similar to *V. vinifera,* except that they had a distinctive blue-green stained hypodermis and endodermis. In this variety, the difference in PAS/TBO staining compared with previously described varieties was striking. Phenolics stained a distinctive blue-green colour compared to the dark navy blue in *V. vinifera* and Ramsey. In most potted vine roots observed the epidermis had collapsed, however where it was intact it contained many blue-green stained globules, while the exodermis was evenly stained blue (data not shown).

V. riparia: Roots from tissue cultured vines (figure 5.5d) had a large number of small vacuoles or vesicles in the epidermis and hypodermis stained dark blue-green, indicating a high phenolic content. There were also dark blue stained globules in the cytoplasm of the cortical cells. In other respects, this variety was similar to those described previously. Potted vine roots were not available for this study.

Börner: Roots from tissue cultured vines (figure 5.5c) had a relatively shallow cortex, the one pictured showing signs of collapse, indicating its relatively advanced state of differentiation (section 5.3.1). The epidermis was stained pale blue throughout, while the hypodermis and endodermis were stained the distinctive blue-green colour as seen in other tissues of Schwarzmann and *V. riparia*. The stele of the section shown contained more developed vascular tissues (metaxylem) than others described so far, with a very high starch content in the phloem parenchyma. The distinct blue-green staining of the endodermis was a consistent feature of primary roots of this variety, irrespective of the state of differentiation. Potted vine roots were not available for this study.

V. rotundifolia: In the epidermis of tissue cultured vine roots (figure 5.5e), vacuoles were stained a unique olive-green colour. This staining was consistently observed in the roots of this variety, but not seen in any other varieties examined. The identity of the compound(s) responsible remains unknown. There was a only small amount of starch in the cortex. The endodermis was either stained pale blue or unstained. Potted vine roots were similar to the tissue cultured vine roots examined, except that small amyloplasts were scattered evenly throughout the cortex of the potted vine roots (not shown).

Summary: Most varieties showed some variation in the staining of phenolic components, especially those where sections of both potted vine and tissue cultured vine roots were included. Consistent differences between varieties included the inability to detect starch in the cortex of Ramsey roots, and the presence of blue-green staining phenolics in the hypodermis and/or endodermis of Schwarzmann, *V. riparia*, and Börner which were distinct from the dark blue staining commonly seen in other varieties. The olive-green stained

compound(s) in the vacuoles of the epidermis of *V. rotundifolia* roots was a unique characteristic of this variety.

Autofluorescent compounds

Sections of the same root samples as those used for PAS/TBO staining were observed by UV excitation, but this technique did not distinguish between the varieties. The results are therefore not presented in this section, although the transverse section of a potted vine root of *V. vinifera* cv. Shiraz shown in Chapter 4, figure 4.6a was typical of the results for all varieties. Autofluorescent compounds, presumably lignin and suberin, were detected in the xylem and endodermis respectively, while other autofluorescent compounds were present in the epidermal cell vacuoles and some cells of the stele parenchyma.

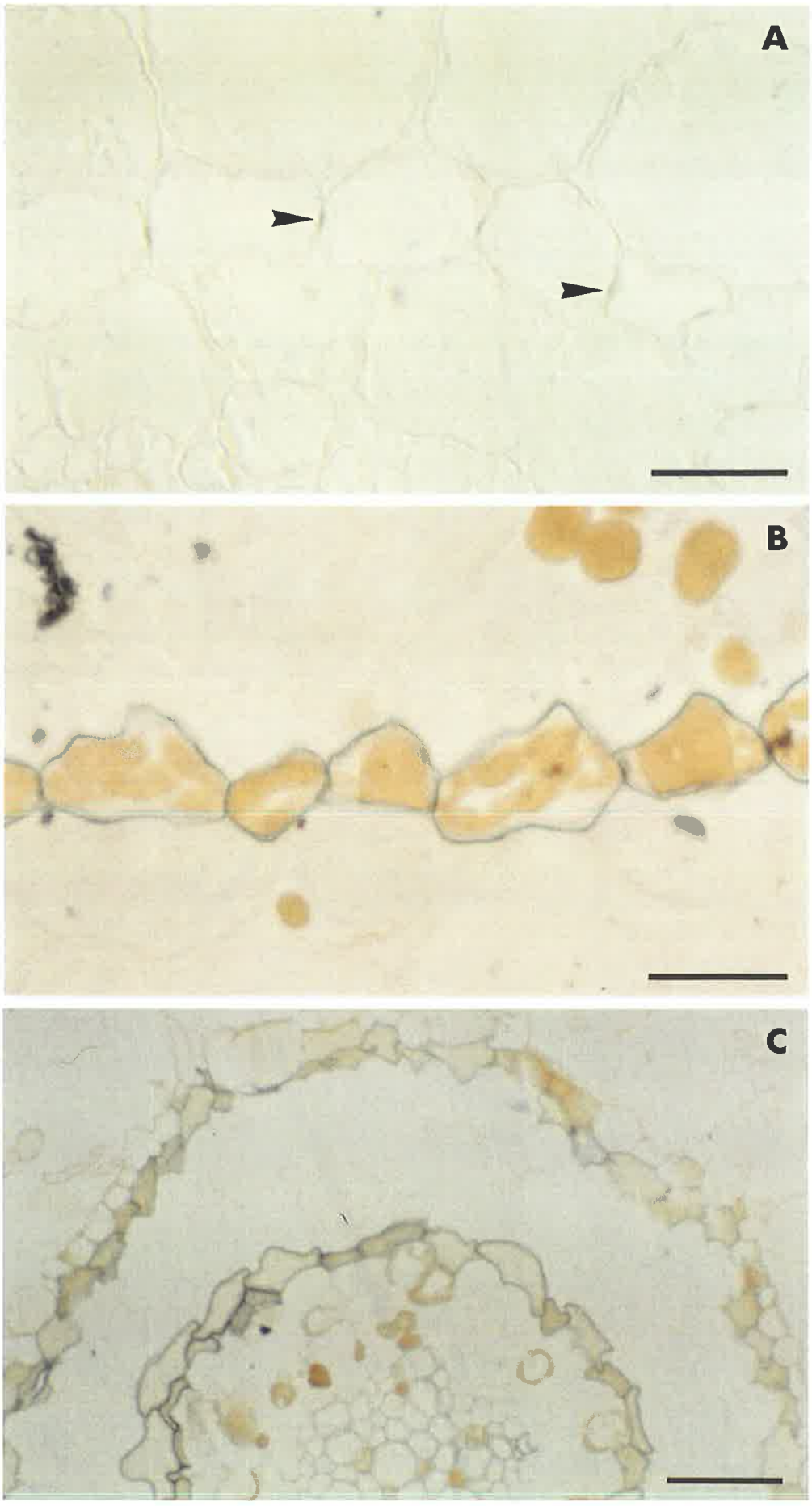
Suberin

The state of suberisation of the endodermis was assessed in roots of pot grown *V. vinifera* cv. Shiraz (not shown), and tissue cultured Ramsey (figure 5.6b) and *V. riparia* (figure 5.6c). In all three varieties the endodermis developed from state one (casparian strip only) to state two (fully developed suberin lamellae) as the root tissues matured, irrespective of resistance to phylloxera. In tissue cultured *V. vinifera* cv. Shiraz, however, state one endodermis was still observed in roots even at some distance from the root tip (figure 5.6a).

Discussion of histochemistry results

The presence of blue-green stained phenolics in the exodermis of Schwarzmann, *V. riparia* and Börner is associated with the occurrence of a necrotic response to phylloxera feeding, and hence with resistance as defined from tissue culture-based bioassays (section 5.2). The tendency of phylloxera to successfully settle along the entire length of the root on *V. vinifera* tissue cultured vines (section 5.2.1) may be related to the characteristic of this variety to retain state one suberisation of the endodermis even at some considerable distance from the root tip. (Phylloxera also settled along the length of roots on Ramsey, which develops state two suberisation of the endodermis. Nodosities of this variety, however, failed to accumulate starch (section 5.4.1) and phylloxera development was poor.) Few other noteworthy differences were seen between varieties except for the olive-green staining compound in the epidermis which was unique to *V. rotundifolia* and may be related to its very strong resistance or possible status as a non-host plant. It was not possible to identify any of the stained phenolic compounds further using techniques available in this study. Various microscopy techniques have been used by other researchers for identification of phenolic compounds in fresh tissue usually using a combination of dyes and fluorescence (Dai *et al.* 1995a, b, Hutzler *et al.* 1998) and these could possibly be applied to studies of the phylloxera-grapevine interaction in the future. It is likely that the distribution and

Figure 5.6 Transverse sections through roots of tissue cultured vines stained with Sudan black. Suberin is stained blue, non-suberised walls are not stained. a) *V. vinifera* cv. Shiraz endodermis with state one suberisation. Arrows mark casparian strip in radial walls of the endodermis. Scale bar equals 20μm. b) Ramsey endodermis with state two suberisation in all walls of endodermis and hypodermis. Scale bar equals 20μm. c) *V. riparia* with state two suberisation in all walls of endodermis and hypodermis. Scale bar equals 50μm.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composition of phenolic compounds in primary roots may play a role in resistance to phylloxera through their involvement in the oxidative browning (necrosis) response to feeding seen in the highly resistant varieties, and/or through direct antimetabolic effects.

5.3.3 *Free phenolic content of primary roots*

A correlation between the presence of certain compounds in plant tissues with the resistance of those tissues to pest attack has, for many plant-pest systems, led to hypotheses regarding the role of these compounds in pest resistance. HPLC has often been used to identify compounds involved in such systems (e.g. Cole 1984, Grayer *et al.* 1992, Cole 1994b, Chen *et al.* 1996, Cole 1996, Chen *et al.* 1997, Nicol and Wratten 1997). For most phloem-feeding aphids, these hypotheses can be further tested through the use of *in vitro* feeding systems in which artificial diets contain the compound of interest (Todd *et al.* 1971, van Helden *et al.* 1995, Rahbé *et al.* 1995, Annan *et al.* 1996, Down *et al.* 1996, Chen *et al.* 1996, 1997, Fragoyiannis *et al.* 1998). No successful artificial feeding system has yet been developed for phylloxera. There remains, however, the possibility of investigating the biochemical composition of grapevine roots from different varieties, and determining the correlation, if any, between the presence of certain compounds and the resistance of the roots to phylloxera feeding and/or nodosity formation.

Determination of the spectrum of free phenolic compounds present in vine roots was selected as the focus of this study. Phenolic compounds are likely candidates for having a role in resistance of roots to pest attack. For example, chlorogenic acid has also been shown, through the use of artificial diets, to prevent reproduction of the aphid *Schizaphis graminum* (Todd *et al.* 1971). Cole (1984) found that the presence of chlorogenic acid in lettuce roots was correlated with resistance to the lettuce root aphid *Pemphigus bursarius*. Speed and quantity of accumulation of chlorogenic acid in tomato roots is also correlated with their resistance to root-knot nematodes (Mote *et al.* 1990).

In addition, *trans*-resveratrol and its derivative stilbenes and stilbenoids are known to be the major phenolic constituents of grapevine roots (Mattivi and Reniero 1992, Korhammer *et al.* 1995, Mattivi *et al.* 1996, Reniero *et al.* 1996). These compounds are generally considered to be biologically active and have been implicated in grapevine defence against a number of fungal pathogens (Langcake 1981, Stein and Blaich 1985, Dercks and Creasy 1989, Sbaghi *et al.* 1995). It is possible that they also play a role in the defence of grapevine roots against phylloxera. Prior to the work reported in this study, varietal differences in root phenolics had not yet been investigated, so no correlation has yet been determined between phenolic composition and resistance to root pests. This section reports on the analysis of free phenolics extracted from the primary roots of six vine varieties using HPLC.

Materials and methods

Extracts for HPLC analysis of free phenolics were made from duplicate root samples harvested from the perlite-based medium cocultivations (Chapter 2 section 2.4.4), and from pot trial four (Chapter 2, section 2.3.4) according to free phenolic extraction method one (Chapter 3, section 3.2.1). Extracts were separated using HPLC chromatography method nine (Chapter 3, section 3.2.2).

Free phenolic profile of primary roots

Absorption profiles obtained at either 280nm or 330nm for free phenolics extracted from roots of tissue cultured vines and separated by HPLC, presented in figure 5.7a, b, were overlayed for comparison between varieties. The major peaks occurring were numbered from 1 to 11. Their retention times and spectral properties are listed in table 5.4.

The free phenolics present in primary roots were found to be primarily derivatives of resveratrol, i.e. stilbenes and stilbenoids, which is in agreement with previous analyses of *Vitis* root extracts (Mattivi *et al.* 1996). There was no evidence of the presence of other free phenolic compounds such as phenolic acids or quercetin, based on comparisons with a wide range of standard compounds (Appendix five, table A5.1).

Peak three appears to be made up of a *trans*-resveratrol glucoside (most likely to be piceid, the 3-β-glucoside of *trans*-resveratrol) as well as another unknown compound eluting slightly later. This identification was partly made from the absorption spectrum (figure 5.8b), which is similar to that of free *trans*-resveratrol (figure 5.8c). Further, treatment of extracts with β–glucosidase, according to the method described in Appendix two, resulted in the disappearance of peak 3 coincidentally with the appearance of a peak with the same retention time and absorbance spectrum of *trans*-resveratrol. To illustrate this, figure 5.8a shows HPLC chromatograms of root extract from *V. rotundifolia* before and after β-glucosidase treatment. The necessity to resuspend the extract in an aqueous buffer prior to treatment resulted in a significant reduction in all peak areas compared with figure 5.7.

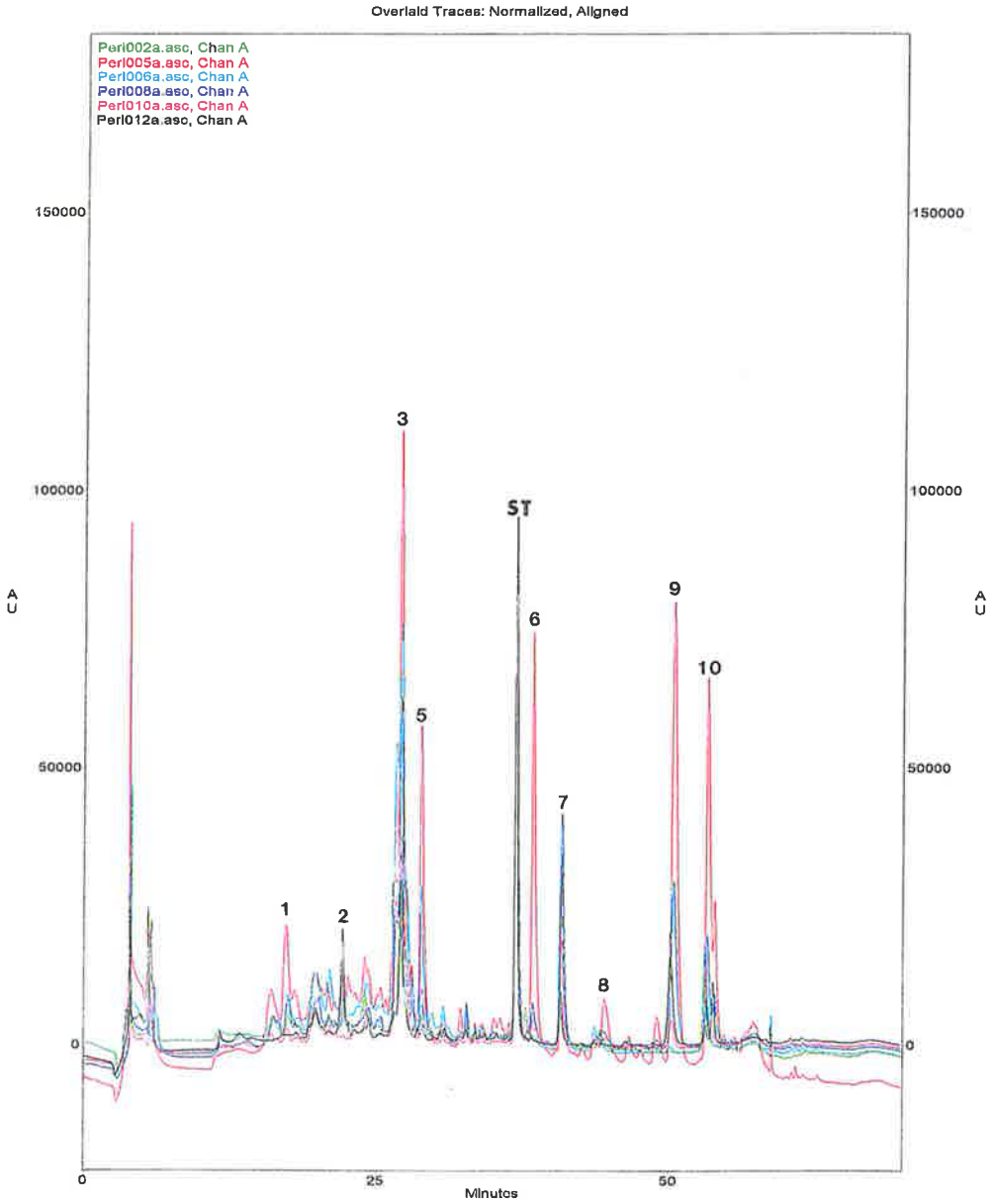


Figure 5.7a HPLC chromatograms showing absorbance at 280nm for tissue cultured roots of six grapevine varieties. Major peaks are numbered 1-11. Putative identification of each numbered peak is given in table 5.4. Internal cinnamic acid standard marked (ST). *V. vinifera* cv. Shiraz, green; Schwarzmann, red; Ramsey, light blue; *V. riparia*, dark blue; Börner, pink; *V. rotundifolia*, black.

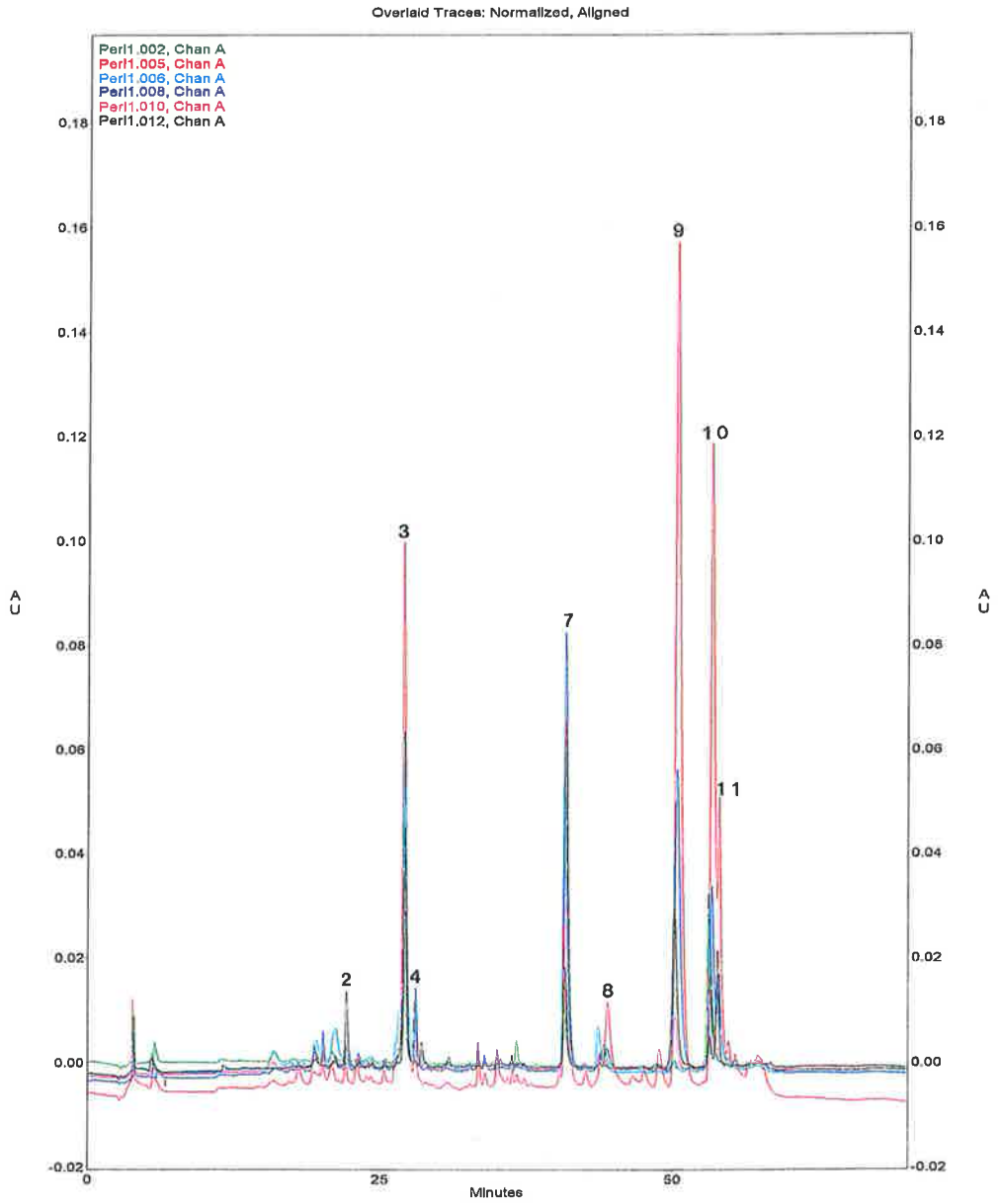


Figure 5.7b HPLC chromatograms showing absorbance at 330nm for tissue cultured roots of six grapevine varieties. Major peaks are numbered 1-11. Putative identification of each numbered peak is given in table 5.4. *V. vinifera* cv. Shiraz, green; Schwarzmann, red; Ramsey, light blue; *V. riparia*, dark blue; Börner, pink; *V. rotundifolia*, black.

Table 5.4 Major peaks in HPLC chromatograms of grapevine root extracts including retention times, spectral properties and putative identities. Numbers in parentheses indicate the position of shoulders on the absorption spectra. Spectra are shown in Appendix seven.

Peak no.	Retention time (min)	λ max (nm)					Putative Identity[1]
1	17.45	206	(228)	278			Unknown stilbenoid
2	22.38	206		297			Unknown resveratrol hexoside[2]
3	27.14	206	248	304	(314)	(366)	*Trans*-resveratrol 3-β-glucoside plus unknown
4	28.14	206	(288)	305	(320)		Methylated resveratrol glucoside[3]
5	28.92	206	(226)	281			Ampelopsin A
6	38.48	206	(226)	282			Unknown
7	40.98	204	(223)		319		ε-viniferin
8	44.54	204	(223)	283	326		r-2-viniferin
9	50.55	204	223	284	326		Gnetin H
10	53.48	204	(223)	(284)	326		r-viniferin
11	54.11	204	(223)	(284)	326		unknown oligostilbene b

[1] Identification of peaks 1, 3, 5-11 was made by F. Mattivi, Instituto Agrario, San Michele all'Adige, Italy, from a Schwarzmann root extract sent from Australia. Data given in Appendix eight. Also noted in the Schwarzmann extract by F. Mattivi (not detectable in this extract using the HPLC systems described in this study) were free *trans*-resveratrol eluting between peaks five and seven, unknown oligostilbenes 'a' and 'd' eluting before peak nine and unknown oligostilbene 'c' eluting after peak eleven (Mattivi *et al.* 1996, Mattivi pers. comm.).

[2] Peak two was putatively identified by mass spectrometry (Appendix six) and a comparison of the absorption spectrum with published data (Mattivi and Reniero 1996, Hillis and Ishikura 1968, K. Markham, pers. comm.).

[3] Peak four was identified by mass spectrometry as described in text.

In addition this treatment removed the 'unknown' component of peak three from the HPLC chromatogram, which suggests that this compound is relatively insoluble in water. The same result was obtained when root extracts of all six vine varieties were treated in the same way with β-glucosidase (data not shown). To further confirm the identity of piceid, ionspray mass spectrometry demonstrated that the ionisable fraction of peak three contained a compound with a mass of 229.2 (see Appendix six), which is the mass that would be expected after protonation of *trans*-resveratrol (mass 228.2) during ionisation. The absorption spectrum obtained for peak three was slightly variable both between varieties and between replicates of root extracts from the same variety (figure 5.9). The reason for this variation is not known. The 'unknown' component of this peak was not ionisable, so could not be identified by mass spectrometry.

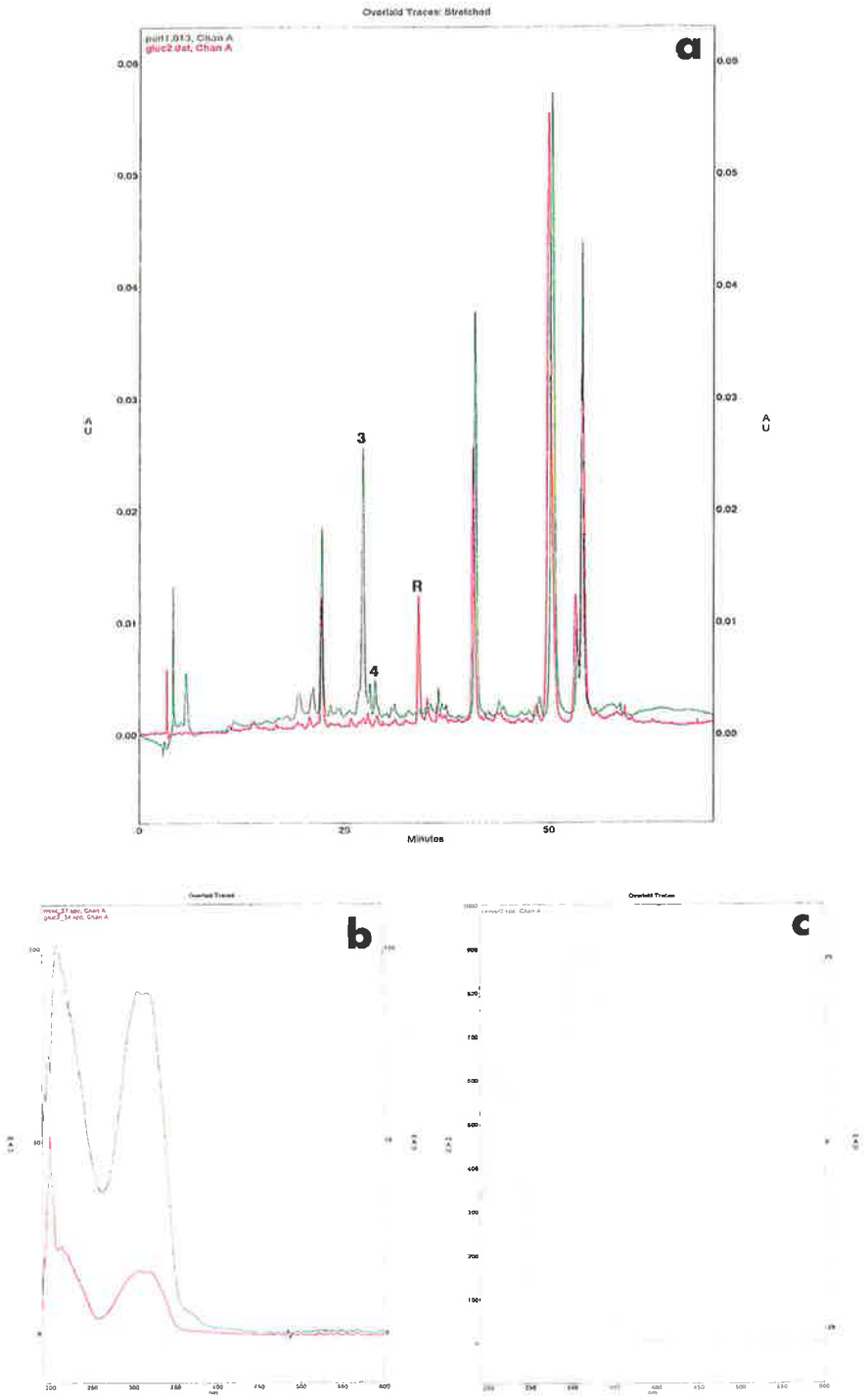


Figure 5.8 a) HPLC chromatogram of *V. rotundifolia* tissue cultured root extract before (black) and following (red) treatment with β-glucosidase. The disappearance of peak three is accompanied by a slight accumulation of free *trans*-resveratrol (R). b) Absorption spectra of peak three from untreated extract (black), and new peak (R)(red) in treated extract. c) Absorption spectrum of commercially available *trans*-resveratrol (Sigma).

Unfortunately, purified preparations of stilbenes other than *trans*-resveratrol were not commercially available and therefore could not be run as standards for comparison with the free phenolics extracted from the roots. An extract made from Schwarzmann roots, which contained all major compounds except number two, was sent to Dr F. Mattivi, Instituto Agrario, San Michele all'Adige, Italy, for identification of phenolic components according to methods published in Mattivi *et al.* (1996) and Mattivi and Reniero (1996). The HPLC chromatogram obtained by Dr Mattivi for this extract is presented in Appendix eight. Some of the compounds identified have not yet been named, but are referred to as 'unknown oligostilbenes' (Mattivi *et al.* 1996). The identities given in table 5.4, with the exception of peaks 2 and 4, are as determined by Dr Mattivi.

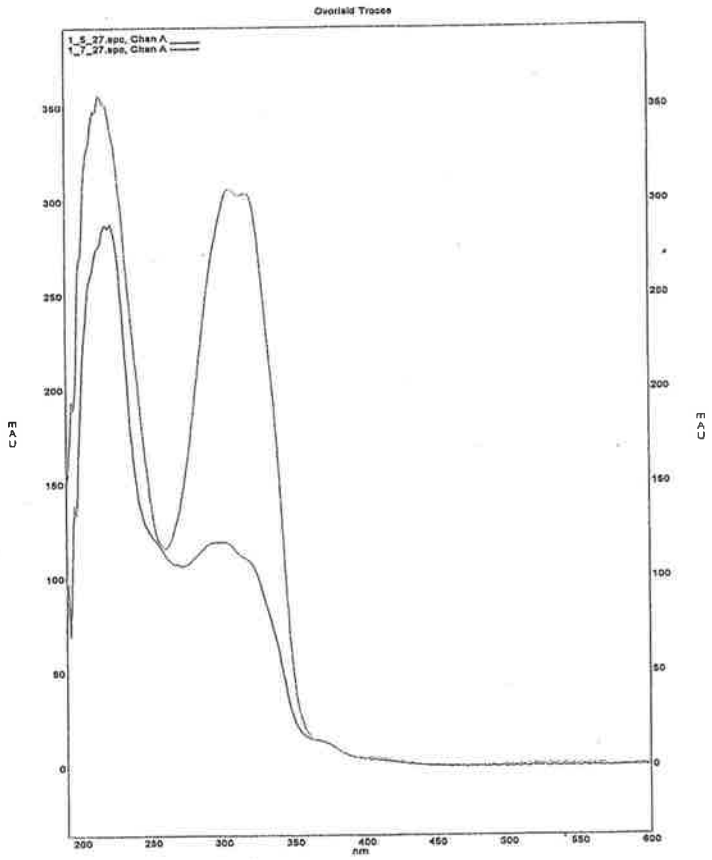


Figure 5.9 Absorption spectra of peak three from extracts of replicate samples of Ramsey tissue cultured vine roots showing the slight variations in spectral properties.

Peak two, which has an absorption maximum at 297nm, was unique to *V. rotundifolia*. Since this variety was rated as immune in this study, the compound present in peak two was felt to be of sufficient interest to attempt further identification. Fresh extracts were prepared from tissue cultured vine roots, and the sample used for ionspray mass spectrometry. The complexity of the absorption profile in the region of peak two made interpretation of mass spectrometry results difficult. Ions of masses 391.6, 373.2 and 229.4 (see Appendix six)

were identified at similar HPLC retention times to peak two, and were unique to *V. rotundifolia*, as determined by comparison with analysis of a Schwarzmann root extract.

These masses suggested that the compound contained in peak two was a hexoside of resveratrol[6]. The absorption spectrum of peak two was very similar to the *cis*- isomer of resveratrol glucoside (Jeandet *et al.* 1997, Mattivi and Reniero 1996), however the published retention time of the *cis* isomer upon HPLC is greater than that of the *trans*, while the retention time of peak two (22.38 minutes) is less than that of the *trans* isomer, (~34 min). The absorption maximum at 297nm could result from a relatively small shift of band two (304nm) of *trans*-resveratrol glucoside (Appendix five, table A5.2), while band one (316nm) may have been reduced to such an extent that it only appears as a slight shoulder (Appendix seven, peak two). It seemed most likely, judging by the combination of mass spectrometry results, retention time and absorption spectrum, that compound two was either the *cis*- or *trans*- isomer of a previously undescribed hexoside of resveratrol (K. Markham, Industrial Research Limited, Wellington, NZ, pers. comm.). There was insufficient root material available to collect the HPLC fraction corresponding to peak two and purify it for further analysis.

The compound present in peak four was shown by ionspray mass spectrometry to have a mass of 259.2 (Appendix six). This is exactly 30 greater than peak three (piceid), and could be accounted addition of a methyl group. The absorption spectrum and retention time were both similar to those of peak three, and, as for peak three, this compound was removed from the HPLC profile by treatment with β-glucosidase (figure 5.8) It is likely therefore that this peak represents a methylated form of piceid.

The presence or absence of peaks 1-11 in the free phenolic extracts from tissue cultured or potted vine roots of each variety was determined using 'Custom Report', a report generation program of Beckman Gold Nouveau version 1.6, where a minimum area was assigned for the automatic recognition of peaks (tables 5.5 and 5.6). All reports were also checked manually by comparing each HPLC trace with its appropriate peak report.

Due to the quantitative variation inherent in the methods used in this study (discussed in Chapter 3, section 3.2.4), the results are only reported as presence or absence of peaks with no attempt at quantitative comparisons.

[6] Protonated ion masses present in peak two are as follows. 391: Ionised glycoside – ionised resveratrol (m.w. 229) plus ionised hexose (m.w. 162). 373: the ionised glycoside minus a water moeity (m.w.18). 229: the ionised aglycone.

Table 5.5 Presence or absence of HPLC peaks 1-11 in free phenolic extracts from tissue cultured vine roots.

vine variety	1	2	3	4	5	6	7	8	9	10	11
V. vinifera	-	-	+	-	+	-	+	+	-	+	-
Schwarzmann	+	-	+	-	+	+	+	+	+	+	+
Ramsey	+	-	+	-	-	+	+	+	-	+	-
V. riparia	-	-	+	+	+	-	+	+	+	+	+
Börner	+	-	+	-	+	+	+	-	+	+	+
V. rotundifolia	-	+	+	-	-	-	+	-	+	+	+

Table 5.6 Presence or absence of HPLC peaks 1-11 in free phenolic extracts from potted vine roots.

vine variety	1	2	3	4	5	6	7	8	9	10	11
V. vinifera	+	-	+	-	+	+	+	+	+	+	-
Schwarzmann	+	-	+	-	+	-	+	-	+	+	-
Ramsey	+	-	+	-	+	-	+	+	-	+	-

Discussion of HPLC analyses of free phenolics

These studies confirmed the presence of considerable levels of free phenolics in grapevine roots. Mattivi *et al.* (1998) calculated that stilbenes and stilbenoids typically occur at concentrations of 17-27mg/g fw and 15-18mg/g fw respectively in primary roots of *Vitis* spp., and constitute approximately 95% (w/w) of the phenolic component of the root cortex. All the free phenolics present in the root extracts prepared here appear to belong to this same class of compounds. Only the compounds represented by peaks two, three and four appear to be present in the form of glycosides. Glycosylated phenolics are stored in the vacuole and may function as non-toxic reserves which may be deglycosylated, and hence 'activated' on, for example, pest attack (Rhodes and Wooltorton 1978).

The quantitative and qualitative variation in phenolic profiles between replicate samples of potted vine roots or tissue cultured vine roots of the same variety and between different varieties suggests that the amount and type of these compounds in roots, as detected by the methods used here, are not highly significant in terms of the interaction with phylloxera. The comparison of extracts from potted vine roots and tissue cultured vine roots has minimised potential misinterpretation of data. For example, the coincidence of certain peaks and resistance to phylloxera which exists for one set of data, does not hold for the other. In extracts from tissue cultured vine roots, peaks 9 and 11 are absent in susceptible *V. vinifera* and resistant Ramsey, but present in highly resistant Schwarzmann, Börner and *V. riparia*. However, in extracts from potted vine roots peak 11 is absent altogether, and peak 9 is present in *V. vinifera* and Schwarzmann, while the interaction of each variety with VWL-1 phylloxera is similar as to the interaction with tissue cultured vines. It is more

likely that similarities between highly resistant vines might result from their common *V. riparia* parentage, as Schwarzmann and Börner, rather than being associated particularly with resistance to phylloxera.

Comparison between the results presented in tables 5.5 and 5.6 and those obtained from analysis of roots harvested from pot trial three (data not shown), further illustrated the apparently high level of natural variation in the composition of free phenolics. In *V. vinifera*, for example, peaks eight and nine were present in roots from pot trial four (table 5.6), but not pot trial three, and only peak eight was present in the tissue cultured vine roots (table 5.5). Similarly, the free phenolic profiles of Schwarzmann roots, while virtually identical between the two pot trials, are missing peaks six, eight and eleven when derived from tissue cultured vine roots (table 5.5). Comparative results from other trials were not available for other varieties.

The one consistent and possibly significant difference detected here was the presence of peak two exclusively in root extracts of *V. rotundifolia*. This compound has been putatively identified as a hexoside of resveratrol. Phylloxera behaviour observed in this study indicated that the immunity of *V. rotundifolia* to phylloxera may involve non-preference (Chapter5, section 5.2.2), possibly suggesting the presence of a chemical deterrent, or absence of a chemical attractant. The compound present in peak two could be a candidate deterrent molecule although this needs to be assessed more rigorously through the use of a system such as artificial diets. *V. rotundifolia* is also reported to be capable of a hypersensitive response in response to attack by other pathogens (Dai *et al.* 1995a, b) and, in some cases to phylloxera's attempts to feed on secondary roots (Boubals 1966a). It is therefore likely that, should a compound conferring non-preference exist, other mechanisms of resistance or immunity may also be present.

In conclusion, the presence of potentially toxic compounds such as ε-viniferin and *trans*-resveratrol glucoside in the roots of all grapevine varieties including *V. vinifera*, suggests that these are neither toxic nor distasteful to phylloxera. However, whilst significant quantitative or qualitative differences in free phenolic composition could not be detected between root extracts of different varieties by the methods used here, this does not eliminate the possibility that particular distributions of phenolic compounds within the roots might play a role in phylloxera resistance. Microscopy results (section 5.3.2) suggest that a high concentration of phenolics in the epidermal layers of the cortex was coincident with browning in highly resistant vine varieties Schwarzmann, Börner and *V. riparia*. These compounds may play a role in resistance to phylloxera, but, as will be discussed in section 5.4, this is most likely to involve either very localised increases in concentration of specific

compounds, and/or their oxidation to more toxic forms (e.g. o-quinones).

5.4 Differences in induced response to phylloxera attack

A combination of histochemistry and HPLC can be a powerful tool for identifying inducible defence responses, as the accumulation of many compounds can be detected by histochemistry, and these may subsequently be identified by HPLC. Langcake (1981) and Dai *et al.* (1995a, 1995b) showed the induction of fluorescent compounds in leaves of resistant varieties of grapevine following attack by downy mildew. Using HPLC, they then identified one of the key fluorescent compounds as *trans*-resveratrol.

5.4.1 Root response to phylloxera attack observed by histochemistry

Histochemistry used for the investigation of plant-pathogen or plant-pest interactions can determine both the location and the chemical nature of defence responses. Typical defence-related responses which have been observed using histochemistry include accumulation of phenolic compounds (Dai *et al.* 1995c, 1996), callose deposition (Shinoda 1993, Kortekamp *et al.* 1997) and lignification (Vance *et al.* 1980, Dai *et al.* 1995a, b). Other than observation of formation of a wound periderm in phylloxera-resistant *Vitis* roots (Boubals 1966a; King and Rilling 1991), there are no published histochemical studies of grapevine-root pest interactions, and none that use chemical-specific stains. Extensive microscopic investigations of plant-parasitic nematode interactions with roots of other host species have largely been based on ultrastructural studies (Jones and Payne 1977, Jones 1981, Wyss *et al.* 1988, Hussey *et al.* 1992, Wyss 1992) or studies of the behaviour of live nematodes (Böckenhoff *et al.* 1996); few such studies have used histochemical techniques. A range of techniques tested (Chapter 3, section 3.3.2) and selected to best investigate the defence response, if any, of resistant vine roots to phylloxera attack were used in this study. The aim was to determine biochemical changes, if any, which occur in response to phylloxera attack in a range of vine varieties.

Materials and methods

Uninfested and infested (nodosities) roots of *V. vinifera* cv. Shiraz, and Ramsey vines were harvested from pot trial four (Chapter 2, section 2.3.4) and perlite-based medium cocultivations (Chapter 2, section 2.4.4). *V. riparia* uninfested roots and nodosities were harvested from perlite-based medium cocultivations (Chapter 2, section 2.4.4). VWL-1 phylloxera were unable to initiate nodosities on other varieties, so infested root material was not available. Harvested roots and nodosities were fixed in 3% (v/v) glutaraldehyde and embedded in GMA (Appendix three).

Microscopy techniques used were PAS/TBO stain for detection of lignin, starch and polyphenolics, UV excitation for observation of the accumulation of autofluorescent compounds such as *trans*-resveratrol or derivatives, as well as for detection of suberin and lignin, and Sudan black stain for observation of suberisation (Chapter 3, section 3.3.2).

In order to test whether accumulation of autofluorescent compound(s) occurred in response to the feeding of phylloxera or merely in response to the wounding of the root by stylet penetration, a wounding trial was conducted. Vines of six varieties (*V. vinifera* cv. Shiraz accession PT23/A.N.61.0020, Schwarzmann accession WA/A.S.74.2257, Ramsey accession A11V2/I.V.63.2065, *V. riparia*, Börner and *V. rotundifolia*) were established in tissue culture as described in Chapter 2, section 2.4.3, except that the culture containers were 2cm deep disposable petri dishes (Sarstedt). After vines were well established (approximately two weeks after transplanting), the roots were wounded longitudinally using a scalpel, resulting in an incision approximately 20mm long. This was done under a dissecting microscope and care was taken not to penetrate deeper than the cortex. Roots were harvested two days after wounding, and fixed and embedded as above. They were sectioned and observed using UV excitation (Appendix three). An initial trial using a micromanipulator and microneedle to mimic the wound created by the stylet of phylloxera was unsuccessful for this purpose since it was impossible to locate the site of wounding after the roots had been fixed and embedded. The injection of a dye or other 'marker' to enable subsequent location of the wound could have been used, but this would have added another variable which could not have been discounted had any response to wounding been observed.

Response of resistant varieties to phylloxera attack

The response of *V. vinifera* cv. Shiraz, Ramsey and *V. riparia* to phylloxera was observed after staining with PAS/TBO (figure 5.10). The typical anatomy of a nodosity on *V. vinifera* (figure 5.10a) included starch accumulation, swelling of the cortex distal to the phylloxera feeding site and non-differentiation of the endodermis (see Chapter 4 for more detail).

In contrast with nodosities formed on *V. vinifera*, those on Ramsey were smaller, and fewer in number (section 5.2.1) with no accumulation of starch in the cortex (figures 5.10b, c). The endodermis was well defined and tissues in the stele well differentiated (figure 5.10c). Sudan black staining, however, showed that the endodermis was not always suberised. In one section the endodermis was found to be suberised only on the side distal to feeding (figure 5.11a) and in another not at all (data not shown), even though it was otherwise well defined. In Ramsey, in contrast to the nodosities formed on *V. vinifera*, an accumulation of phenolics (blue staining) and occasional necrosis at the feeding site was observed. The

Figure 5.10 Transverse sections through nodosities stained with PAS/TBO. a) Nodosity on *V. vinifera* cv. Shiraz: phylloxera (P); undifferentiated endodermis (DD); starch accumulation (St). Scale bar equals 200µm b) Nodosity on Ramsey: epidermis (Ep), hypodermis (H); feeding site (FS). Scale bar equals 50µm c) Nodosity on Ramsey: epidermis (Ep); endodermis (En); stele (S); necrosis (N). Scale bar equals 50µm. d) Nodosity on *V. riparia*: cortex (C); endodermis (En); stele (S). Scale bar equals 20µm.

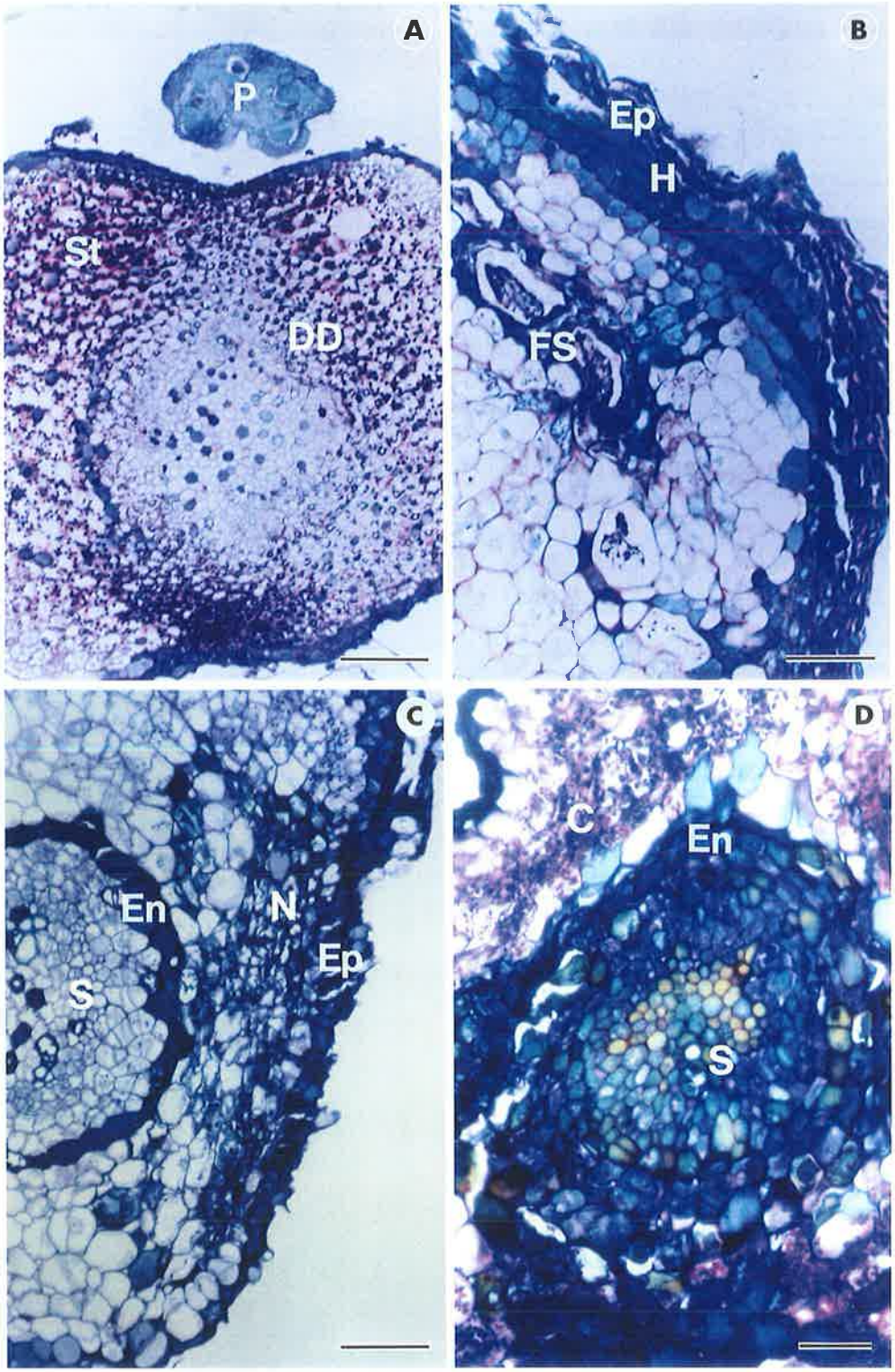
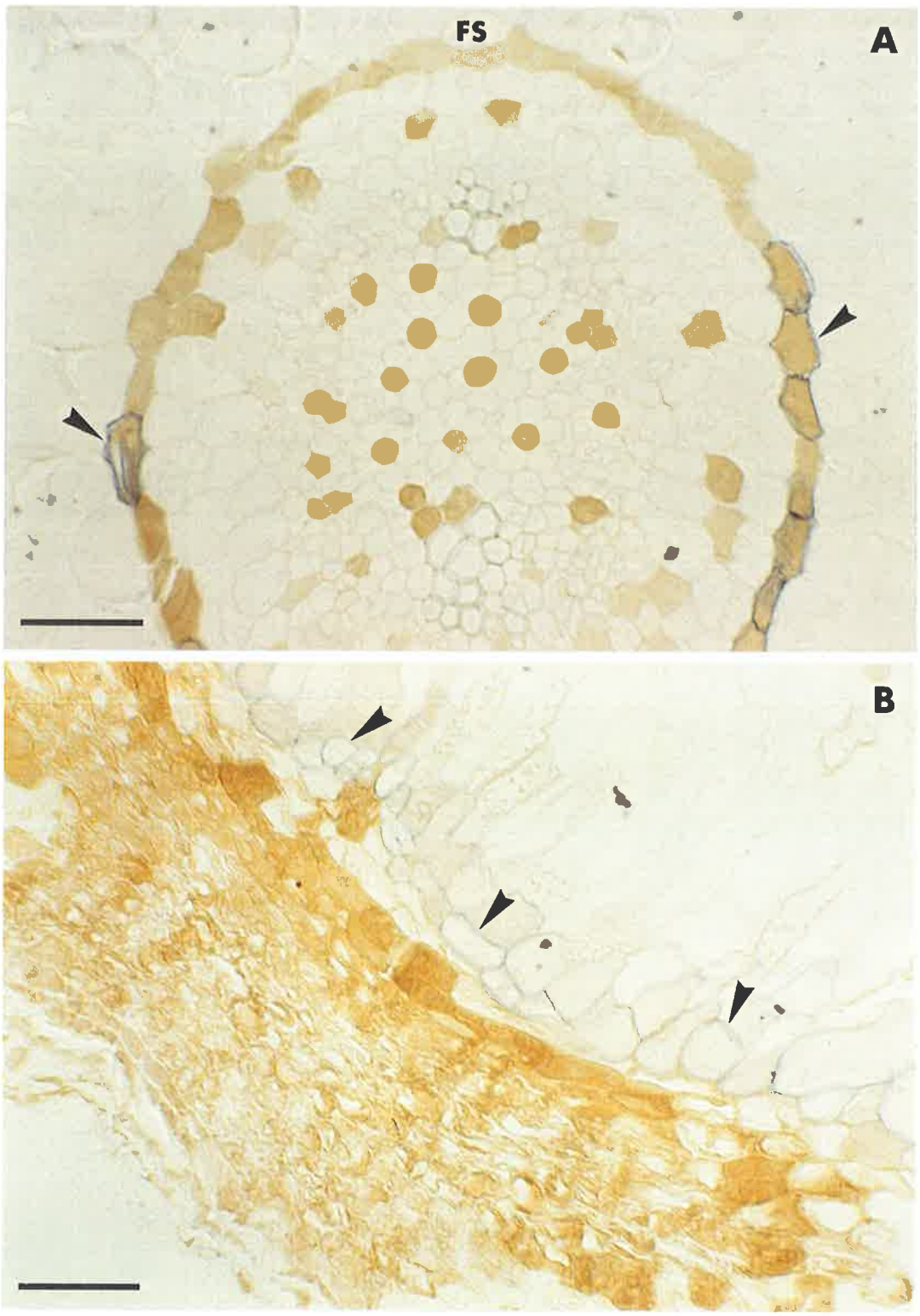

A
P
St
DD
B
Ep
H
FS
C
En
N
Ep
S
D
C
En
S

Figure 5.11 Transverse sections through nodosities on Ramsey potted vines stained with Sudan black. Suberin is stained blue. a) Nodosity with suberin lamella developed in wall of endodermis (arrow heads) only on side distal to site of phylloxera feeding (FS). b) Necrotic region of nodosity showing development of suberin lamella (arrow heads) in walls of cells immediately adjacent to necrosis. Scale bars equal 50μm.

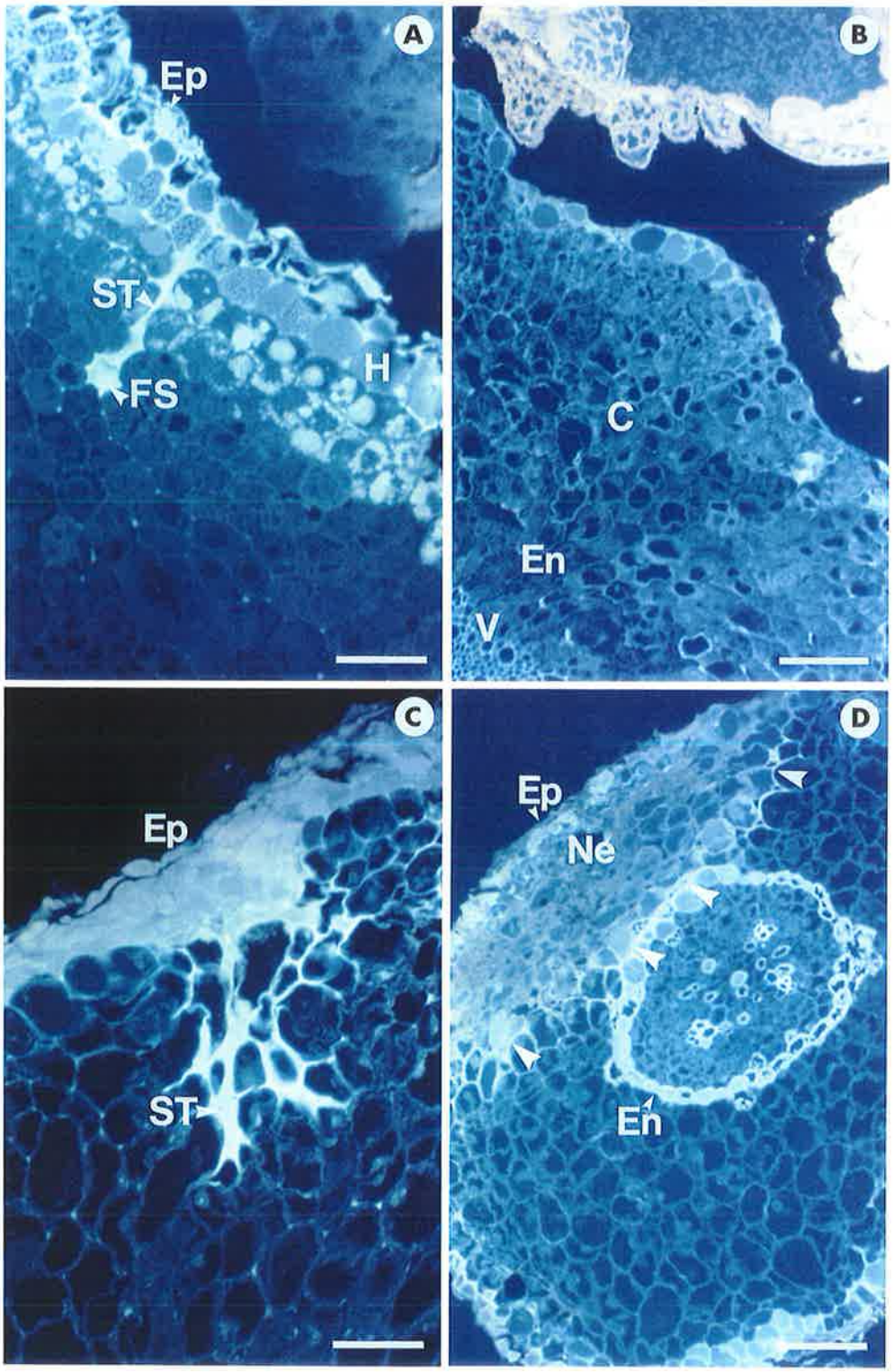

FS
A
B

necrosis could be either very localised (figure 5.10b), or cover a larger region of the root cortex (figure 5.10c), but did not penetrate the endodermis. Similar necrosis of cortical tissue was only observed in nodosities on *V. vinifera* as a delayed response after phylloxera had been feeding from the nodosity for several weeks. Sudan black staining confirmed the presence of a layer of cells with suberin lamellae surrounding regions of necrotic cells (figure 5.11b). A similar induction of suberisation was observed in cortical cells adjacent to the region of delayed necrosis in nodosities of *V. vinifera* (Chapter 4, figure 4.5c), however, in this case, necrosis had penetrated the vascular tissues. The suberisation observed in Ramsey may assist in isolating the necrotic regions from the rest of the root.

V. riparia roots had a striking response to feeding of phylloxera, involving massive accumulation of polyphenolic compounds in the stele (figure 5.10d). This is presumably associated with the observation that, in the tissue culture-based bioassays, nodosities were initiated but rapidly became brown, with the death of crawlers *in situ*. The cortex of the nodosity shown is badly damaged, possibly in response to infestation, but abundant starch grains are still evident. It is possible that some tissue damage occurred during fixation, however as the tissue appeared brown before harvesting, it can be assumed that the tissue damage was already significant. Although several nodosities were initiated on *V. riparia*, most were very necrotic at the time of assessment of the bioassays, and only the one shown was suitable for microscopy. Due to the small size (<2mm long) and poor condition of this nodosity, very few good sections were obtained, so it was not possible to expand on these results with autofluorescence or Sudan black staining.

The response of the primary roots of Ramsey to phylloxera in comparison to that of *V. vinifera* cv. Shiraz was also observed by UV excitation (figure 5.12). A single stylet track in the root of a potted *V. vinifera* vine (figure 5.12a) penetrated five cell layers below the epidermis into the root. The path of stylet penetration appeared to be largely intercellular, with intracellular fluorescence only observed in the immediately affected cell at the terminal point of the stylet track, and not in neighbouring cells. In contrast, a nodosity formed on Ramsey (figure 5.12c) contained multiple stylet tracks, each accompanied by accumulation of a blue-white autofluorescent compound throughout what may be the narrow band of cytoplasm outside each cell's central vacuole. Alternatively this accumulation may be in the apoplastic space, possibly cell wall-bound. Multiple stylet tracks, but never single ones, were observed in several nodosities collected from this variety. Figure 5.12c also shows a very localised necrotic (dull orange in appearance) region at the point of entry of the stylet into the root. A similar response was never seen in *V. vinifera*. A more widespread necrotic region which had also been penetrated by a number of separate stylet tracks was present adjacent to the feeding site shown in figure 5.12c. The stylet tracks observed in figure 5.12c

Figure 5.12 Transverse sections though nodosities under UV excitation. a) Nodosity on *V. vinifera* cv. Shiraz showing stylet track of feeding phylloxera: epidermis (Ep); hypodermis (H); stylet track (ST); feeding site (FS). Scale bar equals 50µm. b) Nodosity on *V. vinifera* cv. Shiraz showing non-differentiation of endodermis: cortex (C); location of endodermis (En); vascular tissue (V). Scale bar equals 100µm c) Nodosity on Ramsey showing stylet track of feeding phylloxera and localised autofluorescence around stylet tracks: epidermis (Ep); stylet track (ST). Scale bar equals 50µm. d) Nodosity on Ramsey showing necrotic response to phylloxera and autofluorescence in surrounding cells: epidermis (Ep); necrotic region (Ne); endodermis (En); autofluorescence (arrow heads). Scale bar equals 100µm.


A
Ep
ST
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H
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V
C
Ep
ST
D
Ep
Ne
En

appear to represent a new feeding site established adjacent to the necrotic area, probably following its abandonment. Figure 5.12d shows a necrotic region similar to the larger one described above. This was surrounded by a layer of cells showing a very similar blue-white autofluorescence as seen in figure 5.12c. This autofluorescence was strongest in the endodermis adjacent to the necrotic region. The necrosis had not penetrated the stele which, apart from accumulation of phenolic compounds in the endodermis on the side adjacent to phylloxera feeding, appeared unaffected by the presence of the phylloxera.

Suberisation of cell walls at the feeding site was not seen in any variety preceding tissue necrosis.

Response of resistant varieties to wounding

The wounded regions of the primary roots of the six varieties tested were visualised by UV excitation (365nm excitation filter, 400nm barrier filter) (figure 5.13). There was no blue-white autofluorescence similar to that noted previously in the roots of Ramsey in response to feeding phylloxera seen in response to wounding. Very little autofluorescence in response to wounding was observed in any variety, however in Börner and *V. rotundifolia* a faint yellow-greenish fluorescence was seen in cells immediately adjacent to the wound. Due to the use of light sensitive tungsten film for photography, this appears in the figure as pale blue.

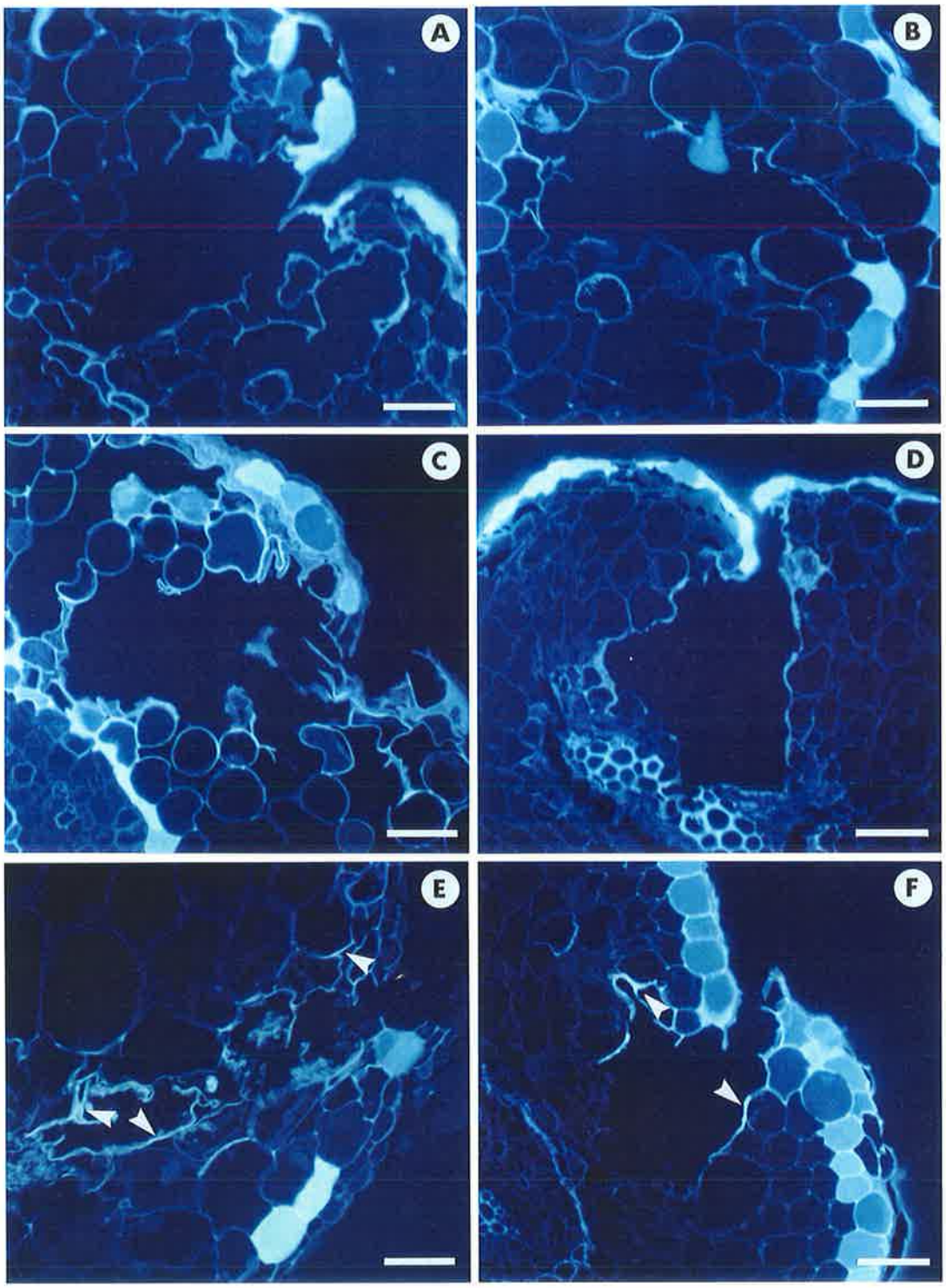
Discussion of histochemistry results

Ramsey

Nodosities formed on Ramsey are characteristically smaller than on *V. vinifera*, and contain necrotic regions, although these do not deter phylloxera from feeding on non-necrotic regions of the same nodosities. No accumulation of starch was detected within the nodosity cortex. The endodermis in Ramsey nodosities always appeared clearly defined, although it was not always fully suberised, as it was in uninfested roots at the same stage of development. Necrosis and lack of starch accumulation coincided with a significant reduction in performance of phylloxera compared with those on *V. vinifera* (table 5.1).

It was a consistent feature of *V. vinifera* nodosities that the endodermis was not fully differentiated and even where it was clearly defined, it was not suberised (Chapter 4, section 4.3.2, figure 4.5b). The hypothesis was put forward in Chapter four that this might facilitate the establishment of a nutrient sink by potentially enhancing apoplastic and/or symplastic transport of solutes from phloem to cortex, which in turn could contribute to the massive accumulation of starch in the cortex of *V. vinifera* nodosities.

Figure 5.13 Transverse sections of roots wounded with a scalpel blade and observed with UV excitation. a) *V. vinifera* cv. Shiraz b) Schwarzmann c) Ramsey d) *V. riparia* e) Börner f) *V. rotundifolia.* Slight autofluorescence in response to wounding marked with arrow heads. Scale bar equals 20μm.


A
B
C
D
E
F

VWL-1 phylloxera were clearly able to obtain sufficient nutrients from Ramsey to feed and reproduce on the roots of this variety, albeit at a lower efficiency than on roots of *V. vinifera*. However, if starch accumulation is a reflection of cortical sugar concentration (Sung *et al.* 1989, Wang *et al.* 1993, Zrenner *et al.* 1995), and hence also of the strength of the photosynthate sink, the lack of starch in nodosities on Ramsey may be an indication of the formation of a weaker sink than that formed in *V. vinifera* nodosities. This might explain both the smaller size of nodosities, and lower reproductive rate of phylloxera noted on Ramsey. Ramsey was also the only variety in which starch was never detected in the uninfested root cortex. It is possible that in Ramsey the overall sink strength of primary roots was not as great as in other varieties, or the sink which did exist was not as sensitive to enhancement by phylloxera feeding. This may be due to lower endogenous activities of enzymes involved in either sucrose metabolism or starch synthesis, or alternatively, to restriction in the pathways of phloem unloading into the root or nodosity.

The observation of multiple stylet tracks at single feeding sites in Ramsey suggests that the phylloxera were not as sedentary as when they were feeding on *V. vinifera,* where a single stylet track was consistently observed. The repeated probing on Ramsey could be due to a number of factors including poor nutrient supply, lack of necessary stimulants or the presence of deterrent or antibiotic compounds.

The autofluorescence surrounding stylet tracks in Ramsey was similar to that reported by Dai *et al.* (1995a, b) in their investigation of the response of grapevine leaves to downy mildew. They used HPLC to demonstrate the correlated accumulation of *trans*-resveratrol and a compound which fluoresces blue-white under UV excitation. This is consistent with results presented here where a similar co-occurrence of induced blue-white autofluorescence and the accumulation of *trans*-resveratrol (section 5.4.2) is found. The distribution of the autofluorescent compound suggests that it may be located in the narrow strip of cytoplasm at the periphery of each cell (cortical cells have a very large central vacuole which occupies much of the cytosol), as would be expected for an aglycone such as free *trans*-resveratrol, or in the apoplastic space. This result suggests that Ramsey is showing a very localised defence response, similar to that seen in leaves by Dai *et al.* (1995a, b). It is possible that the accumulation of *trans*-resveratrol could have a deterrent or antibiotic effect on phylloxera although the toxicity of this compound to insects is unknown.

Parallels can be drawn between the grapevine-phylloxera interactions and the better characterised plant-nematode interactions on the basis of the observations made here. Grundler *et al.* (1997) reported accumulation of autofluorescent compounds and cellular necrosis in the area surrounding an incompatible interaction between *Arabidopsis thaliana* and the cereal cyst nematode *Heterodera glycines*, while in a compatible interaction

between *A. thaliana* and *Heterodera schachtii,* autofluorescence was only seen in the cells actually penetrated by the nematode. Comparable differences were seen between Ramsey and *V. vinifera* in response to VWL-1 phylloxera feeding (figures 5.12a and c).

It is possible that the development of suberin lamellae in the walls of cells surrounding necrotic regions, which occurred both in *V. vinifera* (figure 4.5c), and in Ramsey (figure 5.11b), is functionally related to formation of a wound periderm. In secondary roots of phylloxera tolerant vines this serves to 'seal off' the necrotic regions resulting from formation of tuberosities. The formation of suberin lamellae in primary roots is considerably delayed in *V. vinifera* and does not isolate the necrosis from the stele, however in Ramsey it develops rapidly and may help isolate the necrotic regions, thereby protecting the rest of the root.

In conclusion, the tissue culture bioassay results indicated that feeding on Ramsey results in decreased performance of phylloxera, but not increased mortality *in situ*, suggesting that poor nutrition combined with the relatively rapid (compared to *V. vinifera*) necrosis of the feeding site, might play a significant role in the resistance of Ramsey. The contribution of localised accumulation of phenolics, including *trans*-resveratrol, warrants further investigation.

V. riparia

The observation of starch accumulation in *V. riparia* nodosities is consistent with observations reported by Hofmann (1957). It appears that nodosity initiation by VWL-1 phylloxera involved induction of a strong nutrient sink on this variety, but the massive accumulation and/or oxidation of phenolics resulted in a reaction similar in appearance to a hypersensitive response (HR), and the nodosity development was aborted. Nodosity formation on *V. riparia,* as described by Hofmann (1957) did not invoke an HR, which may indicate that he used a different accession or cultivar to that used in this study. A dramatic variation in phylloxera resistance between accessions of this species was reported by Grzegorczyk and Walker (1998). Alternatively, different phylloxera biotypes may act differently in invoking a defence response.

The death of the crawlers *in situ* may have been caused by a preformed toxic compound, not detected in this study. Alternatively the HR-like response observed in roots of *V. riparia* may have been very rapid and involved the accumulation of toxic compounds. The latter could be produced by oxidation of endogenous phenolics to more toxic polymeric compounds. *V. riparia* was shown by Langcake (1981) to accumulate stilbenes (ε- and α-viniferins) to high concentrations in its leaves in response to downy mildew *Plasmopara viticola* infection. The stilbene ε-viniferin is, however, a major component of the primary

roots of all varieties, including *V. vinifera*, so it is unlikely that this compound plays a direct role in antibiosis towards phylloxera. Unfortunately, an insufficient number of *V. riparia* nodosities were available for HPLC analysis.

It is likely that the ability of this variety to respond rapidly to phylloxera attack with the accumulation of oxidised phenolic compounds is responsible for its high level of resistance to phylloxera as well as to other pathogens.

Wounding

Wounding of plant roots is known to cause accumulation of simple phenolics such as chlorogenic acid, caffeic acid and coumarins, synthesis and deposition of polyphenolic complexes such as lignin and suberin, as well as the oxidation of endogenous phenolic compounds and de-glycosylation of phenolic compounds stored in vacuoles, thus releasing the aglycones for potential oxidation (Rhodes and Wooltorton 1978). Increases in the levels of activity of PAL, chalcone synthase, chalcone isomerase, chitinases and proteinase inhibitors are also associated with wounding of plant tissues (Baron and Zambryski 1995). All these responses have parallels with pathogenesis-related responses.

In the grapevine roots observed in this study, the response of all varieties to wounding was not strong, and of most relevance, did not involve the accumulation of blue-white auto-fluorescent compound(s). Rather, a faint yellow-green autofluorescence was observed in the most phylloxera-resistant varieties. The time between wounding and observation in this trial was the same as that between infection and accumulation of autofluorescent compounds in the histochemical study conducted by Dai *et al.* (1995a, b). These authors reported both blue-white and yellow-green autofluorescence in the leaves of the more resistant species of grapevine in response to downy mildew infection. While the blue-white autofluorescence was attributed to *trans*-resveratrol, the identity of the yellow-green fluorescent compound(s) is unknown. In a subsequent study by the same authors, resistant lines of cotton infected with *Xanthomonas campestris* showed a similar yellow-green autofluorescence which was attributed to either phenolics or terpenoid napthols (Dai *et al.* 1996). Whether the yellow-green autofluorescent compounds in this study and the two studies by Dai *et al.* (1995a, b) can be attributed to the same compound(s), and the identity of these compounds, remains to be determined.

Summary

V. vinifera has no detectable defence response to phylloxera feeding. In contrast, the two resistant varieties examined, Ramsey and *V. riparia*, show strikingly different responses to phylloxera attack, and this may account for the different performance of VWL-1 phylloxera on the two varieties. In the case of Ramsey this could be due to one or more of the observed

responses, which include lack of starch accumulation, localised accumulation of a fluorescent compound (possibly *trans*-resveratrol) at the feeding site, and premature necrosis of the nodosity. The death of phylloxera and early abortion of nodosity formation on the primary roots of *V. riparia* appears to be related to a massive accumulation of oxidised, phenolic compounds which also cause necrosis of the region surrounding the feeding site and may also be toxic to phylloxera. The roots of all six varieties examined lacked a strong wound response, indicating that the accumulation of the autofluorescent compound at the site of phylloxera feeding in Ramsey (and in Schwarzmann in response to SRU-1 phylloxera, see Chapter 6, section 6.3) is a pest-induced response implying recognition of phylloxera, possibly through the stylet sheath or through insect-derived substances injected during root penetration and/or feeding.

5.4.2 Changes in free phenolics in response to phylloxera attack

HPLC analysis was used in an attempt to identify any changes that might occur in the free phenolic profile of resistant varieties following phylloxera attack. It was also hoped to gain evidence for or against the presence of free phenolics which might act as 'gall enhancers' or 'gall inhibitors' as proposed by Denisova (1965).

Materials and methods

The root material analysed consisted of VWL-1 induced nodosities of *V. vinifera* cv. Shiraz and Ramsey, which were the only varieties from which sufficient numbers of nodosities could be obtained, and uninfested roots of all varieties of vines which had been inoculated with phylloxera and were therefore considered to be 'challenged'. 'Challenged' roots were included in order to test for a systemic response by the plant. Roots and nodosities were collected from perlite-based medium cultures (Chapter 2, section 2.4.4) and pot trial four (Chapter 2, section 2.3.4) and analysed using free phenolic extraction method one (Chapter 3, section 3.2.1) and HPLC chromatography method nine (section 3.2.2). HPLC chromatograms were compared between uninfested roots (described in section 5.3.3), 'challenged' roots and nodosities, and between varieties.

Results

Changes in free phenolics in tissue cultured vine roots

Table 5.7 shows the areas of peaks 1-11 detected in HPLC chromatograms of root extracts from the three varieties, *V. vinifera* cv. Shiraz, Schwarzmann and Ramsey. For other varieties tested (*V. riparia*, Börner, *V. rotundifolia*) there were no significant differences between uninfested and 'challenged' roots (data not shown). In all cases there were few differences between treatments within any one variety. Additional minor peaks were

detected in 'challenged' roots of *V. vinifera* (peak eight), Schwarzmann (peaks four and eleven) and Ramsey, (peak six).

Of more interest, peak three, which has been putatively identified as *trans*-resveratrol 3-β-glucoside (piceid) (see section 5.3.3), underwent some noteworthy changes in phylloxera-infested roots. There were in total three different absorption spectra seen for the peak with a retention time of 27 minutes in various root sample types. Two were variants of peak three, and have been described previously (section 5.3.3, figure 5.9). These we found in uninfested roots and in VWL-1 'challenged' roots of all varieties except Ramsey, and will not be discussed further. A new peak with a retention time of 27 minutes, designated peak 3′, was seen in all nodosities examined, i.e. those formed on *V. vinifera* and Ramsey, in response to VWL-1 phylloxera, and on *V. vinifera* and Schwarzmann in response to SRU-1 phylloxera (see Chapter 6, section 6.4.2), and also in VWL-1 'challenged' roots of Ramsey. The absorption spectrum of peak 3′ in comparison with peak 3 is illustrated in figure 5.14.

An additional change in VWL-1 'challenged' roots and nodosities of Ramsey was the detection of a novel compound in peak 3′a, the retention time of which was slightly greater (28 minutes) than of peak 3′ (27 minutes). The absorption spectra of these two peaks (3′ and 3′a) were, however, the same.

In addition to peaks 3′ and 3′a, another novel peak was present in VWL-1 'challenged' roots and nodosities of Ramsey which was not present in uninfested roots (figure 5.15, not listed in table 5.7). Its retention time of ~34 minutes was the same as that of free *trans*-resveratrol (Appendix five). The absorption spectrum was slightly different to that of the *trans*-resveratrol standard, but similar to one variant of peak three mentioned earlier (section 5.3.3, figure 5.9). It is likely therefore that this new peak represents a small accumulation of free *trans*-resveratrol, or a slightly modified form of this compound.

Table 5.7 Areas of major peaks in HPLC chromatograms of uninfested or VWL-l phylloxera 'challenged' roots or nodosities from *V. vinifera* cv. Shiraz, Schwarzmann and Ramsey from perlite-based medium cocultivations (Chapter 2, section 2.4.4). Peak areas are the means of duplicate root samples, and given as $AU \times 10^{-5}$.

Peak no.[1]	*V. vinifera* cv. Shiraz			Schwarzmann		Ramsey		
	uninfested	'challenged'	nodosity	uninfested	'challenged'	uninfested	'challenged'	nodosity
1	0.00	0.00	0.80	2.49	2.28	2.00	4.62	3.27
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	22.34	21.13	-	25.66	17.24	32.23	-	-
3′[2]	-	-	17.60	-	-	-	11.12	18.27
3′a[3]	-	-	-	-	-	-	16.83	32.43
4	0.00	0.00	0.00	0.00	0.57	0.00	0.00	0.00
5	1.04	1.62	1.62	3.89	2.07	0.00	0.00	0.00
6	0.00	0.00	0.00	2.14	1.18	0.00	1.36	0.00
7	0.92	2.64	2.12	5.36	4.39	20.90	18.13	13.84
8	0.00	1.61	0.00	0.00	0.00	1.94	1.74	1.76
9	0.00	0.00	0.00	25.43	11.51	0.00	0.00	0.00
10	1.77	13.55	2.87	20.48	9.49	10.63	7.73	4.08
11	0.00	0.00	0.00	0.00	1.92	0.00	0.00	0.00

[1] Peak numbers refer to numbers assigned according to peak retention time using HPLC chromatography method nine (Chapter 3, section 3.2.2), illustrated in fig. 5.7.

[2] In nodosities of *V. vinifera* and 'challenged' roots and nodosities of Ramsey, the absorption spectrum of the peak with a retention time of 27 minutes is altered compared with peak three. This new peak has been designated peak 3′.

[3] In 'challenged' roots and nodosities of Ramsey, a novel peak with the same absorption spectrum as peak 3′, but with a slightly different retention time of 28 minutes has been designated as peak 3′a

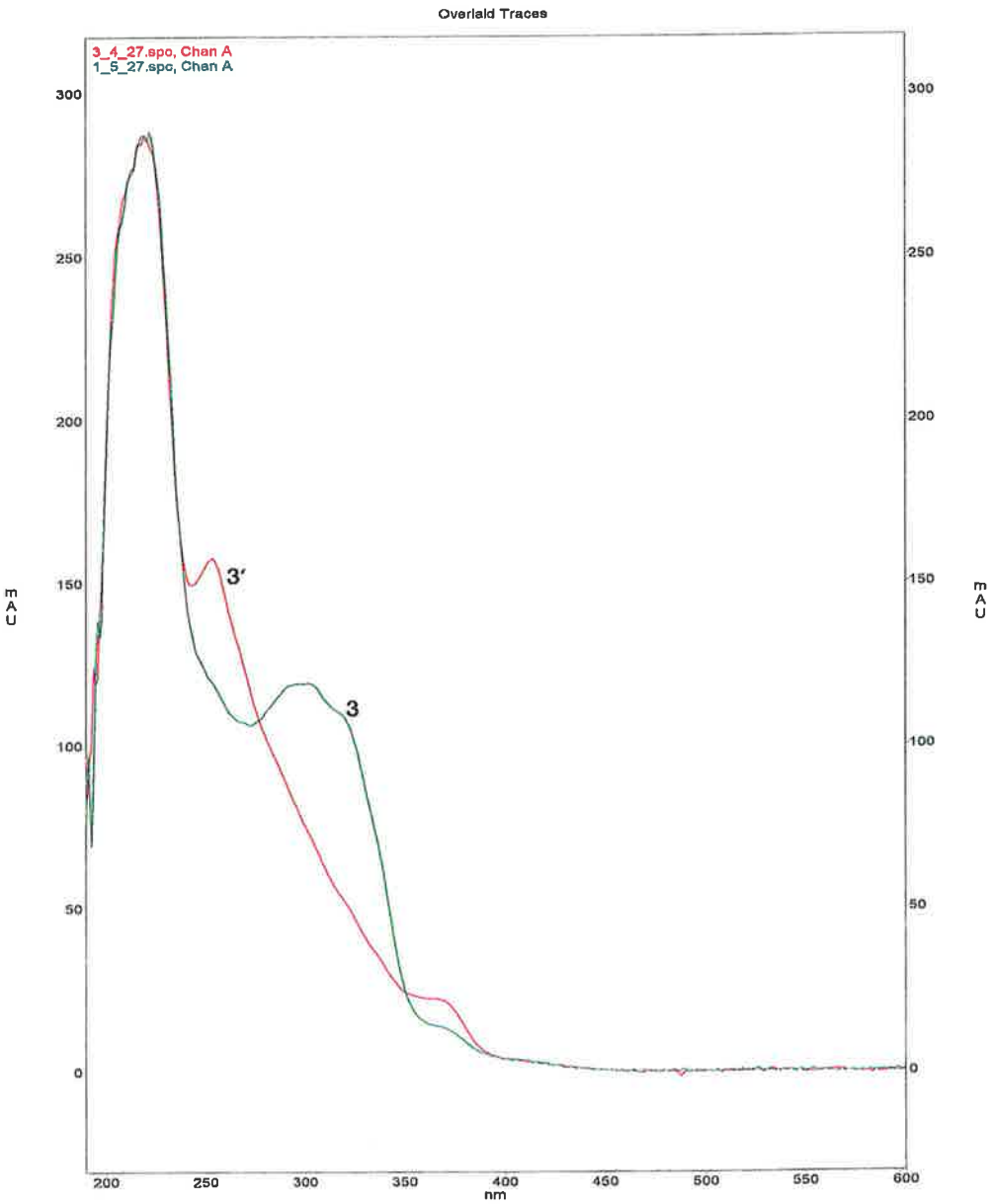


Figure 5.14 Absorption spectra of peak 3 (black) and peak 3′ (red) in extracts from uninfested roots and nodosities of Ramsey.

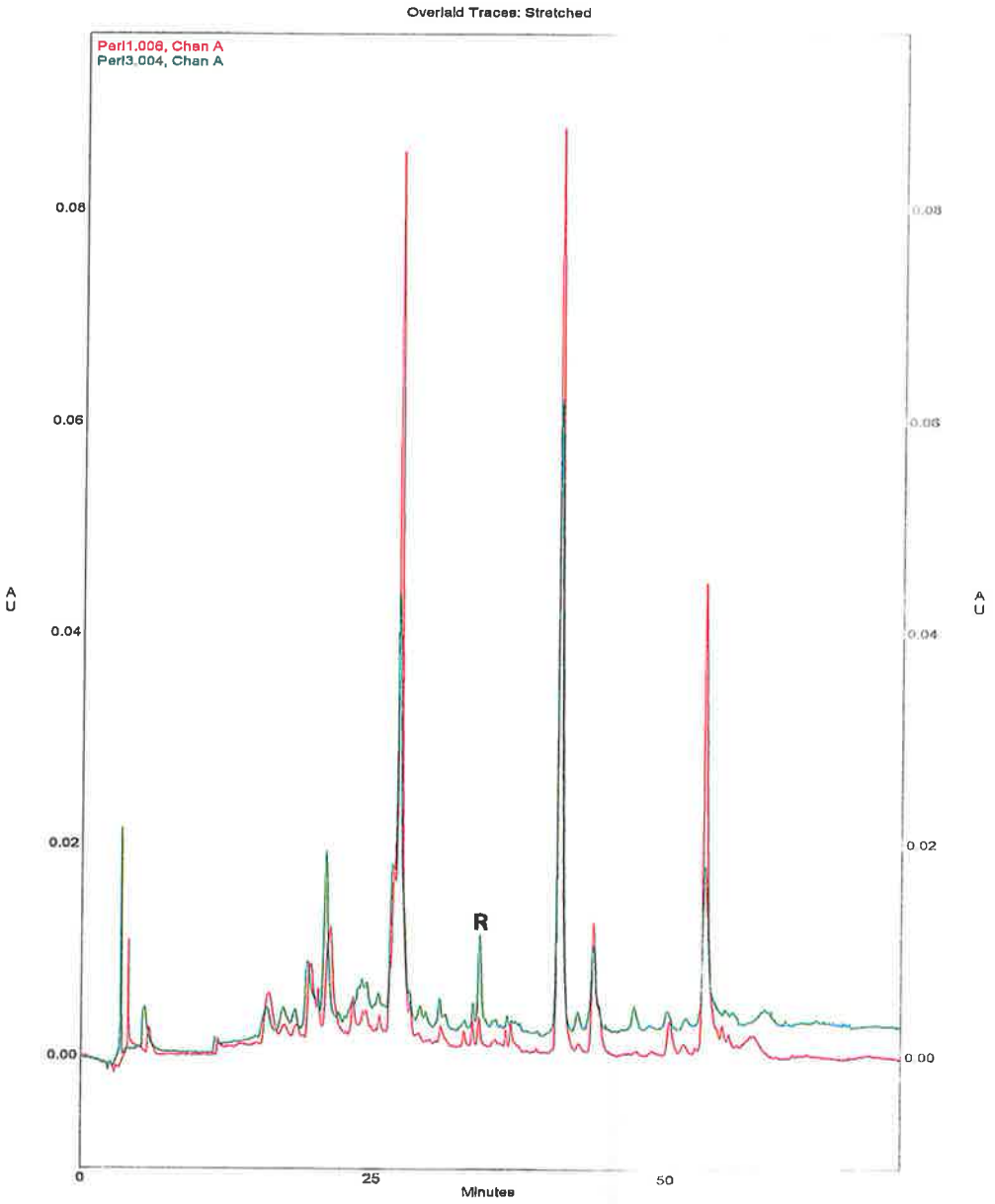


Figure 5.15 HPLC chromatogram of extracts from uninfested roots (green) and nodosities (red) from Ramsey collected from perlite-based medium cocultivations with VWL-1 phylloxera illustrating the appearance of a minor peak (R) which is putatively identified as free *trans*-resveratrol.

Changes in free phenolics in potted vine roots

As previously noted in the HPLC analyses of uninfested roots there were differences between the phenolic profiles obtained for roots and nodosities from potted and tissue cultured vines of each of the three varieties tested (*V. vinifera* cv. Shiraz, Schwarzmann and Ramsey). For this reason, only results which are consistent with those from tissue cultured vines, or otherwise of interest, are reported here.

The change from peak 3 to 3′ occurred in both *V. vinifera* and Ramsey nodosities. In *V. vinifera* nodosities this change was also accompanied by the accumulation of a new minor peak as seen previously in Ramsey nodosities from tissue cultured vines, and likely to be free *trans*-resveratrol, or a slightly modified form of this compound.

In nodosities and 'challenged' roots of Ramsey, the HPLC chromatograms remain relatively unaltered compared with uninfested roots, except for the change from peak three to peak 3′ as described above, plus the appearance of a new minor peak with a retention time of approximately 47 minutes. The absorption spectrum indicates that this compound is possibly 'unknown oligostilbene a' (Mattivi *et al.* 1996, F. Mattivi, pers. comm.).

Schwarzmann 'challenged' roots have an almost identical profile to uninfested roots.

Discussion of HPLC analyses of free phenolics

The differences in free phenolic profiles observed between varieties, the two culture methods and the presence or absence of phylloxera are subtle and complex. It seems, however, that the presence, absence or relative amounts of the majority of compounds is variable, and does not seem to have much, if any bearing on the interaction of these varieties with phylloxera. In particular, the significance of minor peaks detected in 'challenged' roots of *V. vinifera* (peak eight), Schwarzmann (peaks four and eleven) and Ramsey, (peak six) is questionable given the level of quantitative variation observed in the HPLC analyses in this study (discussed in Chapter 3, section 3.2.3).

The only consistent response to phylloxera attack was the change in absorption spectrum of peak 3 to 3′ upon challenge with phylloxera - sometimes accompanied by an (albeit minor) accumulation of free *trans*-resveratrol, or an apparently slightly modified form of this compound. Peak 3′a seen in Ramsey, appears to results from a 'split' of peak 3′, the basis of which is unknown.

The change in absorption spectrum from peak 3 to 3′ was similar to that which occurs on treatment of extracts with β-glucosidase (section 5.3.3, figure 5.8). Peak three appears to contain at least two compounds, one putatively identified as *trans*-resveratrol glucoside (piceid), and the other unknown. Assuming the absorption spectrum of peak three is an

additive result of the two compounds present, the absorption spectrum predicted to result from the removal of piceid matches that of peak 3′. This suggests that the change of peak 3 to peak 3′ involves an *in vivo* deglycosylation of piceid, resulting in accumulation of free *trans*-resveratrol as sometimes observed, or possibly its conversion to other resveratrol derivatives.

In growing grapevine (*V. vinifera*) roots, β-glucosidase activity is generally confined to the meristem, hypodermis and vascular bundle, but in nodosities formed by the dagger nematode *Xiphinema index*, it is highly expressed in all cells (Rumpenhorst and Weischer 1978). The latter cells are also more densely cytoplasmic (without a large central vacuole) than normal cortical cells, and the increased β-glucosidase activity was, in this tissue, attributed to an overall increase in metabolic activity. β-glucosidase activity is also induced in apple leaves on wounding, and gives rise to the production of phenolic aglycones which in turn leads to tissue browning (Rhodes and Wooltorton 1978). A similar increase in β-glucosidase activity in *V. vinifera* nodosities observed here could readily account for the loss of a major glucoside such as piceid.

Only minor accumulation of free *trans*-resveratrol was detected in nodosities of Ramsey tissue cultured vines and *V. vinifera* potted vines (although more distinct accumulation occurred in the Schwarzmann SRU-1 interaction (Chapter 6, section 6.3.2)). The absence in nodosities of a free *trans*-resveratrol peak of equivalent size to peak three suggests that the aglycone may be quickly oxidised to its oligomeric derivatives (stilbenes and stilbenoids). Hoos and Blaich (1988) found that due to such rapid oxidation it was not possible to detect synthesis of free *trans*-resveratrol.

The free-phenolic extraction method used in this study was limited in some respects. It was only suitable for the investigation of free phenolics, and did not enable analysis of wall-bound phenolics (Chapter 3, section 3.2.1). These might be important, as the phylloxera appears to penetrate cell walls in order to feed. The HPLC analyses also only provided a picture of the phenolic content of the nodosity as a whole; a very localised response may be disguised by the complexity of the total HPLC chromatogram. In other studies (e.g. Dai *et al.* 1995a, b) this has been overcome by combining histochemistry with HPLC.

Soluble phenolics, whether present as glycosides or aglycones, represent only intermediate phases in a dynamic system - the end products of which are insoluble compounds that produce, for example, the browning characteristic of necrotic tissues. It has been hypothesised (Miles 1999) that the concentration of these end products, rather than the concentration of short-lived intermediates, may provide a clearer indication of the nature and intensity of a plant's response to an invasive organism. Therefore the concentration of

soluble phenolics may not reflect the amounts of potentially toxic, defensive compounds that are being mobilised and detoxified in tissues affected by invasive organisms.

Summary

In conclusion, the HPLC results presented here suggest it is unlikely that any of the phenolic compounds detected in the nodosities of the resistant variety Ramsey play a significant role in its defence against phylloxera. The roots of all varieties, including *V. vinifera*, contain a high level of potentially biologically active and toxic compounds. Similar changes in response to phylloxera were detected in roots of both *V. vinifera* and Ramsey, although the changes of peak 3 in 'challenged roots' of Ramsey suggest that this variety responds more readily to phylloxera than does *V. vinifera*, possibly including a response systemic in the root system. Rapid oxidation of the high level of endogenous phenolics in the root epidermis of the more resistant *V. riparia* and Börner appears to be involved in their resistance to phylloxera. Insufficient infested material was obtained for HPLC analysis of nodosities or other attempted feeding sites on these varieties. Histochemistry of fresh tissues or peroxidase activity assays would be more appropriate techniques for investigating the potential of some varieties to respond to phylloxera by rapid oxidation of stilbenes if appropriate analytical facilities could be established within a quarantine district.

5.5 Discussion

Tissue culture-based bioassays enabled the generation of resistance ratings based on the criteria of nodosity formation and phylloxera survival. *V. vinifera* cv. Shiraz was susceptible to VWL-1 strain phylloxera, Ramsey resistant, *V. riparia*, Schwarzmann and Börner highly resistant and *V. rotundifolia* immune. The resistance ratings for the different vine varieties also agree with those published elsewhere (Boubals 1966a, Pongrácz 1983, Whiting *et al.* 1987, Becker 1988, Grzegorczyk and Walker 1998). Observations of nodosity morphology were similar to those previously reported by King *et al.* (1982) from potted vine trials, even though different vine varieties and phylloxera populations were used. Tissue culture-based bioassays therefore provide a good system for quickly and effectively assessing the degree of resistance of grapevines to phylloxera. They do not, however, give any indication of phylloxera tolerance, which is primarily a function of secondary roots.

Little has been published regarding the settling and feeding behaviour of phylloxera crawlers. First instar phylloxera are highly mobile after hatching, hence the term 'crawler' is used to describe them. The mechanism by which phylloxera are able to detect a suitable host plant has not been investigated. Crawlers may move constantly, and often apparently aimlessly, for several days before locating and settling at a suitable feeding site. This behaviour makes it

impossible to establish synchronised infestations and also make observations of probing or other feeding site location difficult. The mechanism by which crawlers, once on a root, choose a feeding site is unknown. Possibly they do so using their olfactory sense and/or by probing root tissues with their stylet. Phylloxera will preferentially settle on preformed tuberosities (Omer *et al.* 1995b) and, as observed here and by Forneck *et al.* (1996), they also appear to be attracted to root tips, suggesting an attraction to preformed nutrient sinks or possibly also attraction to root cap exudates. The large numbers of dead crawlers on roots of Schwarzmann vines in perlite-based medium cocultivations suggests that the roots of at least some varieties are attractive to phylloxera even if they are subsequently unable to establish feeding sites. The tendency for crawlers to move off the roots in excised root cultures was particularly strong in *V. rotundifolia,* and no dead crawlers were observed on roots of this variety on perlite-based medium cultures. This suggests that some factor which might cause attraction of phylloxera to roots of other *Vitis* spp. is not present in *V. rotundifolia*, or alternatively that they are actively repelled from feeding on this variety. *V. rotundifolia* is also a poor host for *Xiphinema index*, causing a reduction in population number not seen in highly resistant rootstocks such as Börner (Sopp *et al.* 1998).

With a few exceptions discussed below, grapevine root resistance to phylloxera does not seem to be related to constitutive differences, such as anatomical differences, between varieties, or significant differences in free phenolic content as measured in root extracts. The roots of all varieties contain significant amounts of piceid and other resveratrol derivatives which are candidate defence-related compounds. As they are equally prevalent in all varieties including *V. vinifera*, however, no correlation could be demonstrated between their presence and phylloxera resistance.

The presence of phenolics in the root epidermis is a constitutive feature of primary roots which may be associated with resistance. The highly resistant varieties *V. riparia*, Börner and Schwarzmann all have distinctive blue-green staining phenolics in the epidermis and the immediately adjacent cell layers of the root cortex. The state of root differentiation, particularly the maturation of the stele and extent of suberisation of the endodermis relative to the distance from the root tip is also associated with resistance. The more highly resistant varieties appear to mature closer to the root tip, possibly making them less responsive to gall-inducing agents. The immune variety *V. rotundifolia* possesses both a unique olive-green staining compound in the epidermis and at least one unique phenolic component, the compound present in peak two, in its root tissues. The identity of the olive-green compounds remains unknown, but its location raises the possibility of its olfactory detection by the phylloxera crawlers. This is especially interesting as phylloxera were never seen to attempt to feed on this variety. Peak two appears to represent an undescribed hexoside of resveratrol,

however its role, if any, in the mechanism of immunity of this variety remains unknown. Further identification and characterisation of the novel phenolics in *V. rotundifolia* would therefore be of interest.

The reduced reproductive rate of VWL-1 phylloxera on Ramsey, in combination with their tendency to abandon the nodosities suggests that poor nutrition may contribute to the resistance of this variety. This is probably the result of a weak nutrient sink, which is indicated by the lack of starch accumulation in the cortical cells of the nodosity.

The slight accumulation of free *trans*-resveratrol in nodosities of Ramsey was coincident with the autofluorescence noted at feeding sites. However, in *V. vinifera* nodosities from potted vines, a similar accumulation of *trans*-resveratrol is detected which does not correlate with any observation of autofluorescence. It seems likely that the accumulation of free *trans*-resveratrol detected by HPLC in both varieties is a result of the deglycosylation of piceid during nodosity formation, rather than synthesis *de novo* as part of a defence response localised at the feeding site. The autofluorescent compound is undoubtedly associated with a specific defence response to phylloxera as a similar response is seen neither in wounding of resistant varieties, nor in the susceptible *V. vinifera*, but the exact nature of the response remains to be elucidated.

High levels of peroxidase activity have been associated with the resistance of *Vitis* hybrids to downy mildew (*Plasmopara viticola*), possibly by enabling rapid oxidation of stilbenes to fungitoxic viniferins (Calderón *et al.* 1992). Constitutive peroxidase activity has also been identified as being responsible for the production of resveratrol oxidation products causing tissue browning (Calderón *et al.* 1994). Increases in peroxidase activity in roots of Börner and other rootstocks was proportional to the extent of an HR, and the level of their resistance to the dagger nematode *Xiphinema index* (Sopp *et al.* 1998). Investigation into the level of peroxidase activity in roots of *V. riparia* and Börner after attack by phylloxera might show a similar correlation, as peroxidases could facilitate the rapid accumulation of resveratrol oxidation products, and consequent browning of tissues seen in resistant varieties in response to phylloxera feeding. It was not possible to investigate the level of activity of such enzymes at the time of this study, as quarantine restrictions prevented transport of fresh, infested root material to a suitable analytical facility.

The response of the highly resistant varieties *V. riparia* and Börner to phylloxera feeding supports the hypothesis that an oxidation mechanism is involved in resistance. Both varieties respond by rapid browning of tissues surrounding the feeding site, with an associated antimetabolic effect causing death of phylloxera *in situ*. The compounds which are antimetabolic to phylloxera may be constitutively present in roots or, like the polyphenolic

compounds which cause browning, may be the result of oxidation of less toxic phenolic precursors.

Histochemistry results demonstrate that in *V. riparia* the oxidation of phenolics in response to phylloxera feeding occurs throughout all root tissues, and is correlated with localised death of the root. Observations made in excised root cultures using Börner suggest that for this variety at least, piercing of the epidermis is necessary to invoke such a response, as phylloxera are able to feed for several days at scars left by detached lateral roots without triggering such a response. This implicates the blue-green phenolic compounds located in this tissue in the observed HR-like response. To a lesser extent, a similar browning response is seen in Ramsey, and much later in nodosity development, in *V. vinifera*. The extent and rapidity of tissue browning and necrosis is thus correlated with the resistance of primary roots to phylloxera.

A significant constraint in analysing vine roots for potential mechanisms of phylloxera resistance, is the difficulty in generating sufficient quantities of infested roots or nodosities. Many resistant rootstocks are indeed very resistant and little, if any, infested material can be obtained. It is also not feasible, using currently available techniques, to wait for phylloxera to attempt to feed on roots and then collect individual roots or nodosities at defined stages of development.

Conclusions

The results of this study suggest that resistance to phylloxera can take three forms, not two as suggested by Boubals (1966a). First, antibiosis as seen in Ramsey, and more strongly in *V. riparia*, Börner, and Schwarzmann probably involves oxidation of endogenous phenolics leading to an HR-like response. This would be similar to the mechanisms that grapevines and other plants use as defence against many different pathogens. Second, poor nodosity induction, as seen in Ramsey possibly occurs through weak induction of a nutrient sink. The relative strength of sink formation may be related to the ability of the root tissue to respond to the hormonal stimulus of the feeding insect. Finally, the interaction between at least this accession of the immune species *V. rotundifolia* and phylloxera appears to be one of non-preference. These mechanisms are not mutually exclusive, indeed the complex genetics of phylloxera resistance suggests that combinations of these mechanisms are operating at different levels of efficiency in most rootstocks. Ramsey, for example, exhibits the accumulation of autofluorescent compounds as well as premature necrosis of nodosities, and some accessions of *V. rotundifolia* are reported to show a hypersensitive response to attempted phylloxera feeding. Results from Chapter six further suggest that the efficacy of some of these mechanisms is also dependant on the biotype of phylloxera since, unlike VWL-

1 phylloxera, SRU-1 phylloxera appears little affected by the potential antibiosis of Schwarzmann.

The importance of a good nutrient supply, or formation of a nodosity which is a strong nutrient sink, for phylloxera survival and development has been previously demonstrated by Granett and coworkers (Granett *et al.* 1983). Using excised secondary root bioassays they were able to demonstrate both qualitative and quantitative variation in the resistance mechanisms of the two rootstocks ARG1 (A×R #1), and *V. rupestris* cv. St George to phylloxera. The resistance of ARG1 appeared to be related to poor phylloxera nutrition as adult survivorship was relatively high but fecundity was low, while that of *V. rupestris* cv. St George appeared to be due shortened adult duration resulting in lower total fecundity.

All the evidence available to date serves to illustrate the complexity of the resistance of grapevines to phylloxera, and more research will be necessary before specific mechanisms are determined. Molecular analysis of the root response to phylloxera could play an informative part in these investigations in the future. Such analysis was not included in this study because of the limitations in the amount of infested root material which could be obtained. The use of *in situ* hybridisation would be an ideal method for future investigation as it would require only small numbers of nodosities, and would enable characterisation of even very localised responses to phylloxera feeding.

6. INTERACTION OF *V. VINIFERA* AND SCHWARZMANN WITH A NOVEL BIOTYPE OF PHYLLOXERA, STRAIN SRU-1

6.1 Introduction

Only very recently have studies on the genetics and biotypes of phylloxera in Australia been undertaken. So far, three genetically distinct strains of phylloxera have been identified from four viticultural regions (Corrie *et al.* 1997a). Some of the strains (VWL-1, VNA-1) are biologically similar to Biotype B isolated from Californian vineyards, as defined by Granett *et al.* (1985) (Corrie *et al.* 1997b). Strain SRU-1 is different and unusual in both its relative lack of virulence on *Vitis vinifera* and in its ability to survive and reproduce equally well on the primary roots of Schwarzmann as on *V. vinifera*.

SRU-1 was originally isolated from Schwarzmann vines in a rootstock propagation block at Campbell's vineyard in Rutherglen, Victoria (Corrie *et al.* 1997a). It lives on the roots of the rootstock vines, and in the summer it emerges from the soil and (unless the temperature is too high) can form vast numbers of leaf galls (figure 6.1). It can also be found on the roots of *V. vinifera* vines and those of several rootstocks in a Department of Agriculture rootstock trial at Stanton and Killeen's vineyard, Rutherglen, Victoria where it was found in roughly equal numbers on the roots of *V. vinifera*, Schwarzmann and ARG-1 (Corrie *et al.*, unpublished). Although not recognised until recently, SRU-1's relative lack of aggressiveness on roots of *V. vinifera* correlates with the absence of a noticeable decline in health or productivity of own-rooted *V. vinifera* vines within this phylloxera-infested region over at least two decades (M. Campbell, Campbell's vineyards, Rutherglen, pers. comm.). Good viticultural practices and deep, fertile soil have undoubtedly also contributed to the continued health of this vineyard, which otherwise would have been expected to have experienced some phylloxera-related decline. By comparison, vineyards in the King Valley infested with VWL-1 strain phylloxera have become unproductive within five years of infestation, despite good viticultural practices and conditions.

The population density of SRU-1 on the roots of Schwarzmann vines in Campbell's rootstock block is high. These are also healthy, productive vines and clearly the presence of the phylloxera at relatively high population densities has not had an adverse affect on their growth. The population density on their primary roots, however, is an indication that these vines should be considered tolerant, not resistant to this strain of phylloxera.

The existence of different strains and biotypes of phylloxera has been known in other countries for some time (reviewed in Chapter 1, section 1.1.1). King and Rilling (1985, 1991) emphasise the importance of keeping strains separate in experimental trials as, if they

Figure 6.1 SRU-1 strain phylloxera-induced leaf galls on Schwarzmann mother vines, Campbell's rootstock block, Rutherglen, Victoria.

differ in biotype, they can generate very different results. Experimental results obtained in this study using two phylloxera strains (VWL-1 and SRU-1) have been kept separate to avoid this possibility.

SRU-1 strain phylloxera had, until 1997-98, been maintained on *V. vinifera* vines in the glasshouse at Agriculture Victoria, Rutherglen as the primary experimental strain. In the first year of the study reported here (the 1996-97 season) neither it, nor any other strain from Australian vineyards, had been biotyped in terms of their response to various vine types. SRU-1 was originally adopted for use in this study because of its ready availability from leaf galls and the fact that it was known to infest 'resistant' varieties (including Schwarzmann), thus enabling the production of sufficient quantities of infested resistant root material for analysis. Subsequent to the work presented in this chapter, in which it became clear that SRU-1 was different to a 'classical' biotype A or biotype B phylloxera, and would not provide a typical susceptible/resistant comparison, the decision was made to switch to the VWL-1 strain for the following season.

In the 1996-97 season many logistical problems (outlined in Chapter two) were encountered with both tissue culture and pot-based cocultivations, resulting in very limited production of infested root material using SRU-1 phylloxera, and only from *V. vinifera* cv. Shiraz and Schwarzmann. The root material obtained was sufficient for analysis by microscopy and HPLC. In the 1997-98 season, SRU-1 phylloxera was also included in tissue culture-based cocultivation trials in order to obtain survival data to place previous HPLC and microscopy results in context regarding the resistance status of Schwarzmann compared to *V. vinifera*. This chapter presents these bioassay results and the results of histochemical and HPLC analyses of infested root material generated in both seasons, as well as northern blot hybridisation analysis of leaves from infested vines.

6.2 Tissue culture-based bioassays of SRU-1 phylloxera cocultivated with a range of grapevine varieties

Tissue culture-based cocultivations were used to determine the resistance of various rootstocks to SRU-1 strain phylloxera.

6.2.1 Materials and methods

Phytatray cocultivation trials (described in Chapter 2, section 2.4.3) were carried out in the 1996-97 season. SRU-1 phylloxera were collected directly from Schwarzmann leaf galls, Campbell's vineyard, Rutherglen which was the easiest way of obtaining sufficient quantities of this strain for experiments. Vine varieties were *V. vinifera* cv. Cabernet Sauvignon, 140-Ruggeri and *V. rotundifolia*.

In the following season (1997-98) perlite-based medium cocultivations (Chapter 2, section 2.4.4) and excised root cultures (section 2.4.6) were carried out. Cocultivations with SRU-1 phylloxera were run in parallel with cocultivations with VWL-1 phylloxera. SRU-1 phylloxera was sourced from a small population originally collected from Schwarzmann leaf galls, Campbell's vineyard, Rutherglen and maintained for a year in tissue culture on *V. vinifera* cv. Shiraz vines. The temperature during the 1997-98 summer was above average (mean max. temp. 31.5°C, Agriculture Victoria Rutherglen weather station records, with several periods above 35°C), and no leaf galls were formed in the field, in contrast to the previous year. Because of limited inoculum, grapevine varieties for these trials were limited to *V. vinifera* cv. Shiraz and Schwarzmann.

6.2.2 *Phytatray and perlite-based medium bioassays*

The results from the phytatray bioassays cannot be considered to be reliable because of the problems discussed in Chapter 2, section 2.4.3, especially the high numbers of insects which could not be accounted for. Some data from phytatray trial two (table 6.1) are nevertheless included here to give an indication of the biotype of SRU-1 phylloxera.

Table 6.1 Survival of SRU-1 phylloxera and nodosity formation per phytatray after 21 days cocultivation with three vine varieties in phytatray trial two. Each phytatray was initially inoculated with 100 surface-sterilised eggs.

vine variety	unhatched eggs (±SD)	crawlers found dead (±SD)	total no. phylloxera alive (±SD)	phylloxera actively feeding (±SD)	nodosities initiated (±SD)	phylloxera not accounted for (±SD)
***V. vinifera* cv. Cabernet Sauvignon**	2.67 ± 3.71	27.22 ± 15.44	3.11 ± 4.83	1.33 ± 1.87	1.89 ± 2.20	68.00 ± 19.96
140 Ruggeri	4.28 ± 6.97	36.71 ± 15.14	3.14 ± 3.58	2.86 ± 3.58	1.57 ± 1.72	57.29 ± 15.77
V. rotundifolia	3.13 ± 4.67	25.38 ± 22.92	0	0	0	71.50 ± 25.09

The rates of phylloxera survival and gall formation were similar on both *V. vinifera* cv. Cabernet Sauvignon and 140-Ruggeri, although highly variable between replicates.

The survival data for SRU-1 phylloxera after 30 days in a perlite-based medium cocultivation are shown in tables 6.2 and 6.3. Raw data are presented to show variability in numbers and types of feeding sites formed.

Table 6.2 Survival of SRU-1 phylloxera life stages and feeding site formation on *V. vinifera* cv. Shiraz in perlite-based medium cultures. Each plant was initially inoculated with 100 surface-sterilised eggs.

			no. live phylloxera at each developmental stage after 30 days cocultivation						
rep.	**no. eggs hatched**	**nodosities/ feeding sites initiated**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults**	**nymphs with wing buds**	**total no. survivors**
1	86	1 nod[1], base[2]	>10[ab]	0	0	0	8	0	8[c]
2	76	6 nod, 2 old[3]	2	1	5	3	6	0	17[c]
3	65	4 nod	3	0	0	0	1	0	4[c]
4	64	21 nod, base	>10[ab]	>10[a]	>10[a]	>10[a]	>10[a]	>10[a]	>10[a]
5	84	3 nod, base	0	0	4	0	6	0	10[c]
6	71	15 nod	>10[ab]	>10[a]	>10[a]	>10[a]	>10[a]	0	>10[a]
7	50	split in stem[4]	0	0	1	0	1	0	2[c]
8	80	1 nod	0	0	1	0	3	0	4[c]
9[5]	70	0	-	-	-	-	-	-	-
10	53	7nod, 1 stem gall[6]	>10[ab]	0	0	1	4	0	5[c]

[a] Too many to count but definitely more than ten.
[b] All second generation insects.
[c] Not counting second generation insects.
[1] 'nod' refers to nodosities formed on primary roots.
[2] 'base' refers to colonies established at the base of the main stem.
[3] 'old' refers to nodosities which had been abandoned and were becoming necrotic (not included in 'nod').
[4] Phylloxera were feeding within a split in the outer tissues of the stem.
[5] Replicate nine accidentally lost during trial.
[6] A gall otherwise similar to a nodosity, but formed on the stem of the plantlet.

Table 6.3 Survival of SRU-1 phylloxera life stages and feeding site formation on Schwarzmann in perlite-based medium cultures. Each plant was initially inoculated with 100 surface-sterilised eggs.

			no. live phylloxera at each developmental stage after 30 days cocultivation						
rep	**eggs hatched**	**no. nodosities/ feeding sites initiated[1]**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults**	**nymphs with wing buds**	**total no. survivors**
1	64	15 nod, base	0	0	>10 [a]	2	5	5	>10 [a]
2	84	15 nod	0	0	>10[a]	0	0	>10[a]	>10 [a]
3	68	22 nod	>10[ab]	>10[a]	>10a	>10[a]		>10[a]	>10 [a]
4	44	16 nod, base	>10[ab]		>10[a]	6	7	>10[a]	>10 [a]
5	85	45 nod	>10[ab]	>10[a]	>10[a]	>10[a]	>10[a]	>10[a]	>10 [a]
6	-	contaminated	-	-	-	-	-	-	-
7	-	contaminated	-	-	-	-	-	-	-
8	56	2 nod	0	0	0	0	2	0	2
9	62	1 nod	0	1	1	0	0	0	2
10	46	10 nod	>10[ab]	>10[a]	>10[a]		8	0	>10 [a]

[1] See table 6.2 for definitions of feeding sites.
[a,b] See table 6.2.

Differences between infestation parameters for SRU-1 and VWL-1 phylloxera were observed, and some indication of the biotype of SRU-1 was obtained (table 6.4). Unfortunately, at the time of assessment (30 days after inoculation), many second generation SRU-1 phylloxera had hatched and were thriving on both vine varieties, therefore the survival rate of the initial inoculum could not be determined, nor was it possible to statistically test for any difference in performance of SRU-1 phylloxera on the two vine varieties.

SRU-1 phylloxera was able to initiate nodosities, as well as feed from the base of the main stem on both *V. vinifera* cv. Shiraz and Schwarzmann. In addition, a stem gall and a feeding site in a split through the bark of the stem were formed on *V. vinifera*. The developmental rate was similar on both varieties. The number of nodosities and total number of feeding sites was higher on Schwarzmann than on *V. vinifera*. Typical nodosities formed by SRU-1 phylloxera were equally large, supporting phylloxera populations of a range of developmental stages, on *V. vinifera* cv. Shiraz and Schwarzmann in tissue culture (figure 6.2).

A comparison of infestation parameters for VWL-1 phylloxera from the same cocultivation trial and on the same grapevine varieties (table 6.4) shows differences between treatments despite the lack of statistical differences. In comparison with VWL-1 phylloxera, the developmental rate of SRU-1 phylloxera on *V. vinifera* was quicker. Second generation VWL-1 had not developed past the first instar at the time of assessment. SRU-1 phylloxera also initiated more nodosities and other feeding sites than VWL-1. VWL-1 phylloxera was completely unable to survive on Schwarzmann.

6.2.3 Excised root culture bioassays

Table 6.5 presents a summary of results from the excised root cocultivation trial. Survival rates were low, but SRU-1 phylloxera was able to reproduce on both *V. vinifera* and Schwarzmann, although nodosities were only formed on Schwarzmann. SRU-1 phylloxera performed slightly better than VWL-1 phylloxera on *V. vinifera*, with one insect surviving to reproductive stage, although this was settled in the scar of a detached lateral root, rather than on a nodosity. Several other SRU-1 insects were observed to be feeding on *V. vinifera* during the course of the cocultivation, especially at the root tips, but they did not progress through to the 4th instar. After 25 days cocultivation many of the nodosities formed on Schwarzmann had been abandoned.

Figure 6.2 Nodosities formed on (a) *V. vinifera* cv. Shiraz and (b) Schwarzmann in perlite-based medium cultures. Note the presence of several life stages on each nodosity, including nymphs with wing buds (arrow) on Schwarzmann. Scale bar equals approximately 5mm.

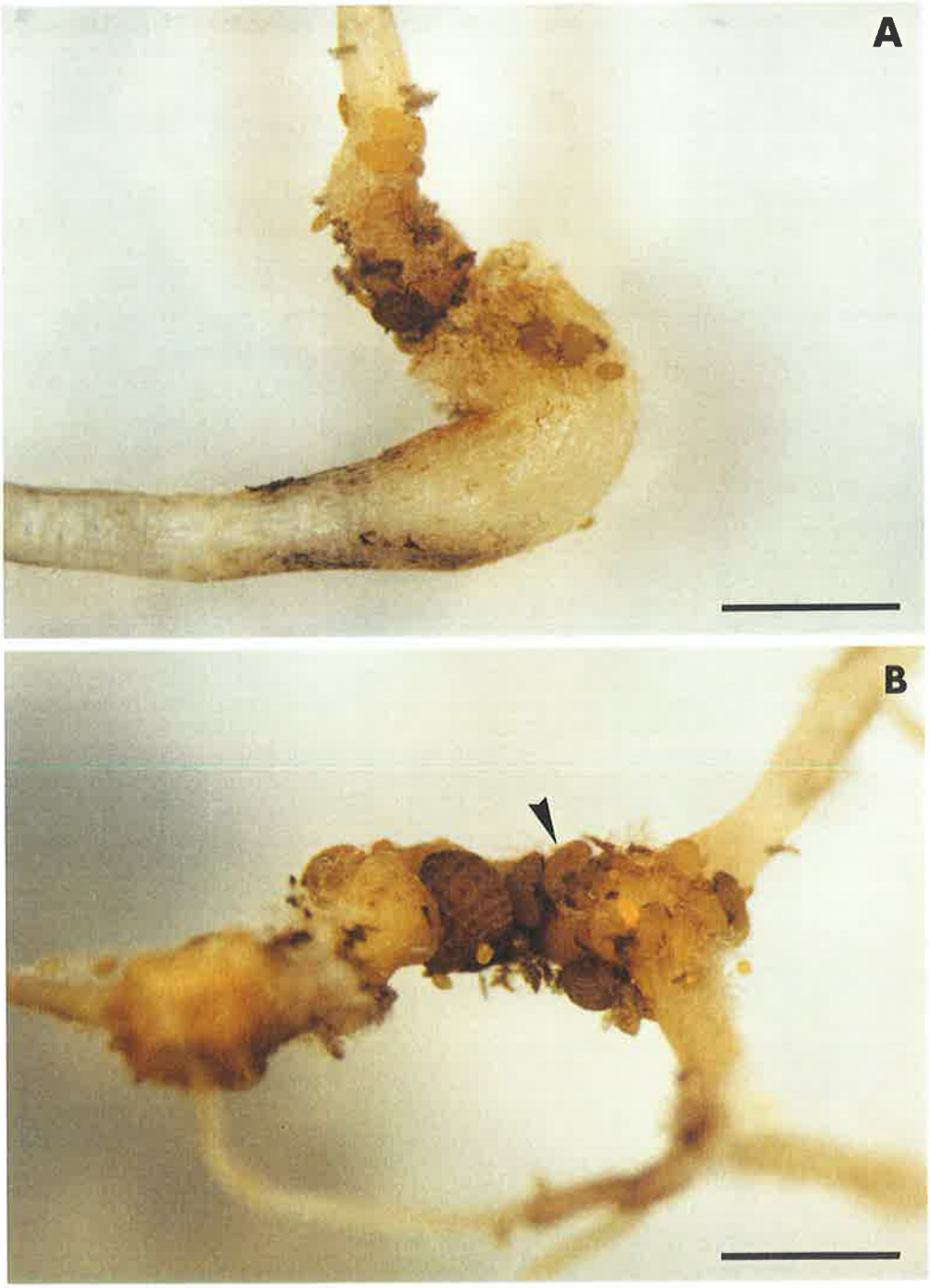

A
B

Table 6.4 Survival of SRU-1 and VWL-1 phylloxera life stages and nodosity formation on *V. vinifera* cv. Shiraz and Schwarzmann in perlite-based medium cultures. VWL-1 data from table 5.1; SRU-1 data from tables 6.2 and 6.3.

			mean no. live phylloxera at each developmental stage after 30 days cocultivation						
vine variety/ phylloxera strain	**eggs hatched (±SD)**	**nodosities formed (±SD)**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults**	**nymphs with wing buds**	**total no. survivors (±SD)**
***V. vinifera*/ VWL-1**	86.63 ± 5.93	4.43 ± 3.87	2.29	2.29	3.14	0.71	6.43	2.28	17.14 ± 7.51
Schwarz./ VWL-1	83.75 ± 11.37	0	0	0	0	0	0	0	0
***V. vinifera*/ SRU-1**[1]	69.90 ± 12.2	6.44 ± 7.10	>10	?	?	?	?	?	?
Schwarz./ SRU-1[1]	63.32 ± 15.34	15.75 ± 13.81	>10	?	?	?	?	?	?

[1] Live phylloxera includes second generation insects which could not be differentiated from original inoculum. It was therefore not possible to calculate means for much of these data, however in many of the replicates the number of live phylloxera at many of the developmental stages was greater than ten.

Table 6.5 Survival of VWL-1 and SRU-1 phylloxera life stages and nodosity formation on *V. vinifera* cv. Shiraz or Schwarzmann on excised root cultures. Twelve roots of each variety were each inoculated with ten surface-sterilised eggs.

			mean no. live phylloxera at each developmental stage after 25 days cocultivation					
vine variety/ phylloxera strain	**eggs hatched (±SD)**	**nodosities initiated (±SD)**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying**	**total no. survivors (±SD)**
***V. vinifera*/ VWL-1**	3.33 ± 1.97	0.08 ± 0.29	0.25	0.67	0.08	0	0	1.00 ± 1.54
Schwarz./ VWL-1	3.58 ± 0.90	0.00 ± 0.00	0	0	0	0	0	0
***V. vinifera*/ SRU-1**	3.66 ± 1.50	0.00 ± 0.00	0.27	0.09	0.09	0.00	0.09[1]	0.55 ± 0.69
Schwarz./ SRU-1	3.58 ± 2.39	0.67 ± 0.78	0	0	0.33	0.08	0.17	0.50 ± 0.80

[1] Only one adult was found laying eggs; it was settled in the scar of a detached lateral root; no nodosities were initiated.

6.2.4 *Discussion of bioassays*

Perlite-based medium bioassays

SRU-1 phylloxera has previously been found to have lower survival rates and fecundity than VWL-1 phylloxera (Corrie *et al.* 1997b, Corrie *et al.* unpublished). However, in the perlite-based medium cocultivation, despite a slightly lower hatch rate, SRU-1 performed better than VWL-1 in terms of number of nodosities initiated, survival and reproduction on both *V. vinifera* cv. Shiraz and Schwarzmann. This assay therefore shows that SRU-1 phylloxera can be the more aggressive strain, at least on primary roots.

In 'classical' root bioassays (conducted by Corrie *et al.* unpublished, using excised secondary roots as per Granett *et al.* 1985) the results show the same trend, although different data were obtained due to the presence of secondary roots, lateral primary roots and callus as potential feeding sites. SRU-1 in secondary root bioassays had higher percent survival on Schwarzmann than on *V. vinifera*. It also had similar percent survival on Schwarzmann as did VWL-1 on *V. vinifera* (a compatible interaction). If only primary roots and callus are considered, the number of SRU-1 feeding sites on Schwarzmann was considerably higher than the number of VWL-1 feeding sites on *V. vinifera*, while the number of SRU-1 feeding sites on *V. vinifera* was similar to the number of VWL-1 feeding sites on this variety.

It is not possible to say from the perlite-based medium cocultivation trial whether SRU-1 phylloxera was more aggressive on *V. vinifera* cv. Shiraz or Schwarzmann primary roots due to confusion of data by second generation insects. At least twice as many nodosities were formed on Schwarzmann than on *V. vinifera*, however, indicating the greater ability of SRU-1 to establish feeding sites on this variety.

Excised root culture bioassays

As discussed in section 2.4.6, survival rates using excised root cocultivations were low, so no statistical analysis of data was performed. Nevertheless the data reflect trends seen in perlite-based medium bioassays. SRU-1 phylloxera were able to reproduce on excised roots of both *V. vinifera* cv. Shiraz and Schwarzmann, while VWL-1 phylloxera were not. SRU-1 phylloxera also performed slightly better, and initiated more nodosities, on Schwarzmann than on *V. vinifera*. Many of the nodosities were abandoned, i.e. they were not able to support continued development and reproduction of phylloxera, indicating that while SRU-1 phylloxera were able to initiate gall formation, their reproductive success was still limited. This was in contrast to large, productive nodosities formed in perlite-based medium cocultivations, and is most likely to have been a result of the culture conditions.

SRU-1 as a novel biotype

Bioassay data collected to date (including the observations made by Corrie *et al.* unpublished) point to a preference of SRU-1 phylloxera for primary roots of Schwarzmann over those of *V. vinifera*, and also for primary roots over secondary roots. There are at least two possible explanations for the former. First, this strain of phylloxera may always have been intrinsically better suited to American *Vitis* species. Alternatively, SRU-1 phylloxera which were collected from Schwarzmann vines may have recently evolved to become better 'adapted' to this rootstock. Kimberling and Price (1996) suggest that phylloxera populations do not become adapted to host genotypes within a wild *Vitis* population, nor are they necessarily better suited to the host from which they have been sourced. How this hypothesis applies to adaptation of phylloxera to vine varieties in the monoculture system of a commercial vineyard is not known. According to Granett *et al.* (1985), however, the evolution of new biotypes could take place within a time frame of several decades. Schwarzmann has been commonly used in Rutherglen for less than two decades, and the possibility of this strain becoming adapted to this rootstock within this time frame remains doubtful.

6.3 Root response to SRU-1 phylloxera attack observed by histochemistry

As SRU-1 is unusual in its ability to thrive on the roots of a 'resistant' rootstock, the response of vines to its attack is of particular interest. Interactions of SRU-1 phylloxera with primary roots of *V. vinifera* and Schwarzmann must both be considered 'compatible', since nodosities are successfully formed. The vine varieties differ in that *V. vinifera* is susceptible to SRU-1 phylloxera while Schwarzmann is tolerant, so their physiological responses to attack might be expected to be different. The aim of this study was to document histochemical changes in response to SRU-1 phylloxera attack in *V. vinifera* cv. Shiraz and Schwarzmann.

6.3.1 Materials and methods

Nodosities were collected from *V. vinifera* cv. Shiraz and Schwarzmann vines in pot trial three (Chapter 2, section 2.3.3) and Schwarzmann nodosities were also collected from pot trial five (Chpater2, section 2.3.5). They were fixed, embedded in GMA and sectioned. As described previously (Chapter 5, section 5.4.1), sections were stained with either PAS/TBO, Sudan black or examined under UV excitation to observe the response to phylloxera. Sample preparation, staining and microscopy protocols are described in Appendix three.

6.3.2 *Root response to SRU-1 phylloxera*

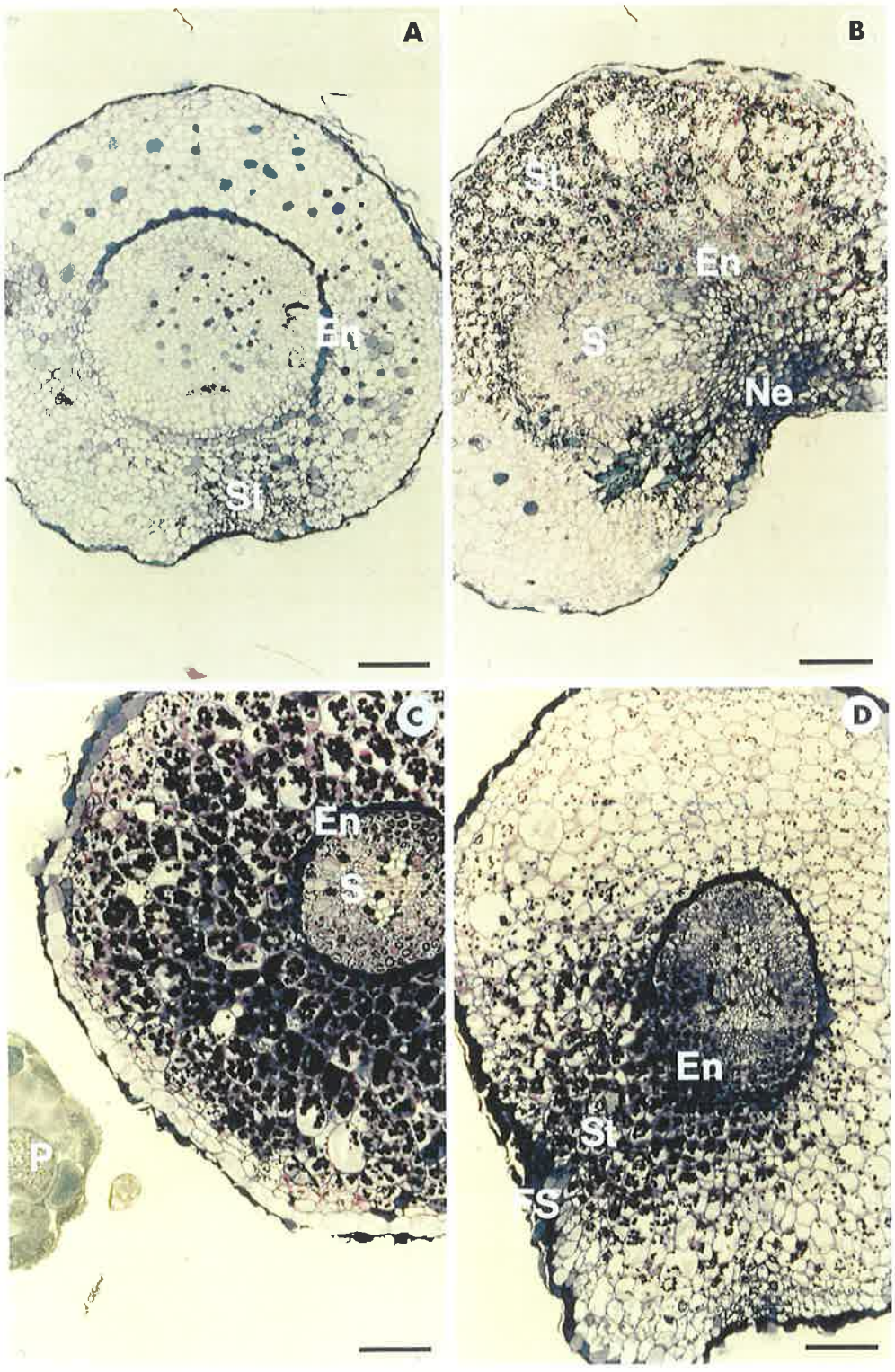
The most striking difference between nodosities of the two vine varieties detected by PAS/TBO staining was the size and abundance of the amyloplasts in Schwarzmann. Neither variety had any detectable starch in the cortex of uninfested roots of similar age from the same vines (not shown). The *V. vinifera* nodosity pictured in figure 6.3a appears relatively young, with a little starch accumulation surrounding the feeding site, and uneven differentiation of the endodermis, indicated by differential staining. In a very mature nodosity (figure 6.3b) (although not immediately apparent from this section, it was becoming necrotic through age) the endodermis appears almost completely undifferentiated, and starch has accumulated throughout much of the cortex.

Very large and abundant amyloplasts in the cortex of the Schwarzmann nodosity (figure 6.3c) contrast with the much smaller amyloplasts in the nodosities on *V. vinifera*. Schwarzmann nodosities varied from being small, with relatively even hypertrophy of cortical cells and unaltered endodermis and stele (figure 6.3c), to being more similar to *V. vinifera* nodosities with the endodermis partly undifferentiated on the side of phylloxera feeding, and swelling distal to the feeding site (figure 6.3d). The accumulation of starch in the latter section was greatest immediately adjacent to the undifferentiated region of the endodermis and within the stele, although there were small amyloplasts scattered throughout the cortex.

The endodermis of uninfested Schwarzmann roots typically had state two suberisation (figure 6.4a), while in nodosities initiated close to the root tip suberin could not be detected histochemically (figure 6.4b. This is a section of the same nodosity as in figure 6.3c). Abundant amyloplasts were visible in the stele, endodermis and cortex of nodosities even when unstained (figure 6.4b). In one case a nodosity initiated further behind the root tip, where the endodermis already had developed state two suberisation, was examined. In this example amyloplasts were visible, however their distribution in the root was limited to tissue within the stele (data not shown). This example further suggests the effectiveness of endodermal cell wall suberisation in restricting movement of solutes into the cortex.

Under UV excitation there was no autofluorescent response to SRU-1 phylloxera in *V. vinifera*, even when infested by a number of insects (although these are early instars) (figure 6.5a). Bright blue-white autofluorescence accumulated in or between cells immediately adjacent to a region in a *V. vinifera* nodosity becoming necrotic (figure 6.5b) suggesting either that *V. vinifera* can, however, respond to necrosis by an accumulation of autofluorescent compounds, or possibly that this accumulation precedes the development of tissue necrosis. The cell layer immediately outside the region of autofluorescence was

Figure 6.3 Transverse sections through nodosities on both *V. vinifera* cv. Shiraz and Schwarzmann stained with PAS/ TBO. a) *V. vinifera*: differential staining of endodermis (En); early starch accumulation (St). b) *V. vinifera*: relatively undifferentiated stele (S) and endodermis (En); accumulation of phenolic compounds in necrotic region (Ne); accumulation of starch (St) throughout the cortex. c) Schwarzmann: strongly differentiated stele (S) and endodermis (En); massive accumulation of starch in cortex, endodermis and stele (dark grains) as well as some accumulation of phenolics (blue staining cytoplasm adjacent to phylloxera (P)). d) Schwarzmann: indistinct endodermis (En) on side of feeding (FS); accumulation of starch (St) in the cortex and stele on the side of feeding, and the accumulation of some phenolics (blue staining). Scale bars equal 200μm.


A
En
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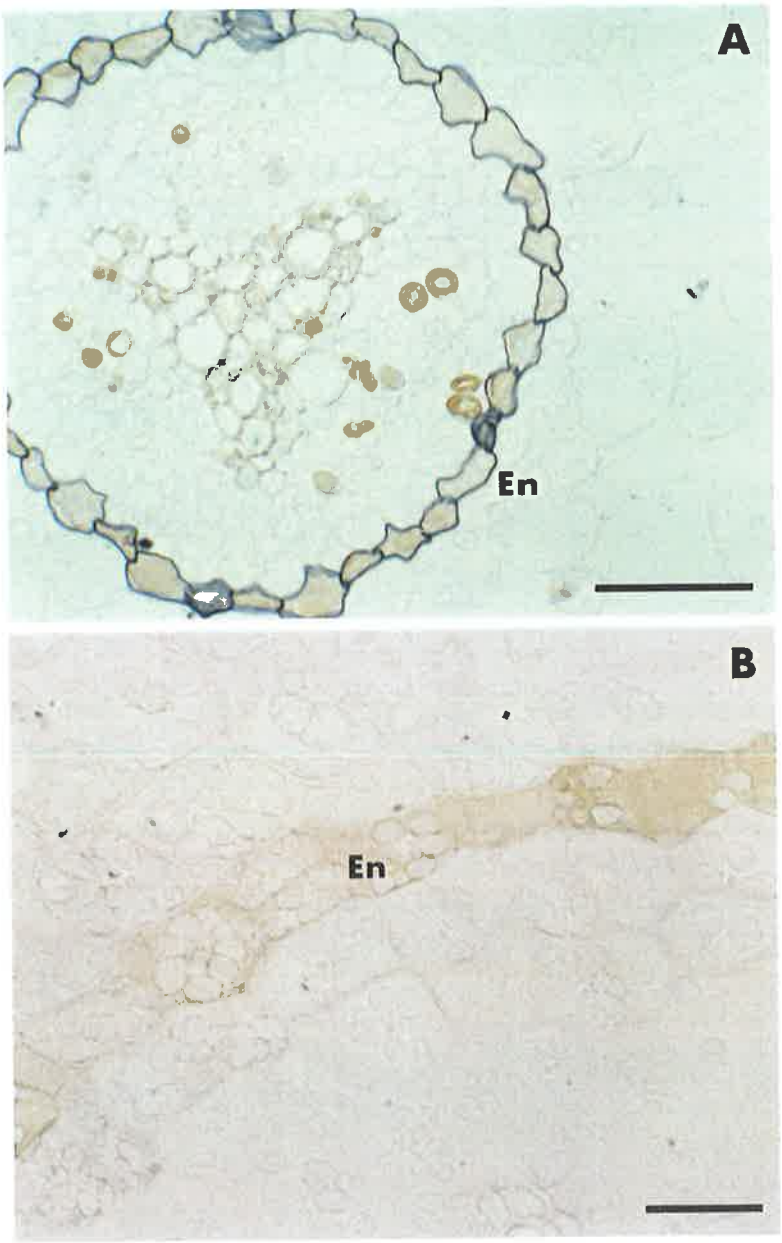
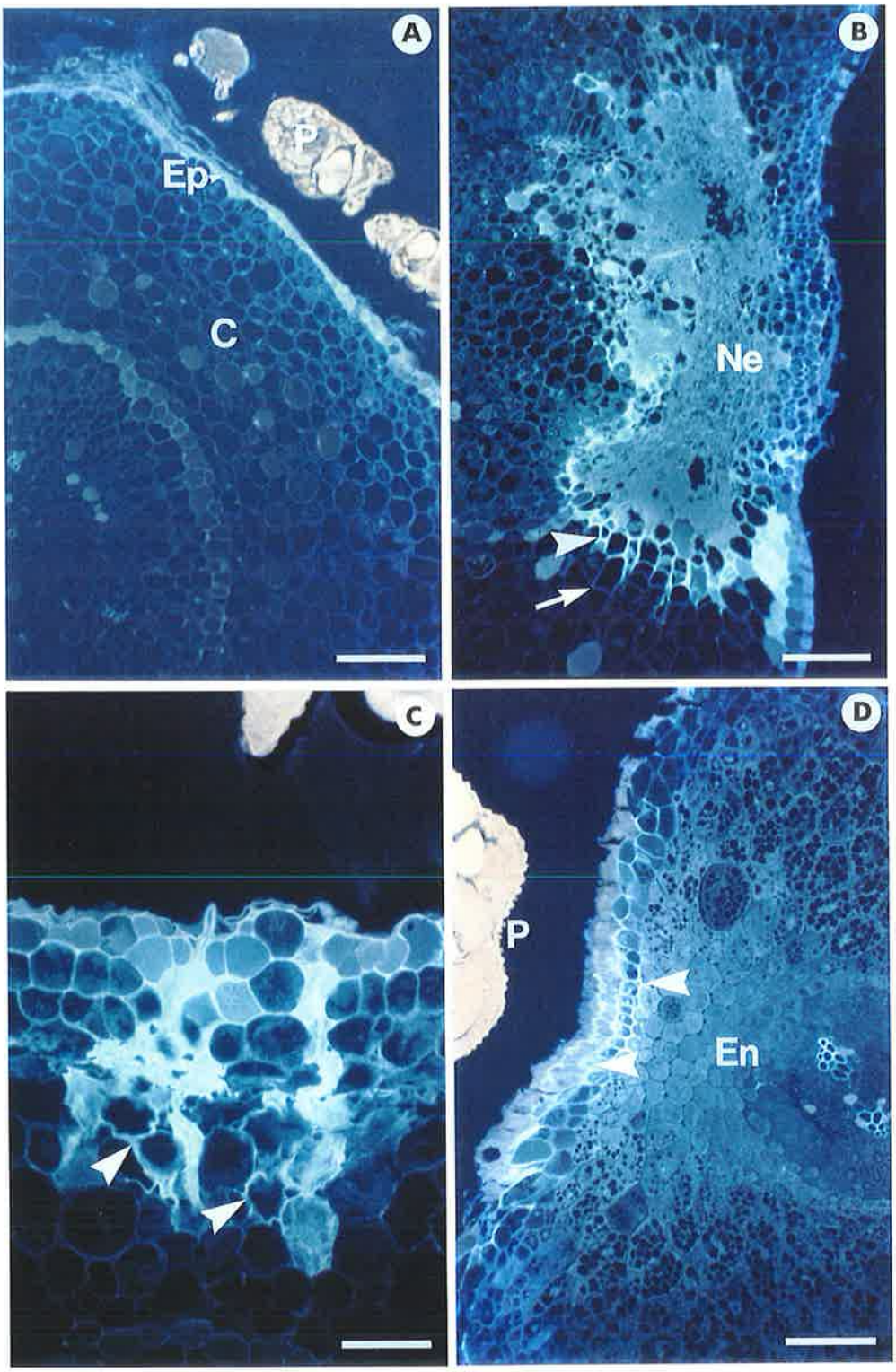


Figure 6.4 Transverse sections through Schwarzmann roots stained with Sudan black. Suberin is stained blue-black. a) Endodermis (En) of an uninfested root with strong state two suberisation of all cells. Scale bar equals 50μm. b) Endodermis (En) of nodosity. No suberin is visible. Scale bar equals 20μm.

Figure 6.5 Transverse sections through nodosities of both *V. vinifera* cv. Shiraz and Schwarzmann under UV excitation a) *V. vinifera*: phylloxera (P); epidermis (Ep); cortex (C). Scale bar equals 100µm. b) *V. vinifera*: note necrotic region (Ne) surrounded by blue-white autofluorescence (arrowhead) and royal blue autofluorescence (suberin - arrow). Cells in the necrotic area are filled with phenolics, and this region penetrates beyond the endodermis. Scale bar equals 100µm. c) Stylet tracks of phylloxera feeding on Schwarzmann. Note blue-white autofluorescence adjacent to stylet tracks (arrow heads). Scale bar equals 50µm. d) Schwarzmann: note lack of distinct endodermis (En) on side of phylloxera (P) feeding, massive accumulation of starch granules (dark) and accumulation of phenolics (opaque, pale blue cell contents). Most striking is the accumulation of blue-white fluorescent compound (arrow heads) adjacent to the feeding site of phylloxera. Scale bar equals 100µm.


A
Ep
P
C
B
Ne
C
D
P
En

suberised (previously described in Chapter 4, section 4.3.3, and confirmed by staining with Sudan black, figure 4.5c), indicated here by a faint royal blue autofluorescence (figure 6.5b).

By contrast, in nodosities on Schwarzmann, there was accumulation of a blue-white autofluorescent compound, not only in cells penetrated by the stylet (figure 6.5c), but also in the region adjacent to those penetrated. This was similar to the autofluorescence observed in Ramsey in response to VWL-1 phylloxera (Chapter 5, figure 5.12c). A much less localised example of this response is illustrated in figure 6.5d. Here, accumulation of a blue-white autofluorescent compound appeared associated with cells of the hypodermis adjacent to the feeding phylloxera. The pictured nodosity was large, with anatomical features typical of nodosities normally seen in *V. vinifera,* including hypertrophy of cortical cells distal to the feeding site and poorly differentiated endodermis adjacent to it. Also, typical of the Schwarzmann/SRU-1 interaction, was a strong accumulation of starch in the cortex (amyloplasts absorb UV light and appear dark). The cortical and stele cells were filled with a substance which appears a dull, opaque pale blue.

6.3.3 Discussion of histochemistry results

A relationship was proposed in Chapter four (section 4.4) between the non-differentiation of the endodermis and starch accumulation in VWL-1 induced nodosities on *V. vinifera* cv. Shiraz. Some Schwarzmann nodosities (figures 6.3c, 6.4b) accumulate significant quantities of starch while having an apparently properly differentiated endodermis. This indicates that differentiation of the endodermis as such does not inhibit successful feeding site formation and associated starch accumulation, at least in this variety. Sudan black staining shows, however, that the endodermis of Schwarzmann nodosities are not suberised (figure 6.4b), thus supporting the hypothesis that lack of suberisation may facilitate the unloading of photosynthates into the nodosity.

The multiple stylet tracks of phylloxera seen in figure 6.5c suggest that feeding site establishment on Schwarzmann was not immediately successful and/or that phylloxera was not as sedentary a feeder on this variety as it is on *V. vinifera*. This may be due to the presence of a repellent substance, possibly even the blue-white autofluorescent compound. Alternatively, the phylloxera may have needed to search for a cell which would respond appropriately to stimuli for feeding site establishment. Multiple stylet tracks were never seen in *V. vinifera*.

The accumulation of the blue-white autofluorescent compound adjacent to the feeding site of SRU-1 phylloxera in Schwarzmann nodosities is typical of a grapevine defence response to an invading pathogen (Dai *et al.* 1995a, b). This occurred here despite this being a

'compatible' interaction, implying that SRU-1 phylloxera was either unperturbed by, or somehow able to detoxify this compound. The difference in extent of accumulation of the autofluorescent compound seen in figure 6.5c and d may be accounted for by the source of infested root material. These nodosities were harvested from pot trials five and three respectively, which used different accessions of Schwarzmann. Another possible explanation is that the nodosity pictured in figure 6.5d was more mature than that in figure 6.5c, possibly allowing more time for accumulation of this compound.

6.4 Changes in free phenolics in response to SRU-1 phylloxera attack

HPLC analysis was used to compare the changes in phenolic profile of primary roots in response to SRU-1 phylloxera attack, as described previously for VWL-1 phylloxera (Chapter 5, section 5.4.2).

6.4.1 HPLC materials and methods

Uninfested roots and nodosities of *V. vinifera* cv. Shiraz were harvested from pot trial three (Chapter 2, section 2.3.3), and Schwarzmann nodosities were also harvested from pot trial five (Chapter 2, section 2.3.5). In addition to this, nodosities were harvested from Schwarzmann rootstock accession WA/A.V.70.2252 in the rootstock trial block at Stanton and Killeen's vineyard, Rutherglen. Free phenolic extracts were made using extraction method one (Chapter 3, section 3.2.1) and HPLC analysis carried out using HPLC chromatography method nine (Chapter 3, section 3.2.2). All extractions and HPLC runs were performed in duplicate.

6.4.2 Free phenolics in SRU-1 phylloxera induced nodosities

The basic free phenolic composition of the uninfested roots of each vine variety was discussed in detail in Chapter 5, section 5.3.3.

HPLC chromatograms obtained at 330nm from material harvested from pot trial three (1996-97) are shown in figure 6.6a. Two novel compounds appeared as major peaks (marked A and B) almost exclusively in extracts from Schwarzmann nodosities. Peak A (figure 6.6b), had an absorption profile typical of a hydroxycinnamic acid. The retention time and absorption spectrum of peak B (figure 6.6c) indicated that it represents free *trans*-resveratrol. The retention time of peak B was also the same as that of the peak proposed to represent free *trans*-resveratrol inVWL-1 induced *V. vinifera* nodosities from potted vines, and Ramsey nodosities from tissue cultured vines (Chapter 5, section 5.4.2). Peak B was also present as a minor peak in extracts from *V. vinifera* nodosities and Schwarzmann uninfested roots (figure 6.6a).

In the following season (1997-98) HPLC analysis of extracts from Schwarzmann nodosities (harvested either from pot trial five, or from field-grown vines) was repeated with the intention of preparing sufficient extract to enable isolation and identification of compound A by mass spectrometry, or if necessary, NMR analysis. When the HPLC profiles from this new material were compared to those from 1996-97, however, compounds A and B were no longer detectable in significant quantities in extracts of Schwarzmann nodosities (data not shown).

In free phenolic extracts from SRU-1 induced nodosities compared with uninfested roots of both *V. vinifera* cv. Shiraz and Schwarzmann, and from both pot trials, peak 3 underwent a similar transformation to peak 3′ as was seen in extracts of VWL-1 induced nodosities (Chapter 5, section 5.4.2).

6.4.3 Discussion of HPLC analyses of free phenolics

The retention time of peak A does not match any readily available standard phenolic compounds (Appendix five), however its absorption spectrum is characteristic of the hydroxycinnamic acids (e.g. chlorogenic acid, ferulic acid and caffeic acid, see Appendix five, figures A5.1, A5.2). It is therefore likely that it represents a hydroxycinnamic acid (possibly either ferulic or caffeic acid) in a previously undescribed conjugation (K. Markham, Industrial Research Limited, Wellington, NZ, pers. comm.).

The accumulation of free *trans*-resveratrol (peak B) seen previously in nodosities of *V. vinifera* cv. Shiraz, Ramsey and particularly in those of Schwarzmann here suggests this may be a general response to phylloxera infestation. This accumulation may result from *de novo* synthesis of resveratrol or from deglycosylation of compound 3 (piceid) as was proposed to occur in VWL-1 induced nodosities (Chapter 5, section 5.4.2). Accumulation of free *trans*-resveratrol is correlated with grapevine resistance to fungal pathogens (as previously discussed in Chapter 1, section 1.4.1). Its role in the interaction with phylloxera, if any, remains to be determined.

The discrepancy in composition of free phenolic extracts between the two seasons is difficult to explain. It is not likely that the presence of peaks A and B in the first season were artefacts of the HPLC method, as the preparation of extracts from these samples was repeated several times during the optimisation of the HPLC method, and peaks A and B were consistently present. It is possible therefore, that fundamental differences between the two cocultivation trials have resulted in the difference in rootstock response to phylloxera.

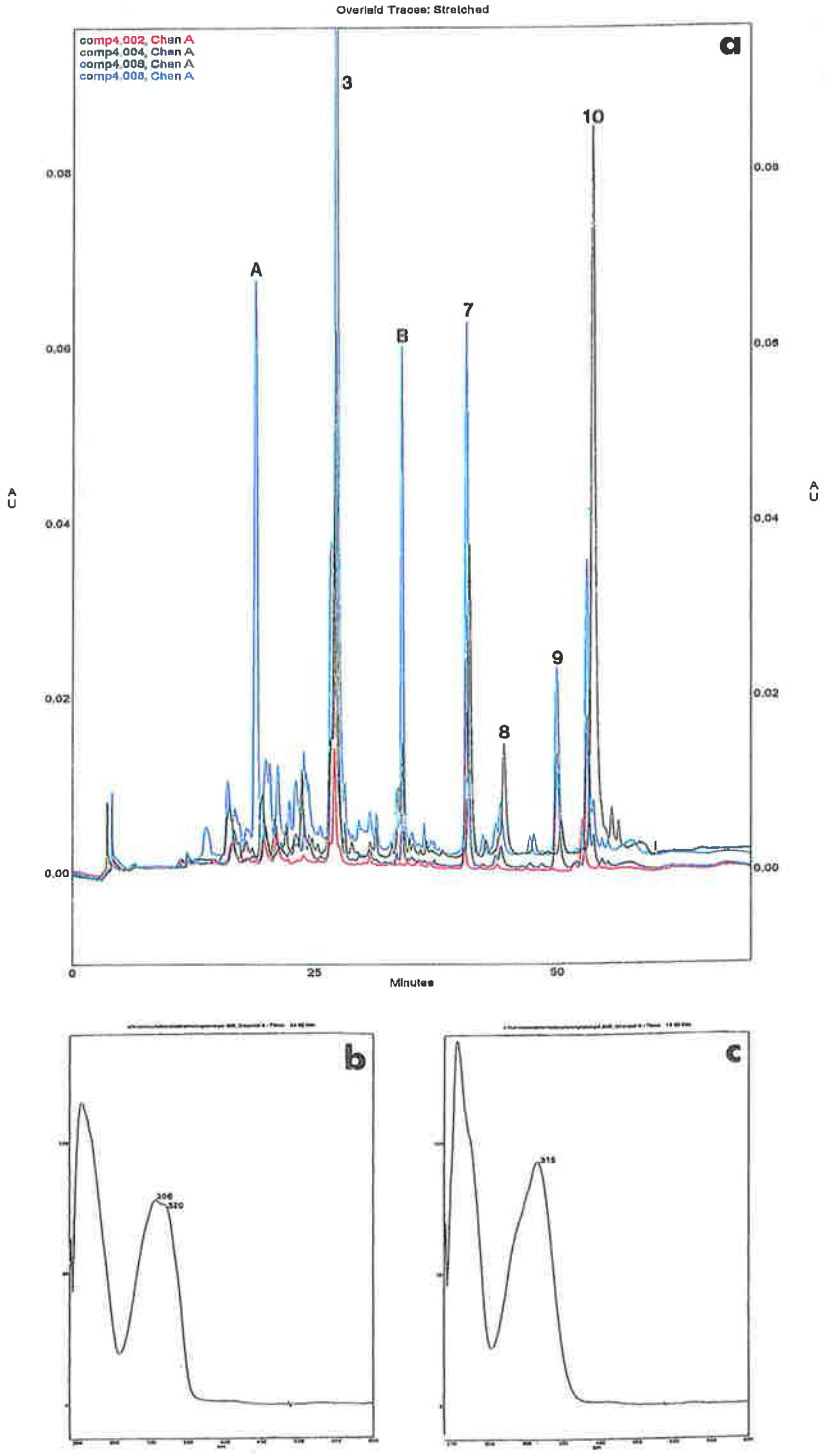


Figure 6.6 a) HPLC trace showing absorption profiles at 330nm for *V. vinifera* uninfested roots (red) and nodosities (green) and Schwarzmann uninfested roots (purple) and nodosities (aqua). Note the presence of two peaks exclusive to the nodosities in the rootstock Schwarzmann (marked A and B). b) Absorption spectrum of peak A. c) Absorption spectrum of peak B.

The Schwarzmann accession used in the pot trial three (WA/A.V.70.2252) was different to the accession used in pot trial five (WA/R317A), but the same as that in the rootstock trial at Stanton and Killeen (J. Whiting, Agriculture Victoria, pers. comm.). When the HPLC profiles from the cocultivation trials were compared, compound B (*trans*-resveratrol) could clearly be detected at a low level in uninfested roots in the extract from pot trial three, but it was not detectable in extract from pot trial five, or from the rootstock trial. This raised the possibility that a secondary pathogen may have been involved in pot trial three, and been responsible for eliciting resveratrol accumulation. This matter remains speculative. In any case, SRU-1 phylloxera was able to become established equally readily on both accessions of Schwarzmann in the pot trials (section 2.4.3, 2.4.5), suggesting that the presence or induction of peaks A and B, while interesting in itself, does not play a key role in the interaction between the rootstock and phylloxera.

6.5 Changes in gene expression in leaves in response to SRU-1 phylloxera attack on roots

Insufficient infested root material was available for northern blot hybridisation analyses, however RNA isolated from leaves of infested and uninfested plants were analysed in an attempt to detect a systemic defence response.

6.5.1 Materials and methods

Leaves were harvested from *V. vinifera* cv. Cabernet Sauvignon vines (either uninfested, or with roots which were heavily infested with SRU-1 phylloxera) from pot trial three (Chapter 2, section 2.4.3). RNA was extracted using extraction method three, and northern blot hybridisation carried out according to methods described Appendix two. Various cDNA clones of defence-related genes (PAL, StSy, VvTL1, VvTL2 and VvPR4a) were used as probes. These clones were used previously, as described in Chapter 4; details of each are given in table 4.4.

6.5.2 Gene expression in leaves

PAL was expressed at a low level in leaves of uninfested vines, and slightly upregulated in infested vines (figure 6.7b), while stilbene synthase was not expressed in either (figure 6.7c). A low level hybridisation signal was detected with the VvTLl probe (figure 6.7d), but it is possible that this was a result of cross hybridisation with the VvTL2 transcript which was expressed more strongly (figure 6.7e). VvTL2 was clearly expressed in leaves of both uninfested and infested vines, and was upregulated in leaves of infested vines. VvPR4a was not expressed in either sample (figure 6.7f).

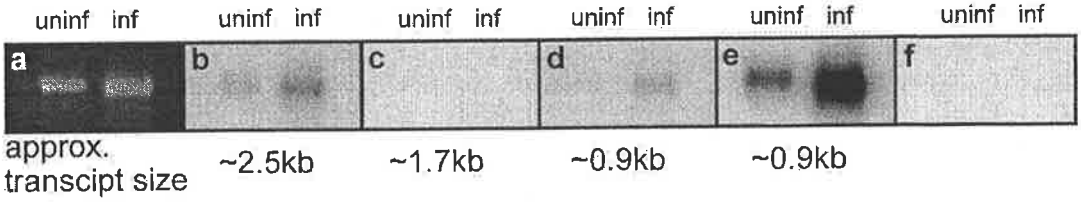


Figure 6.7 Northern hybridisation analyses, with defence-related gene probes, of *V. vinifera* cv. Cabernet Sauvignon leaf total RNA from uninfested and infested plants. For each hybridisation: left lane 10µg uninfested vine leaf RNA, right lane 10µg infested vine leaf RNA. a) Ethidium bromide stained lanes showing relatively even loading of RNA. b) PAL cDNA clone pBS204. c) Stilbene synthase cDNA clone pSV696 d) Thaumatin-like protein cDNA clone VvTL1. e) Thaumatin-like protein cDNA clone VvTL2 f) PR4 type protein cDNA clone VvPR4a.

6.5.3 Discussion of gene expression analyses

Of the defence-related transcripts analysed in this study, VvTL2 was the only one clearly upregulated in leaves in response to phylloxera attack on the root system. VvTL2 is also the only transcript of those investigated with known induction on pathogen invasion of grapevines (Jacobs *et al.* 1999). Interestingly, whilst its expression was upregulated here in leaves in response to SRU-1 phylloxera, it was not upregulated in leaves (data not shown) or roots of VWL-1 phylloxera infested vines (Chapter 4, section 4.3.7). The reason for the discrepancy between leaf northern results is not known (although it may be due to the use of different vine cultivars as well as different phylloxera strains), however the results shown here suggest that *V. vinifera* is, in some cases, capable of responding to the presence of the pest. *V. vinifera* has been demonstrated to be capable of SAR type responses (Busam *et al.* 1997b). It is possible that this defence response may be suppressed in the roots. Alternatively, leaves may express this defence-related gene in response to stress indirectly incurred by the presence of the phylloxera. It should be noted that the roots of the vines studied, while heavily infested, were in relatively good health, and the vines had been infested for only a few months. Stressed roots, have, however been shown to be able to send signals to shoots, causing the shoot to respond even though it is not, itself, under stress (Jackson 1997).

Similar phenomena have been noted in nematode-infested plants by other researchers. Leaves of potato plants infested with the potato cyst nematode *Globodera rostochiensis* accumulated novel proteins and translatable mRNA species, although the roots did not

(Hammond-Kosack 1989, Hammond-Kosack *et al.* 1990). Rahimi *et al.* (1996) also showed that the activity of PR proteins was increased in the leaves, but not roots of potato infected with *G. rostochiensis*. The authors suggested that expression of these PR protein genes may be deliberately upregulated in leaves as part of a strategy by the nematode to enhance host plant defence against other pathogens. Rahimi's results, however, were interpreted by Ohl (1997) as an SAR (systemic acquired resistance) type defence response which was suppressed throughout the roots by the nematode. This type of mechanism was also suggested by Ogallo and McClure (1996), who demonstrated that prior inoculation of tomato with a compatible species of root-knot nematode, *Meloidogyne hapla*, increased the reproductive success by four times relative to controls of challenge *Meloidogyne incognita,* which was normally incompatible with tomato. This 'suppression' hypothesis is supported by observations made by other researchers of down-regulation of a large number of transcripts, including PAL, in nematode-infested roots (Goddijn *et al.* 1993, Williamson and Hussey 1996).

While *V. vinifera* has been shown to be capable of an SAR (Busam *et al.* 1997a, b), suppression of a defence response in roots by phylloxera has not been reported. Omer *et al.* (1995) described reproductive advantages conferred on phylloxera by preformed tuberosities, raising the possibility of suppression of root defence mechanisms at feeding sites, however, as this was a strictly localised effect, any suppression involved could not have been systemic. Northern analysis of VWL-1 phylloxera induced nodosities in this study (Chapter 4, section 4.3.7) did not suggest down-regulation of PAL or other defence-related transcripts compared with uninfested roots. It is more likely then, that reproductive advantages arising from feeding on preformed galls actually results from the preformed nutrient sink established, rather than the suppression of a defence response.

6.6 Discussion

The existence of different phylloxera biotypes was suggested as early as 1914 by Börner (Börner 1914). The implications of this for rootstock resistance ratings have subsequently been confirmed in a number of studies (King and Rilling 1985, Granett *et al.* 1987, King and Rilling 1991, de Benedictis *et al.* 1996) where it has been shown that resistance ratings vary depending on the biotype of phylloxera. In this study, Schwarzmann, which is generally classified as highly resistant to phylloxera (Whiting *et al.* 1987), has proven only tolerant to SRU-1 strain phylloxera. It is clear therefore that rootstock resistance ratings should always be made with specific reference to the biotype of phylloxera used in making this assessment.

Subsequent to this study, a report was published describing a German strain of phylloxera and a range of Hungarian strains from root and leaf galls which are 'adapted' to the rootstocks

SO4 and Teleki 5C, both *V. berlandieri* × *V. riparia* (Kocsis *et al.* 1999). These strains preferentially established feeding sites on primary roots or callus of these rootstocks, and were less aggressive than biotype A phylloxera on *V. vinifera*. These characteristics are similar to those observed here and by Corrie *et al.* (unpublished) for SRU-1 phylloxera, suggesting that strains of this type are not uncommon.

Unlike VWL-1 phylloxera, which was unable to survive on Schwarzmann roots (Chapter 5, section 5.2), SRU-1 phylloxera fed successfully on Schwarzmann primary roots, indicating that it can overcome or otherwise avoid the mechanism which prevented feeding of the VWL-1 strain. The formation of nodosities and the reproductive success of SRU-1 on Schwarzmann further indicated that in at least some rootstocks the physiological mechanisms required for successful gall formation were able to be activated.

In the assays presented here, SRU-1 strain phylloxera established more feeding sites on the roots of Schwarzmann than on *V. vinifera*. This 'improved performance' may have resulted from its ability to induce a stronger photosynthate sink in Schwarzmann, as suggested by the relative extent of starch accumulation. Omer *et al.* (1995) showed that biotype B phylloxera induced tuberosities which improved the fecundity of the less aggressive biotype A. It is possible that this also resulted from formation of a stronger nutrient sink in biotype B-induced galls from which other biotypes could subsequently benefit.

The generation time of SRU-1 phylloxera in tissue culture was considerably shorter than that of VWL-1 phylloxera, even on *V. vinifera*. No correlation was found between this and the extent of starch accumulation in nodosities, which was similar in *V. vinifera* for both phylloxera strains.

The observed response of Schwarzmann roots to SRU-1 phylloxera resulting in accumulation of a blue-white autofluorescence and free *trans*-resveratrol suggests that, despite this interaction being 'compatible' in terms of phylloxera survival and reproduction, the phylloxera has still elicited a response from the roots which might be expected of an incompatible invading pest. Whether the accumulation of the autofluorescent compound(s) and/or *trans*-resveratrol constitute part of phenylpropanoid-based defence response, or whether they are merely indicative of a pest recognition event remains to be determined. A wounding trial described in Chapter five (section 5.4.1) demonstrated that such accumulation is not a general response to wounding.

The accumulation of phenolics must also be involved in the rapid 'suberisation' response of secondary roots of tolerant grapevines which stops, or significantly limits the formation of damaging tuberosities (Boubals 1966a, King *et al.* 1982). Whether the observed

accumulation of phenolics around the feeding site in nodosities of resistant vines reflects the capacity of that vine to produce a rapid suberisation response in secondary roots also remains to be determined. In any case, similar accumulation was not observed in *V. vinifera* in the studies reported here.

Conclusions

The differential resistance of Schwarzmann primary roots to SRU-1 and VWL-1 phylloxera demonstrated in these studies is of great interest and potentially provides a means of dissecting out the basis of phylloxera resistance in this rootstock and other vine varieties. SRU-1 phylloxera may have the ability to withstand the effects of a defence response to which VWL-1 phylloxera is highly susceptible. This could involve the metabolism of potentially toxic phenolic compounds to non-toxic forms by SRU-1 phylloxera. Alternatively, SRU-1 phylloxera, whilst apparently being recognised as a pest, as discussed above, may still be able to limit the scope of the defence response invoked such that this response is insufficient to inhibit gall formation and the growth and reproduction of this phylloxera strain. The ability of phylloxera to suppress natural defence responses was suggested by observations in this study on the interaction with *V. vinifera,* where induction of gene expression of a PR protein was observed in leaves, but not in roots of infested vines. The susceptibility of *V. vinifera* could be further compounded by its general inability to mount a defence response as has been observed in other studies against fungal pathogens (Dai *et al.* 1995a, b).

7. CONCLUSIONS AND FUTURE DIRECTIONS

This study has involved characterisation of some interactions between primary grapevine roots and phylloxera. The development of experimental methods for working with phylloxera and grapevines, and for analysis of the roots was described in Chapters two and three. The interaction between a susceptible vine *V. vinifera* cv. Shiraz and VWL-1 strain phylloxera, as characterised by microscopy, HPLC and northern blot hybridisation analysis, was presented and discussed in Chapter four. The resistance of a number of vine varieties to VWL-1 strain phylloxera was assessed through the use of an *in vitro* cocultivation system as described in Chapter five. These interactions were further characterised by microscopy and HPLC analysis of free phenolics. Finally the interaction between two vine varieties and SRU-1 strain phylloxera was described in Chapter six. Conclusions which can be drawn regarding the grapevine root-phylloxera interaction, by considering, as a whole, the results from Chapters four, five and six are presented below.

Phylloxera galls as nutrient sinks

Phylloxera have been shown to feed from the cortex of the root, not more than seven cell layers below the epidermis. They are able to obtain all their nutrition from a relatively localised region of one or a few cortical cells. Experimental data support the hypothesis that nodosities function as sinks for nutrients including photosynthates, amino acids and amides. It was not possible from this study, however, to determine the strength of this sink relative to that of an uninfested growing root tip.

The mechanism by which such a sink might be induced remains to be elucidated. Several studies have been published suggesting that phloem unloading is under hormonal control. Gershani *et al.* (1980) demonstrated that the application of various hormones, particularly IAA applied to the basal end of cut hypocotyls of *Phaseolus vulgaris,* can enhance movement of sucrose towards the cut end. These effects, observed within three to eight hours, were believed to be the result of positive hormonal feedback. Further, Hayes and Patrick (1985) showed that exogenous application of IAA to stems of *P. vulgaris* could increase photosynthate transport. Blee and Anderson (1998) showed that expression of transcripts encoding sucrose metabolising enzymes was enhanced during mycorrhizal arbuscule formation, but only in arbusculated cells, suggesting they were involved in establishment of a sucrose sink. The authors hypothesised a role for plant growth regulators in the upregulation of this gene expression.

IAA has previously been implicated in the initiation of insect-induced galls (Forrest 1987). It is possible therefore that IAA accumulation is involved in both the initiation of nodosities and

in the establishment of a nutrient sink. A previous study showed, however, that phylloxera preferentially settled on pre-formed tuberosities, but not on similar root swellings induced by exogenous application of IAA (Granett 1990). Further, HPLC analysis of ethanol-soluble root extracts in this study did not detect the presence of IAA. The hypothesis that IAA is involved in sink induction in nodosities thus remains unsubstantiated, but warrants further investigation.

Assuming that enhanced phloem unloading occurs in nodosities, enhanced transport of solutes from the vascular bundle to the root cortex and then to the phylloxera feeding site is possible. This could be facilitated by the reduced suberisation of the endodermis in nodosities as seen in this study. Reduced suberisation could allow apoplastic flow of solutes across the endodermis and /or enhanced symplastic flow, as suberisation of cell walls constricts plasmodesmatal size.

Results presented in Chapters five and six showed some indication of a correlation between short phylloxera generation time and extent of starch accumulation (which is possibly indicative of sink strength), although this needs to be tested more rigorously. This trend was particularly reflected in the Schwarzmann/SRU-1 interaction, characterised by massive starch accumulation and short generation relative to the *V. vinifera*/VWL-1 interaction. Conversely, in the Ramsey/VWL-1 interaction, low rates of survival and reproduction coincided with no detectable starch accumulation.

The inability to detect changes in gene expression in nodosities compared with uninfested roots, as shown by northern blot hybridisation analysis, suggested that if any changes do take place, they might occur only very transiently or are localised to one or a few cells. The use of *in situ* hybridisation and PCR amplification of specific sites (Gurr 1991) would be useful in detecting such localised changes in gene expression.

Plant defence responses and potential mechanisms of resistance

Oxidation of free phenolics

HPLC data suggest that any particular ethanol-soluble phenolic compounds in primary roots which are detectable by this method are not directly involved in the grapevine root-phylloxera interaction. Soluble phenolics may, however, represent only intermediates in the production of oxidised, toxic end products. Resistance of the highly resistant rootstocks *V. riparia* and Börner appears to involve a hypersensitive-like response. The necrosis observed in Ramsey roots could represent a weaker form of the same reaction. The accumulation of an autofluorescent compound(s) at the site of feeding in 'resistant' varieties suggests the involvement of phenolic compounds, possibly stilbenes or their derivatives. It is possible that

observed differences in the presence or activity of oxidising enzymes, for example peroxidases, might be responsible for varietal differences in the rate and extent of necrosis in response to phylloxera attack, rather than the presence of particular phenolic precursors. Analysis of endogenous oxidase activities in the roots of resistant varieties would indicate the potential for this type of mechanism to exist.

The deglycosylation of piceid occurred in nodosities of all varieties, including *V. vinifera* cv. Shiraz, implying that the release of free *trans*-resveratrol probably plays no direct role in a defence mechanism. This deglycosylation might, however, serve as a first step in the oxidative process by supplying the substrate for peroxidase activity. Rhodes and Wooltorton (1978) proposed a model whereby β-glucosidase activity, induced by wounding or pest attack, causes the release of aglycones which are subsequently oxidised to form toxic quinones and the polymeric phenolic compounds responsible for antibiosis and browning respectively (figure 7.1).

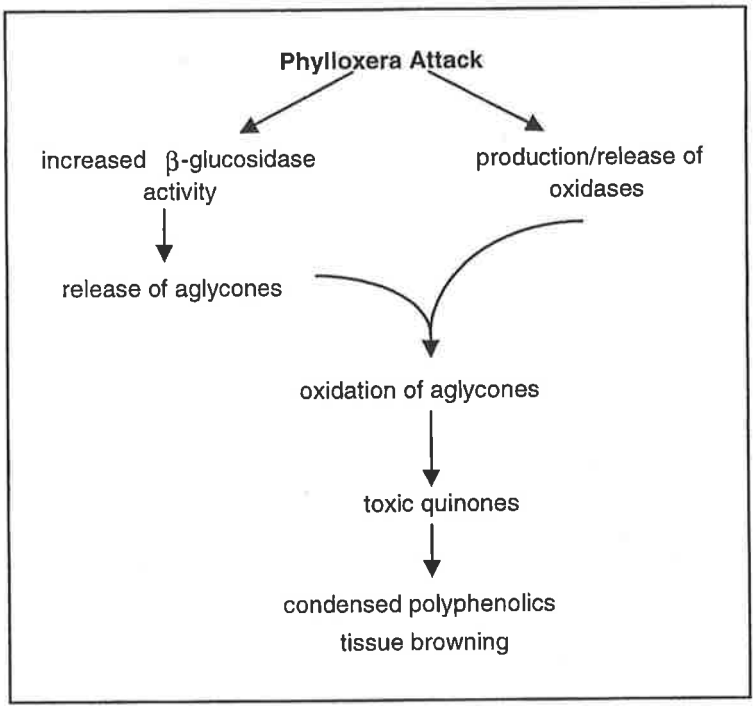


Figure 7.1 Diagram showing a possible pathway leading from insect attack to deglycosylation of phenolic glycosides (e.g. piceid) and production of polyphenolic compounds causing tissue browning and possibly contributing to insect death and tissue necrosis. Based on Rhodes and Wooltorton 1978, Miles 1990.

The failure to detect accumulation of autofluorescent compound(s) at the feeding site in *V. vinifera*, together with the reportedly low constitutive activity of isoperoxidases in this

species (Calderón *et al.* 1992) suggests that *de novo* synthesis and/or metabolism of phenolics, and their oxidation, may not be rapid enough to form an effective defence mechanism. Thus, whilst the steady state levels of free phenolics might be similar in nodosities of *V. vinifera* and resistant varieties (as determined by HPLC in this study), the flux through the pathways involved in synthesis of glycosylated precursors, release of aglycones, and their subsequent oxidation and polymerisation could be much higher in the latter, leading to the resistance response. In the 'compatible' Schwarzmann/SRU-1 interaction, such oxidation or polymerisation steps may be inhibited in some way and/or SRU-1 phylloxera may be insensitive to any toxic compounds such that, while accumulation of an autofluorescent compound was still observed, SRU-1 phylloxera were able to thrive and root necrosis is not observed.

Expression of defence-related transcripts

Lack of upregulation of gene expression observed for a number of PR protein transcripts suggests that PR proteins are not induced in the roots of *V. vinifera* in response to phylloxera attack. Similarly, the expression of genes encoding PAL and stilbene synthase did not appear to be upregulated in *V. vinifera* nodosities, suggesting that this species, which is susceptible to phylloxera, does not mount any defence response in the roots at all. Analysis of the expression of these genes in nodosities of a resistant variety might show more difference in levels of expression.

VvTL2 transcript expression was demonstrated to be upregulated in leaves of *V. vinifera* vines infested with SRU-1 phylloxera. Similar phenomena have been noted in a number of plant-nematode interactions in which defence-related transcripts or proteins are upregulated in leaves but not roots of infested plants (Hammond-Kosack 1989, Hammond-Kosack *et al.* 1990, Rahimi *et al.* 1996). The explanation for this remains unknown, but it suggests the possibility of some type of systemic response to phylloxera in at least some cases. A similar response in leaves was not observed in the *V. vinifera*/VWL-1 interaction (data not shown). Repetition of this experiment might demonstrate whether upregulation of the VvTL2 transcript in leaves is a strain-specific response.

Other potential mechanisms of resistance

The lack of starch accumulation in nodosities formed on Ramsey suggests this variety is unable to respond to signals involved in nutrient sink induction or responds in some other way not identified here. This could act as a mechanism of resistance which, in combination with premature necrosis of nodosities, deters phylloxera feeding.

No evidence was found for the presence of a constitutive resistance mechanism to phylloxera, with the possible exception of *V. rotundifolia,* which showed some characteristics of a non-host. The compounds detected specifically in *V. rotundifolia* i.e. the olive-green stained compound(s) located in the epidermis, and 'compound two' detected by HPLC may have a role in deterrence. Alternatively, it is possible that specific stimulants required by phylloxera for probing as well as for feeding are not present in *V. rotundifolia.*

Implications of biotypes for grapevine resistance

The distinct differences between performance of VWL-1 and SRU-1 phylloxera on Schwarzmann indicate the specificity of the interaction between phylloxera and its hosts. Histochemistry results demonstrated a defence response involving the accumulation of an autofluorescent compound near the feeding site in response to both phylloxera strains, however, SRU-1 phylloxera was apparently not deterred by this response. SRU-1 phylloxera may produce antioxidant compounds which effectively detoxify an oxidative-based defence reaction. Alternatively, Schwarzmann may recognise VWL-1 phylloxera, but not SRU-1 phylloxera, via some form of ligand, and respond with an additional defence reaction, possibly a localised HR.

It is possible that the two strains of phylloxera examined in this study have unique requirements for stimulants to initiate feeding, and this may play a role in the differential responses seen between phylloxera strains on different vine varieties. Unfortunately, the requirements of phylloxera for phagostimulants is as yet unknown. Alternatively, a system involving recognition by the vine of a strain-specific signal which causes the induction of nodosities might exist. This may not be active in the VWL-1/Schwarzmann interaction.

Future directions

There are several different directions in which the research undertaken in this study could proceed. Valuable insights have been gained into many aspects of the grapevine-phylloxera interaction in this study, several of which warrant further investigation, including:

- Ultrastructural investigation of the feeding site and/or use of phloem-mobile tracers

  These could help to determine how phylloxera are able to enhance movement of nutrients to the feeding site within the gall. Ultrastructural investigation of plasmodesmatal size and structure would possibly require the use of freeze substitution or other alternative methods of resin embedding, as standard methods used in this study appear to be incompatible with the tissue surrounding the feeding site.

- Further investigation of the nodosity as a nutrient sink.

  A simple investigation of the timing and development of starch deposition could help elucidate the importance of this phenomenon for phylloxera development. Relative sink strength could be investigated through the use of radiolabelled CO_2 experiments (e.g. Thorpe *et al.* 1998), which would allow comparison of competition for photosynthates between a healthy growing tip and nodosity. The role of IAA in regulation of sink strength could be tested directly with such an experimental system. Analysis of the expression of sucrose synthase and other sucrose metabolism or starch synthesis-related genes might also help elucidate the mechanism by which sink establishment occurs.

- Use of *in situ* hybridisation, immunohistochemistry or PCR techniques to investigate localised patterns of gene expression within a nodosity, particularly in resistant varieties.

- Investigation of the oxidative potential of roots of resistant varieties.

  Assays of both constitutive and potentially induced levels of activity of PPO or peroxidase (particularly 4-hydroxystilbene-oxidizing isoperoxidases) would give an indication of the potential for an oxidative-based defence response in resistant varieties.

- Development of an artificial diet for phylloxera.

  This could be used to investigate possible roles for endogenous compounds in antibiosis or non-preference. For example purification and characterisation of 'compound two' from *V. rotundifolia* could be followed by screening using an artificial diet. Use of an artificial diet would also be valuable as a preliminary step for screening potentially bioactive compounds in the development of transgenic resistant vines.

Some of these experiments require specialised laboratory facilities within a phylloxera-infested regions (i.e. within a quarantine zone), as fresh infested root material and/or live phylloxera are involved.

APPENDIX ONE - MAJOR REACTIONS IN THE STRESS-INDUCED PHENYLPROPANOID PATHWAY IN PLANTS

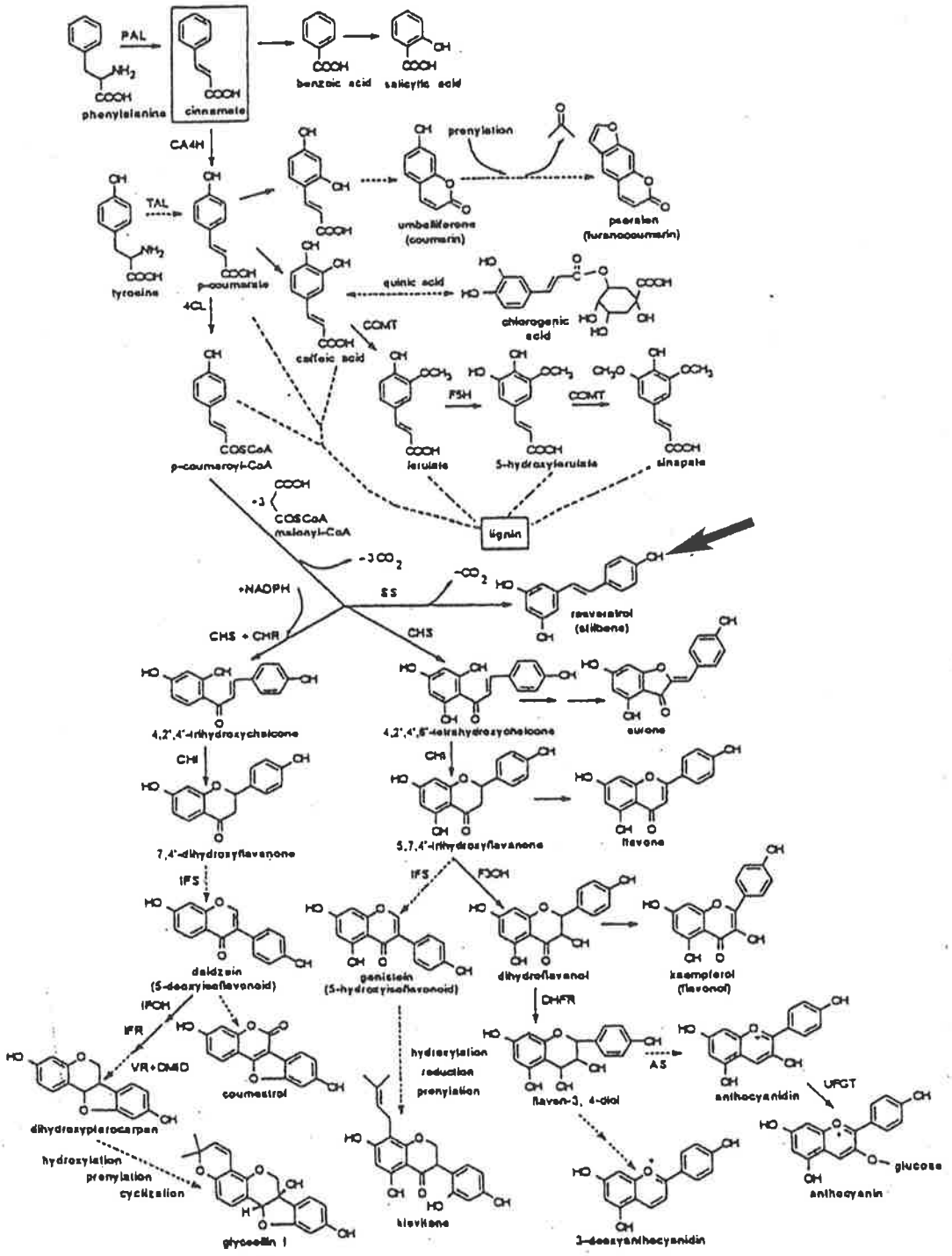


Figure A1.1 Diagram of part of the stress-induced phenylpropanoid pathway in plants from Dixon and Paiva (1995). Arrow indicates the stilbene resveratrol. This compound is the precursor for stilbenes and stilbenoids in grapevine.

APPENDIX TWO - BIOCHEMICAL AND MOLECULAR METHODS

β-glucosidase treatment of HPLC extracts

HPLC extracts were prepared using extraction method one (Chapter 3, section 3.2.1). 50µl extract was dried down under vacuum and resuspended in 50mM sodium acetate pH5.2. To each extract 10µl β-glucosidase (from almond, Sigma) was added (as 0.5U/µl stock solution in 50mM sodium acetate pH 5.2). Extracts were incubated at 37°C overnight, then dried once again under vacuum. Pellets were resuspended in 50µl 95%(v/v) ethanol, centrifuged for 15min at 24500g. 25µl extract was diluted into 475µl solvent A and separated by HPLC using chromatography method nine (Chapter 3, section 3.2.2).

Soluble protein extraction and SDS-PAGE

Extraction of soluble proteins was as follows. Extraction buffer: 100mM TrisCl pH 8.0, 10% (v/v) glycerol, 0.5% (v/v) Tween-20, 0.1g/ml PVPP, 5mM NaDIECA, 2mM DTT. Frozen root tissue was ground to a fine powder in a mortar and pestle and extracted in 10ml buffer per gram tissue. After mixing powder with buffer, the extract was transferred to a centrifuge tube and centrifuged at 12000g, 4°C for 30 min The supernatant was retained and stored at -70°C.

Proteins were separated by SDS-PAGE as adapted from Fling and Gregerson (1986). Buffers were as listed. Separation buffer: for 40ml add in the following order 9.7ml Acryl/bis (Acrylamide 50g: bis-acrylamide 1.35g), 10.2ml H_2O, 19.7ml Tris pH 8.8, 400µl 10% (w/v) SDS, 24µl TEMED, 200µl 10% (w/v) Ammonium persulphate (AMPS). Stacking buffer: for 20ml add in the following order 2ml Acryl/bis, 15.1ml H_2O, 2.5ml 1M Tris pH 6.8, 200µl 10% (w/v) SDS, 15µl TEMED, 200µl 10% (w/v) AMPS. Running buffer: in 5L add 30.5g Tris, 71.3g Glycine, 5g SDS. 5X sample buffer: in 20ml add 1.542g DTT, 2.0g SDS, 8ml glycerol, 6ml 1M TrisCl pH 6.8, bromophenol blue as required. Protein samples were denatured by boiling with sample buffer for 3-5 min, loaded and electrophoresed at 20mAmps for 1 hr, then the gel stained with Coomassie blue.

Extraction of free amino acids

0.5g frozen root or gall tissue was ground under liquid nitrogen in a mortar and pestle and placed in a 2ml eppendorf tube. The following was added to the tube: 600µl methanol, 250µl chloroform, 150µl MilliQ water, 100µl 1% (w/v) homoarginine hydrochloride in MilliQ water (internal standard). Each tube was shaken briefly then placed on a rotating

wheel for 20 min. Tubes were centrifuged at 24000g for five min at 4°C. The aqueous phase was transferred to a fresh tube. 100ml was transferred to a fresh tube, diluted 1:5 in 0.0131% (w/v) carboxymethyl cysteine (2nd internal standard) in borate buffer (pH 8.5) and centrifuged at 23500g for 30min

Supernatant was analysed by Holger Gockowiak at AWRI laboratories, Waite Campus, Adelaide. The HPLC system was as follows: Hardware configuration: GBC Aminomate HPLC comprising LC1150 Multisolvent Delivery System (with Column Oven), LC1650 Advanced Autosampler & External Needle Wash Station Option, LC1250 Fluorescence and LC 1210 UV/Vis Detectors. WinChrom Chromatography Management System, on-line vacuum degasser.

HPLC Conditions: Hypersil column for amino acid analysis 150mm × 4.6mm internal diameter, Solvent A 30mM ammonium phosphate (pH 6.5) in 15% (v/v) aqueous methanol, Solvent B 15% (v/v) aqueous methanol, Solvent C 90% (v/v) acetonitrile.

Solvent gradient:

Time (min)	%A	%B	%C
0.00	17.00	68.00	15.00
32.00	10.80	43.20	46.00
32.05	0.00	0.00	100.00
33.50	0.00	0.00	100.00
35.00	17.00	68.00	15.00

Equilibration time 5 min, flow rate 1.00ml/min, temperature 45°C, injection volume 10µl. Derivatising reagent: 6mg 9-fluorenylmethyl chloroformate in 1.5 ml acetonitrile. Cleavage reagent: 680µl 0.85M NaOH, 3.4mM EDTA, 300µl 0.5M hydroxylamine hydrochloride, 20µl 2-(methylthio)-ethanol. Quench reagent: 0.5ml acetonitrile.

RNA extraction and analysis

RNA extraction method one

This is a lithium dodecylsulphate based method modified from Levi *et al.* (1992). It was published for extraction of RNA from Pecan fruits and leaves, and is designed to suppress the interaction of polyphenols with RNA.

The extraction buffer contains 1.5% (w/v) lithium dodecylsulphate (Sigma), 300mM LiCl (BDH), 1.5% (v/v) Nonidet P-40 (Sigma), 1.5% (w/v) sodium deoxycholate, 200mM Tris

pH 8.5. This mixture was autoclaved, then the following added fresh before use: 10% (w/v) soluble PVP, 2% (w/v) insoluble PVP, 1% (v/v) 1M DTT, 0.5% (v/v) β-Mercaptoethanol (β-Me).

The extraction procedure was as follows: 1g of tissue was frozen in liquid nitrogen, ground to a fine powder, and mixed with 5ml extraction buffer in a 15ml centrifuge tube. This mixture was extracted for 5-10 min with 5ml chloroform, until milky in appearance, then centrifuged at 1075g, 4°C for 25min, after which the aqueous layer was re-extracted with 5ml chloroform. The aqueous layer was removed, and 1/10 vol. 3M NaCl, then 2 vol. ice-cold ethanol added. After 2-3 hr at -20°C, the mixture was centrifuged at 4300g, 4°C for 15 min. The pellet was resuspended in 1.5ml TE1 (50mM Tris HCl, 10mM EDTA, pH 8.0) and transferred to an eppendorf tube. This was centrifuged at 12000g for 10 min. The supernatant, containing RNA, was transferred to a new tube and RNA precipitated with 1/10 vol. 3M NaCl plus 2 vol. isopropanol at -20°C for 1 hr. RNA was pelleted by centrifuging at 14000g for 10 min, and the supernatant discarded. The pellet was washed in cold ethanol and resuspended in 300µl TE1. RNA was precipitated with 100µl 8M LiCl at 4°C overnight, and pelleted by centrifuging as above. The RNA pellet was resuspended in 300µl TE1, precipitated with 450µl KAc for 5 hr on ice, and pelleted by centrifuging. Pellet was resuspended in 300µl TE1, precipitated with 30µl 3M NaCl, and 660µl EtOH, for 1 hr at -20°C, and pelleted as above. Pellet was rinsed with 70% EtOH, centrifuged and air dried before resuspending in TE. This final suspension was centrifuged as above for 5 min, and the supernatant transferred to a fresh tube.

RNA extraction method two

This is a lithium dodecylsulphate/sodium deoxycholate based method adapted from Pawlowski *et al.*(1994). It has been successfully used for isolation of RNA from *Casuarina* roots which contain high levels of polyphenolic compounds.

The extraction buffer contains 200mM Tris-HCl pH 8.5, 300mM LiCl, 10mM EDTA, 3% (w/v) LiDS, 2% (w/v) Sodium deoxycholate, 2% (v/v) NP-40. This was autoclaved then the following added fresh to 50ml extraction buffer before use: 3g polyclar AT (Serva), 90µM β-Me and 25mM DTT. The buffer was stirred on ice.

The extraction procedure was as follows: 4g of tissue was frozen in liquid nitrogen, and ground to a fine powder in a mortar and pestle. The frozen powder was sprinkled into 50ml extraction buffer and stirred for a further 10 min. 42ml 3M ammonium acetate (containing 0.4mM EDTA, 0.36mM NaOH) was added and mixed. Cell debris was pelleted by centrifuging at 5000g, 4°C for 25 min, and the supernatant filtered through Miracloth™.

Nucleic acids were precipitated by adding 1/10 vol. 3M sodium acetate (pH not adjusted) and one vol. isopropanol, and pelleted by centrifuging as above. Pellet was resuspended in 20ml H_2O, and extracted with phenol/chloroform (1:1) until no interphase was left, then extracted once with chloroform. RNA was precipitated with 1/10 vol. 3M sodium acetate (pH 5.2) and 2.5 vol. EtOH at -20°C overnight, centrifuged, and resuspended in 200µl TE.

RNA extraction method three

This is a sodium perchlorate based method adapted from Davies and Robinson (1996). It was designed for extraction of RNA from grape berries, but has since has been successfully used on all tissues of grapevine.

16ml of extraction buffer contains 0.3M Tris-HCl pH 8.3 (from 3M stock solution), 2% (w/v) PEG 4000, 5M Na perchlorate (from 9M autoclaved stock solution), 1% (v/v) SDS (from 10% (w/v) stock solution), 8.5% (w/v) insoluble PVP, 1% (v/v) β-Me, 1ml H_2O all added in the given order to an autoclaved beaker with a rapidly stirring flea.

The extraction procedure was as follows: 4g of tissue was frozen in liquid nitrogen, and ground to a fine powder in a mortar and pestle. The powder was added to stirring buffer (16ml), and stirred at room temperature for 30 min. While stirring, 10ml disposable syringes, with the plunger removed (two per 16ml buffer) were packed with a glass wool plug and supported in 50ml polypropylene centrifuge tubes, sterilised with 0.1N NaOH, and rinsed four times with sterile water. The extract was poured into the syringes in fresh 50ml centrifuge tubes, and centrifuged for 10 min at 500rpm in a swing bucket bench top centrifuge. Supernatant was transferred to 30ml centrifuge tubes (Nalgene), and RNA was precipitated with 2.5 vol. ethanol at -20°C for 20 min, then pelleted by centrifugation at 7650g. The pellet was washed with 70% (v/v) EtOH and air dried. It was resuspended in 1ml TE pH 8.0, 0.2% (v/v) β–Me and extracted twice with phenol/chloroform (1:1) and once with chloroform. RNA was precipitated with 1 vol. 3M sodium acetate pH 5.3, 2.5 vol. ethanol at -20°C for 20 min. RNA was pelleted by centrifuging as above, and the pellet rinsed with 70% (v/v) ethanol, air dried, and resuspended in 300µl TE pH 8.0.

RNA extraction method four

This method used Trizol Reagent (Gibco Life Technologies) as recommended by the manufacturer except that tissue (250mg) was ground to a fine powder under liquid nitrogen in a mortar and pestle, then the powder was mixed in 2ml Trizol solution and incubated at 20°C for 5 min. The solution was extracted with 0.4ml chloroform by shaking vigorously for 15 sec then incubating at 20°C for 3 min. Samples were centrifuged at 12000g, 4°C for

15 min. Aqueous phase was transferred to a separate tube and precipitated with 0.5 vol. isopropanol for 10 min, then centrifuged as above. Pellet was rinsed with 2ml 75% (v/v) ethanol, then centrifuged at 7500g, 4°C for 5 min. Pellet was air dried and resuspended in 100µl H_2O.

For all extraction protocols, RNA yield and quality was measured by absorbance at 260nm and 280nm using a UV spectrophotometer (Beckman DU5). Integrity of RNA was further assessed by electrophoresis in a denaturing formaldehyde-agarose gel, and staining with ethidium bromide.

Denaturing formaldehyde-agarose gel electrophoresis

RNA was separated by denaturing formaldehyde agarose gel electrophoresis as adapted from Ausubel *et al.* (1999), as follows: 5X MOPS buffer: 0.1M MOPS pH 7.0, 40mM Na acetate, 5mM EDTA pH8.0. Denaturing gel: 1.5% (w/v) DNA grade agarose (Progen), 6.5% (v/v) formaldehyde, 1X MOPS buffer. Sample buffer: 1 part 37% (v/v) formaldehyde, 2 parts 5X MOPS buffer, 1 part loading dye (glycerol/TE 1:1 plus 0.05% (w/v) bromophenol blue), 10 parts deionised formamide (ethidium bromide (1µl/ml of 10µg/ml stock solution) was optional). Samples (10µg each) were made up to 10µl with RNase free water, mixed 1:1 with sample buffer and denatured at 65°C for 15 min then chilled on ice. The gel was loaded including RNA molecular weight markers (Gibco BRL) and electrophoresed at 7 volts/cm at 4°C.

Northern blot hybridisation

The gel was rinsed in 5X SSC for 5 min then blotted onto Hybond N (Amersham) nylon membrane overnight according to the manufacturer's instructions. The blot was rinsed in 5X SSC then air dried before UV crosslinking using a Biorad UV crosslinker.

Membranes were prehybridised in 5X SSC, 0.5% (v/v) SDS, 5X Denhardts reagent, 100µg/ml freshly denatured, sheared salmon testes DNA (Sigma) for at least 1 hr at 65°C. cDNA probes were labelled using a Megaprime labelling kit (Amersham) according to the manufacturer's instructions. Radioactive isotope was α-^{32}P dCTP (Bresatec (Geneworks) cat. no. ADC-2). Hybridisation was in the same solution as prehybridisation at 65°C overnight. Blots were washed at 65°C twice in 2X SSC, 0.1% (w/v) SDS, once in 1X SSC, 0.1% (w/v) SDS and once in 0.1X SSC, 0.5% (w/v) SDS. Blots were exposed using a Molecular Dynamics phosphorimager, and images converted to tiff files for preparation of figures.

Enzymatic Starch Assay

Insoluble starch content of uninfested roots and nodosities harvested from potted vines cocultivated with phylloxera was determined using an enzymatic assay (K. Trafford, John Innes Institute, UK, pers. comm.) as follows. Each tissue type was sampled in duplicate and each sample was assayed in duplicate to ensure the reliability of the assay method. 100mg frozen root tissue was ground to a fine powder in a mortar and pestle. The powder was mixed with 1.5ml 80% (v/v) aqueous ethanol, and kept on ice for 20 min The extract was centrifuged at 3000g for 10min, and the supernatant discarded. The pellet was washed by resuspending in 2ml 80% (v/v) aqueous ethanol and centrifuging as above. This wash was repeated once. The pellet was then resuspended in 2ml H_2O and the volume of the total measured accurately. Duplicate 0.5ml aliquots were placed in screw-capped eppendorf tubes and the starch was solubilised by autoclaving at 121°C for 15 min. To each tube the following was added: 0.5ml 100mM Na acetate pH 5.2, 2µl α-amylase (Boehringer cat. no. 161 764) at 10 mg/ml, 8µl amyloglucosidase (Sigma cat. no. A-3514) at 14U/ml, followed by incubation at 37°C for 4 hr ('digested starch'). The resulting glucose concentration was determined spectrophotometrically by mixing the following in a 1ml cuvette: 925µl (100mM Bicine pH7.7, 5mM $MgCl_2$, 0.5mM NAD), 50µl 20mM ATP, 2.5ml hexokinase (Boehringer cat. no. 1426 362) at 200U/ml and 25µl 'digested starch'. The OD was read at 340nm and 2.5µl glucose 6-phosphate dehydrogenase (Sigma cat. no. G8404) at 1000U/ml added to the cuvette. After mixing, the OD was monitored at 340nm until stable. The initial starch concentration was calculated as follows: ΔOD×1/6.22=µmol glucose per 25µl digested starch aliquot×dilution (from total measured volume of starch and water). 1µmol glucose=162µg starch.

APPENDIX THREE - MICROSCOPY METHODS

Preparation of samples for GMA embedding and semi-thin sectioning

Root samples 1cm in length were fixed in FAA (5 parts formaldehyde; 5 parts acetic acid: 90 parts ethanol) or glutaraldehyde fixative (3% (v/v) glutaraldehyde in 0.2M phosphate buffer pH 7.0) for at least 48 hr. They were dehydrated through alcohol series as follows: ethanol 2 hr, propanol 2 hr, butanol 2 hr. Samples were embedded in GMA (2-hydroxyethyl methacrylate, 93mls; polyethylene glycol 400 7mls; benzoyl peroxide 0.6g) as follows: 1part butanol:1 part GMA 2 hr, GMA 2 days, fresh GMA for a further 2 days, fresh GMA in gelatine capsules, heated to 60°C, two days or until set. Sections of 4-5 μm were cut using a Reichart-Jung Supercut microtome fitted with a glass knife.

Preparation of samples for araldite resin embedding and ultrathin sectioning for TEM

Root samples 2mm in length were fixed in 4.0% (v/v) glutaraldehyde, 1.25% (w/v) paraformaldehyde, 4% (w/v) sucrose in PBS pH7.2, for 4-6 hr then washed twice in PBS, 4% (w/v) sucrose for 30 min each wash. Samples were post fixed in 1% (w/v) OsO_4 in PBS for 2 hr. They were then dehydrated through the following alcohol series: 70% (v/v) ethanol 3×20 min, 90% (v/v) ethanol 3×20 min, 95% (v/v) ethanol 3×20 min, 100% ethanol 3×20 min, 100% ethanol 1×60 min Samples were then embedded in Procure araldite-epoxy resin as follows: 1 part ethanol:1 part resin 8 hr, 100% resin 3×8 hr, embed in fresh 100% resin at 70°C in a vacuum oven.

Survey sections of 0.5μm were cut with a Reichart-Jung Ultracut microtome fitted with a glass knife, or ultrathin sections (70nm, for TEM) with a Reichart-Jung Ultracut microtome fitted with a diamond knife.

Light Microscopy

Light microscopy was carried out with an Olympus BH-2 compound microscope fitted with BH2-RFCA excitation cube. UV excitation filters: 365nm excitation plus 400nm barrier. Blue light excitation filters: 420nm excitation plus 515-560nm barrier.

Stains and staining schedules

Aniline blue

Purpose: Callose fluoresces yellow under UV or blue light (O'Brien and McCully 1981; Sedgley 1982).

Stain: 0.1% (w/v) aniline blue was dissolved in potassium 0.1N phosphate buffer [for 100ml: 0.1g aniline blue, 0.69g K_3PO_4], mixed overnight and filtered, adjusted to pH 11.0 with KOH, stored at 4°C, in the dark (Sedgley 1982).

Slides were mounted in aniline blue stain and observed immediately under UV excitation.

Autofluorescence

Purpose: *Trans*-resveratrol fluoresces blue-white under UV excitation (Dai *et al.* 1995a). Lignin fluoresces light blue, suberin fluoresces royal blue (O'Brien and McCully 1981). Terpenoid napthols and some phenolics fluoresce green-yellow (Dai *et al.* 1995c).

Slides were mounted unstained in either 70% (v/v) glycerol or surgipath mounting medium and observed under UV excitation (Dai *et al.* 1995a,b).

Auramine O

Purpose: Aphid stylet tracks fluoresce yellow under blue light (460nm) excitation (Cook and Davies 1994). Epidermis, endodermis and xylem also fluoresce yellow.

Slides were mounted in the stain (0.01% (w/v) aqueous solution) and observed under blue light excitation (Cook and Davies 1994).

DMACA

Purpose: Flavan-3-ols (proanthocyanidins) stain blue (Gutmann and Feucht 1991).

Stain: Make up 0.5M sulphuric acid in 1-butanol. After cooling, add 0.1% (w/v) *p*-dimethylaminocinnamaldehyde and dissolve.

Slides were placed in glass petri dish and covered with 2ml stain. They were heated in a microwave oven (next to a bowl of water) until the solution started to boil (about 10 sec), removed and rinsed repeatedly in absolute ethanol. Slides were dried and mounted in surgipath mounting medium and observed with phase contrast optics. Adapted from Gutmann and Feucht (1991).

Neu's reagent

Purpose: Flavonoids fluoresce yellow at 365nm, lemon yellow at 420nm; gallic acid derivatives fluoresce blue at 365nm (Dai *et al.* 1995a,b).

Stain: 1% (w/v) 2-aminoethyldiphenyl borinate (Fluka) in absolute methanol.

Slides were immersed in stain for 1-5 min. Excess stain was drawn off taking care not to the lose sections. Slides were mounted in the stain, and observed under UV excitation. Adapted from Dai *et al.* (1995a,b).

Periodic Acid/Schiff's reagent with Toluidine Blue O counterstain (PAS/TBO)

Various compounds are stained as follows: Cell wall polysaccharides and starch red or magenta; callose and cellulose are not stained, some phenolics red, polyphenolic compounds blue to blue-green, lignin aqua, starch red and primary cell walls (pectins) pink (O'Brien and McCully 1981).

Solutions: 2,4-DPH - 0.5g 2,4-dinitrophenylhydrazine was dissolved in 15% (v/v) acetic acid, mixed for 1hr at RT and filtered; sodium metabisulphite solution - 5ml 10% (w/v) sodium metabisulphite, 5ml 1N HCl, 90 ml H_2O.

Slides were stained in 2,4-DPH for 30 min then rinsed for 1 hr in running water. They were stained in 1% (v/v) periodic acid for 30 min and rinsed in running water for 5 min; placed in Schiff's reagent (BDH) for 1 hr followed by three 2 min washes in sodium metabisulphite solution, then rinsed with ddH_2O. Finally they were counter stained with TBO (see below) for 5 min, then rinsed in running water till the colour was washed out of the background resin (1-7 hr). Slides were dried and mounted in Surgipath mounting medium, and observed under bright field. From O'Brien and McCully (1981).

Phloroglucinol-HCl

Purpose: Lignin is stained pink, 'wound gum' red (Jensen 1962).

Stain: 10g phloroglucinol dissolved in 95ml absolute ethanol.

Slides were immersed for 5 min in the stain. They were quickly flooded with 50% (v/v) HCl, excess drained off and the slide mounted in stain and coverslip sealed. The slide was immediately examined under bright field with polarising optics. Adapted from Dai *et al.* (1995a,b).

Sudan black

Purpose: Lipid containing tissues are stained blue/black (Bronner 1975).

Stain: A fresh saturated solution is needed. 0.5g Sudan Black was dissolved in 150ml 70% (v/v) ethanol. The solution was kept in a closed container at 37°C for 12 hr then filtered. It was placed at 60°C for 30 min in a closed container before being used.

Slides were laid flat and flooded with stain. They were incubated at 60°C for 30 min then rinsed clear in 70% (v/v) ethanol. The sections were dried and slides mounted in surgipath mounting medium, and observed under bright field. From Bronner (1975).

TBO

For GMA sections:

Purpose: Stains RNA purple, DNA blue/blue-green, pectic acid red or reddish purple, polyphenols and lignin green or blue-green (O'Brien and McCully 1981).

Stain: 0.05% (w/v) toluidine blue in benzoate buffer, pH 4.4 (0.29g sodium benzoate, 0.25ml benzoic acid, 200ml H_2O), mix for 30 min, stand overnight, filter.

Sections were stained for 1-2 min, then rinsed clear in running water (several hours). Slides were mounted in Surgipath mounting medium and observed under bright field.

For araldite sections:

Purpose: Stains most features purple (O'Brien and McCully 1981).

Stain: 0.5% (w/v) Toluidine Blue O in 0.1% (w/v) sodium carbonate (pH 11.1), filtered.

Sections were stained on a hotplate for 30 sec, then rinsed in running water. Slides were mounted in Surgipath mounting medium and observed under bright field.

Both from O'Brien and McCully (1981).

Transmission electron microscopy

Ultrathin sections (70nm) were collected onto copper grids and stained for 20 min in a saturated solution of uranyl acetate in 70% (v/v) ethanol in the dark. They were rinsed extensively in 50% (v/v) ethanol followed by distilled water, and dried. Sections were then stained in a saturated solution of lead citrate in a CO_2 free environment for 5-10 min, washed extensively in distilled water, then dried.

Transmission electron microscopy was with a Philips 420 TEM run at 80kV.

APPENDIX FOUR - COCULTIVATION OF VWL-1 PHYLLOXERA WITH EXCISED ROOT CULTURES

Grapevine varieties

V. vinifera cv. Shiraz accession PT23/A.N.61.0020, Ramsey accession A11V2/I.V.63.2065, Börner, *V. rotundifolia.*

Phylloxera strain

VWL-1 collected from *V. vinifera* roots, Brown Brothers Whitlands vineyard, King Valley, Victoria. Population multiplied on excised *V. vinifera* secondary root pieces.

Cocultivations

1. *V. vinifera* cv. Shiraz, Börner, *V. rotundifolia* with VWL-1 phylloxera (table A4.1).
2. *V. vinifera* cv. Shiraz, Ramsey, Börner, *V. rotundifolia* with VWL-1 phylloxera (table A4.2).

Table A4.1 Survival of VWL-1 phylloxera life stages and nodosity formation on excised root cultures of three vine varieties. Twelve roots of each variety were initially inoculated with ten surface-sterilised eggs.

			mean no. live phylloxera at each developmental stage 25 days after inoculation					
vine variety	**no. eggs hatched (±SD)**	**nodosities formed (±SD)**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults**	**total no. survivors (±SD)**
V. vinifera cv. Shiraz	4.00 ± 1.91	0	0.42	0.33	0	0	0.08	0.83 ± 1.03
Börner	5.58 ± 2.78	0	0.67	0.42	0	0	0	1.09 ± 0.87
V. rotundifolia	5.66 + 2.19	0	0.08	0.08	0	0	0	0.16 ± 0.29

Table A4.2 Survival of VWL-1 phylloxera life stages and nodosity formation on excised root cultures of four vine varieties. Twelve roots of each variety were initially inoculated with ten surface-sterilised eggs.

			mean no. live phylloxera at each developmental stage 8 days after inoculation[1]					
vine variety	**no. eggs hatched (±SD)**	**nodosities formed (±SD)**	**1st instar**	**2nd instar**	**3rd instar**	**4th instar**	**egg laying adults**	**total no. survivors (±SD)**
V. vinifera cv. Shiraz	5.58 + 2.10	0	2.90	0	0	0	0	2.90 ± 2.30
Börner	4.42 ± 1.73	0	1.50	0	0	0	0	1.50 ± 1.31
V. rotundifolia	4.42 ± 1.88	0	1.25	0	0	0	0	1.25 ± 1.36
Ramsey	5.33 + 1.50	0	1.83	0	0	0	0	1.83 ± 1.46

[1]Contaminated medium meant this cocultivation was aborted after eight days. This period is short enough for phylloxera to survive without feeding.

APPENDIX FIVE - RETENTION TIMES AND SPECTRAL CHARACTERISTICS OF HPLC STANDARDS AND RESVERATROL DERIVATIVES

Spectral characteristics and retention times of commercially available standard phenolic compounds separated using chromatography method nine as described in Chapter 3, section 3.2.2 (table A5.1). Spectral characteristics of resveratrol derivatives from published data (table A5.2). Absorption spectrum of chlorogenic acid (figure A5.1) and of ferulic acid (figure A5.2). Spectra were obtained using chromatography method nine, and are shown to illustrate typical spectra of cinnamic acids.

Table A5.1 Absorption characteristics and retention times of free phenolic acids and other commercially available standard phenolic compounds obtained using HPLC chromatography method nine.

Compound	Absorption maxima (nm)					Retention time (min)
acetylsalicylic acid	200		229	277		27.93
benzoic acid	199		229			27.78
caffeic acid	205	217	(224)[1]	(300)	322	19.50
chlorogenic acid	205	217	(224)	(300)	325	18.79
cinnamic acid	205	216		277		36.93
m-coumaric acid	205		(227)	277	325	30.27
o-coumaric acid	211		(231)	279		27.25
p-coumaric acid	207		(225)		308	23.91
ferulic acid	216		234	(296)	323	25.93
gentisic acid	211		(237)		329	17.41
gallic acid	215		270			6.84
IAA	217		280			30.47
protocatechuic acid	205	(216)	261	292		12.19
quercetin	203		254		371	36.70
quercetin glucoside	205		255		352	unbound[2]
***trans*-resveratrol**	216		307	318		34.03
rutin	205		256		354	27.82
salicylic acid	205		237	303		31.20
sinapic acid	206		225	310		26.36
syringic acid	216		275			20.05
vanillic acid	206	217	260	290		18.72
vanillin	205	230	279	309		22.13

[1]Numbers in parenthesis represent the position of shoulders on absorption spectra.

[2]Did not bind to column. Absorption maxima obtained from peak eluting at start of HPLC run.

Table A5.2 Absorption characteristics of resveratrol derivatives from published data.

Compound	Absorption maxima (nm)						Reference
ampelopsin A		(230)[1]	280				Mattivi and Reniero 1996
gnetin H		224	284	326			Mattivi and Reniero 1996
heyneaol A			284	320			Li *et al.* 1996
hopeaphenol		(228)	280				Mattivi and Reniero 1996
pterostilbene			307-8				Jeandet *et al.* 1997
***cis*-resveratrol**		(230)	282				Mattivi and Reniero 1996
***trans*-resveratrol**	218	(235)	(297)	306	320	(336)	Langcake and Pryce 1977
***trans*-resveratrol glucoside**		(230)		304	316		Mattivi and Reniero 1996
***cis*-resveratrol glucoside**		(230)	284				Mattivi and Reniero 1996
***trans*-4-OH-stilbene**		224	300	312			Mattivi and Reniero 1996
α-viniferin		(225)	(281)	286	(293)		Pryce and Langcake 1977
c-viniferin		224			322		Mattivi and Reniero 1996
***trans*-ε-viniferin**		224	(286)	310	324	(354)	Langcake and Pryce, 1977
***cis*–ε-viniferin**	(262)	274	283	(294)	(319)		Langcake and Pryce 1977
r-2-viniferin		(224)	285.4		329		Korhammer *et al.* 1995
r-viniferin		(226)	287		322.4		Mattivi and Reniero 1992
r-viniferin		(226)	287		326		Mattivi and Reniero 1996
unknown oligostilbene a		(228)	282		(320)		Mattivi and Reniero 1996
unknown oligostilbene b		(224)	(286)		326		Mattivi and Reniero 1996
unknown oligostilbene c		222	286		326		Mattivi and Reniero 1996
unknown oligostilbene d		(228)	284				Mattivi and Reniero 1996
unknown oligostilbene e		(228)	280				Mattivi and Reniero 1996

[1]Numbers in parenthesis represent the position of shoulders on absorption spectra.

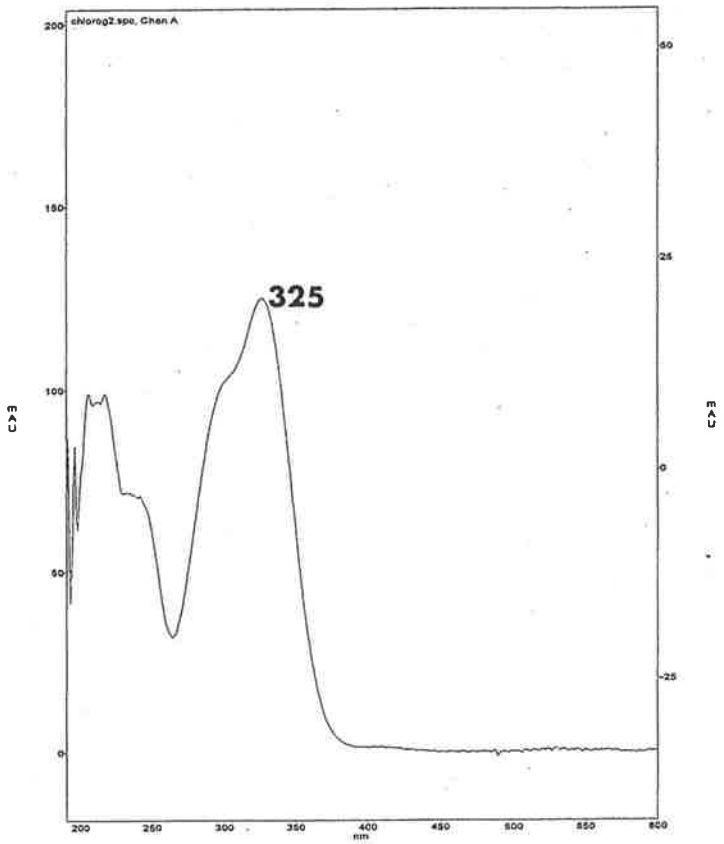


Figure A5.1 Absorption spectrum of chlorogenic acid.

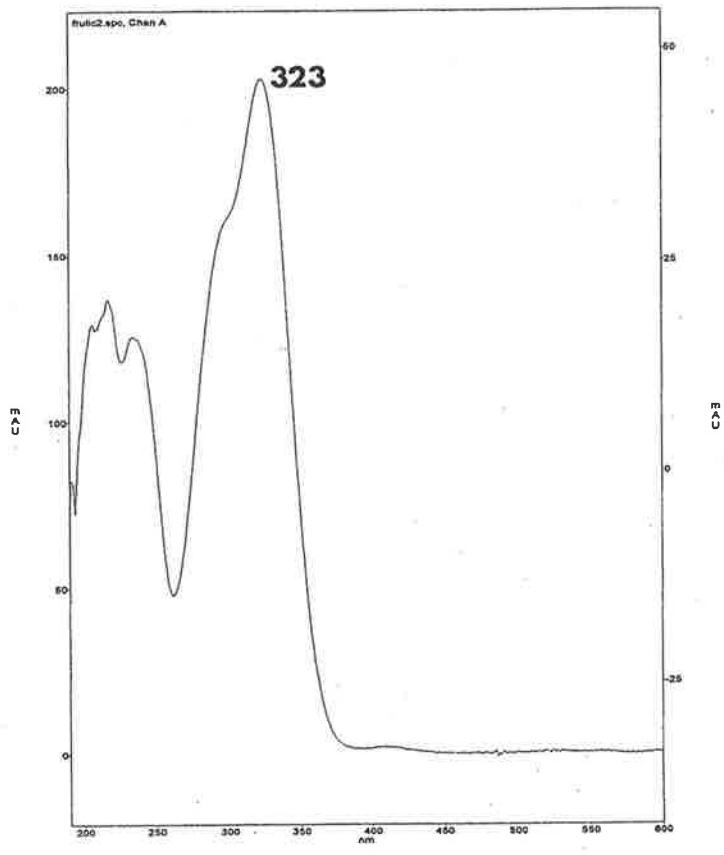


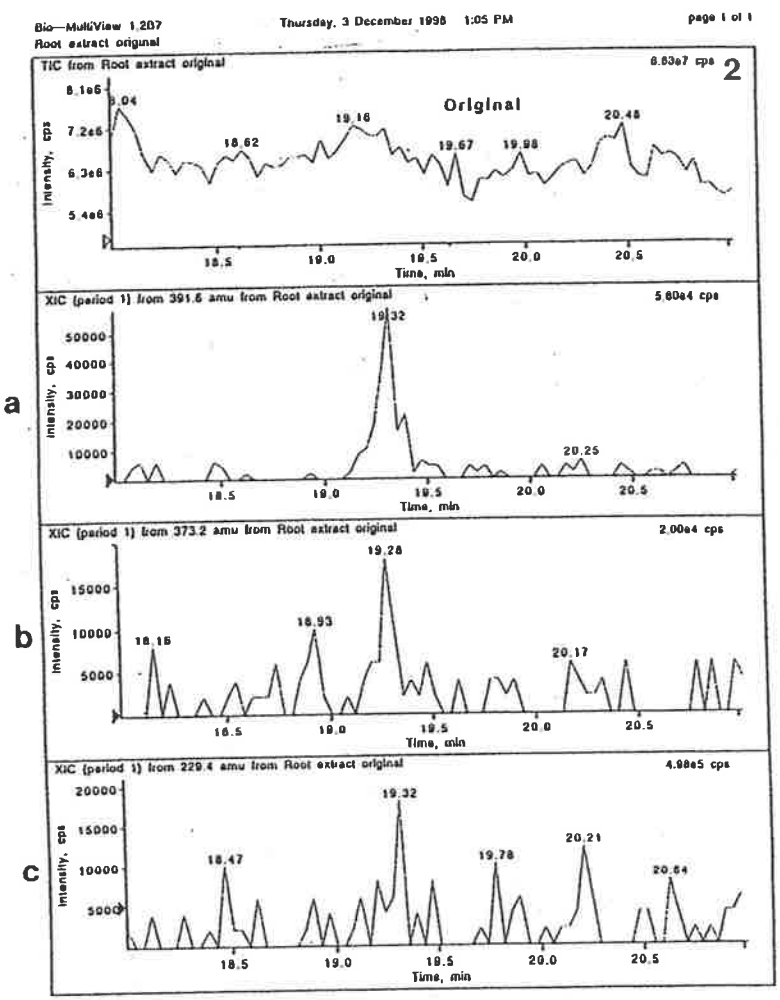
Figure A5.2 Absorption spectrum of ferulic acid.

APPENDIX SIX - MASS SPECTROMETRY DATA FOR IDENTIFICATION OF HPLC PEAKS 2,3 AND 4.

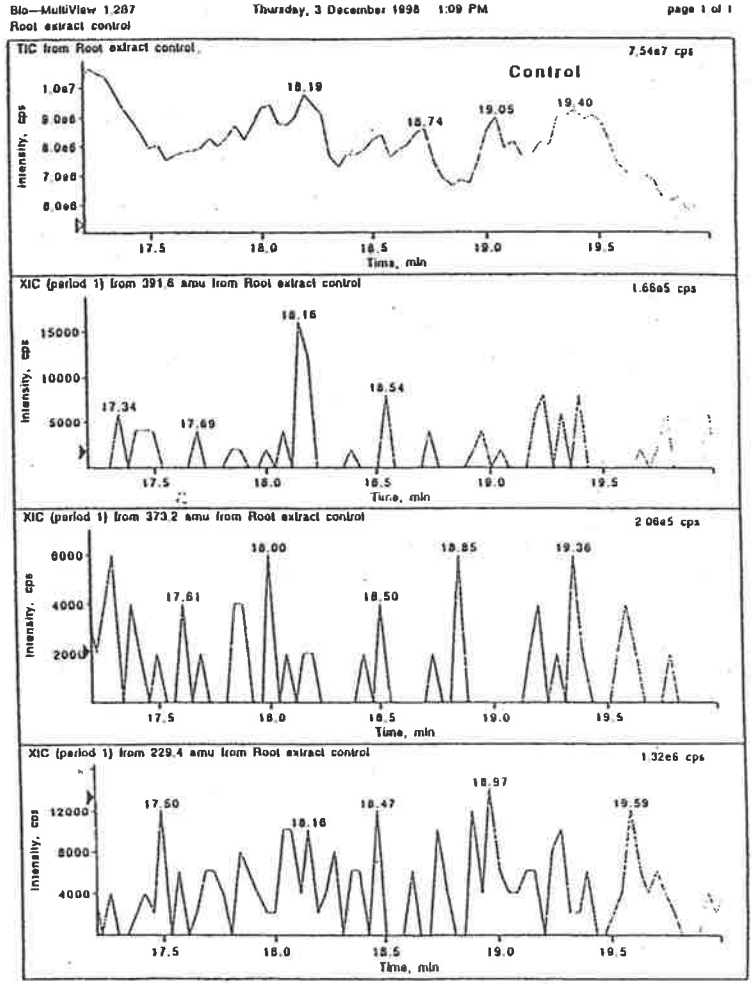
Mass spectrometry was conducted by Yoji Hayasaka, AWRI laboratory, Urrbrae, Adelaide.

The ionspray mass spectra of the compounds were obtained using an API-300 mass spectrometer coupled with an ionspray interface (PE Sciex, Thornhill, Canada). The ion spray and orifice potentials were 5kV and 30V for the positive ion mode, and -4.5kV and -30V for the negative ion mode respectively. The curtain (nitrogen) and nebuliser (air) were set at 8 and 10 units respectively. The sample was injected using the flow injector with a 5µl loop and delivered to a C18 reversed phase HPLC column (2mm × 150mm, Nova-Pak, Waters) at a flow rate of 100µl/min using the syringe pump. All data of mass spectra were processed using Bio-Multiview software 1.2β3 (PE Sciex).

The first page shows comparisons of root extracts from Schwarzmann and *V. rotundifolia* at the approximate retention time of peak two, illustrating ions (marked a, b and c) unique to the *V. rotundifolia* extract. The second page shows the total profile of ionisable compounds at this time point from the *V. rotundifolia* extract. Ions a, b and c are marked. The third and fourth pages show the ionisable compounds in peaks three and four respectively. Peak numbers are marked in the upper right corner of each figure.

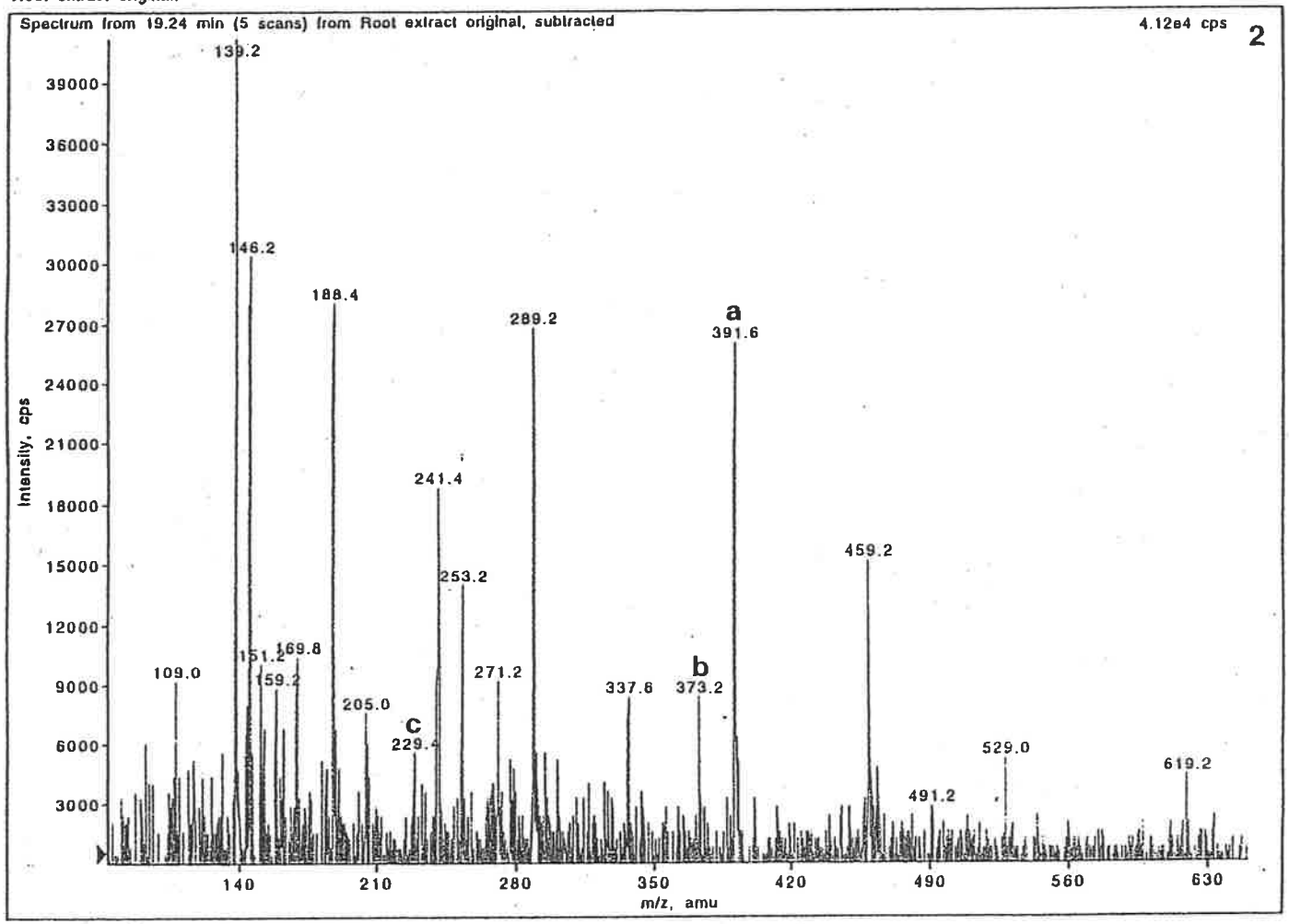

Bio—MultiView 1.2B7
Thursday, 3 December 1998 1:05 PM
page 1 of 1
Root extract original
TIC from Root extract original
8.83e7 cps
2
Original
Intensity, cps
Time, min
XIC (period 1) from 391.6 amu from Root extract original
5.60e4 cps
a
XIC (period 1) from 373.2 amu from Root extract original
2.00e4 cps
b
XIC (period 1) from 229.4 amu from Root extract original
4.98e5 cps
c

V.rot

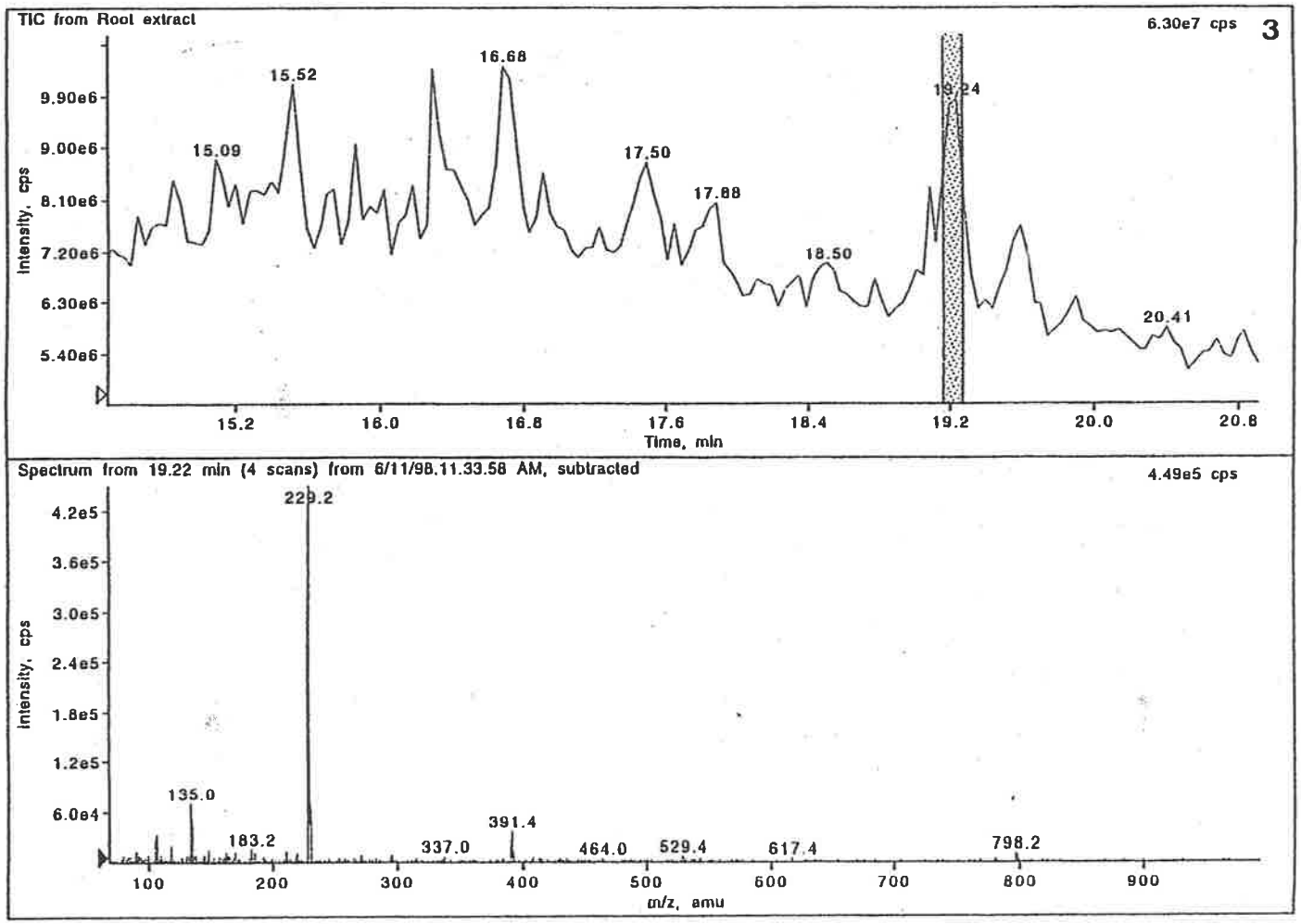

Bio—MultiView 1.2B7
Thursday, 3 December 1998 1:09 PM
page 1 of 1
Root extract control
TIC from Root extract control
7.54e7 cps
Control
Intensity, cps
Time, min
XIC (period 1) from 391.6 amu from Root extract control
1.66e5 cps
XIC (period 1) from 373.2 amu from Root extract control
2.06e5 cps
XIC (period 1) from 229.4 amu from Root extract control
1.32e6 cps

Schw

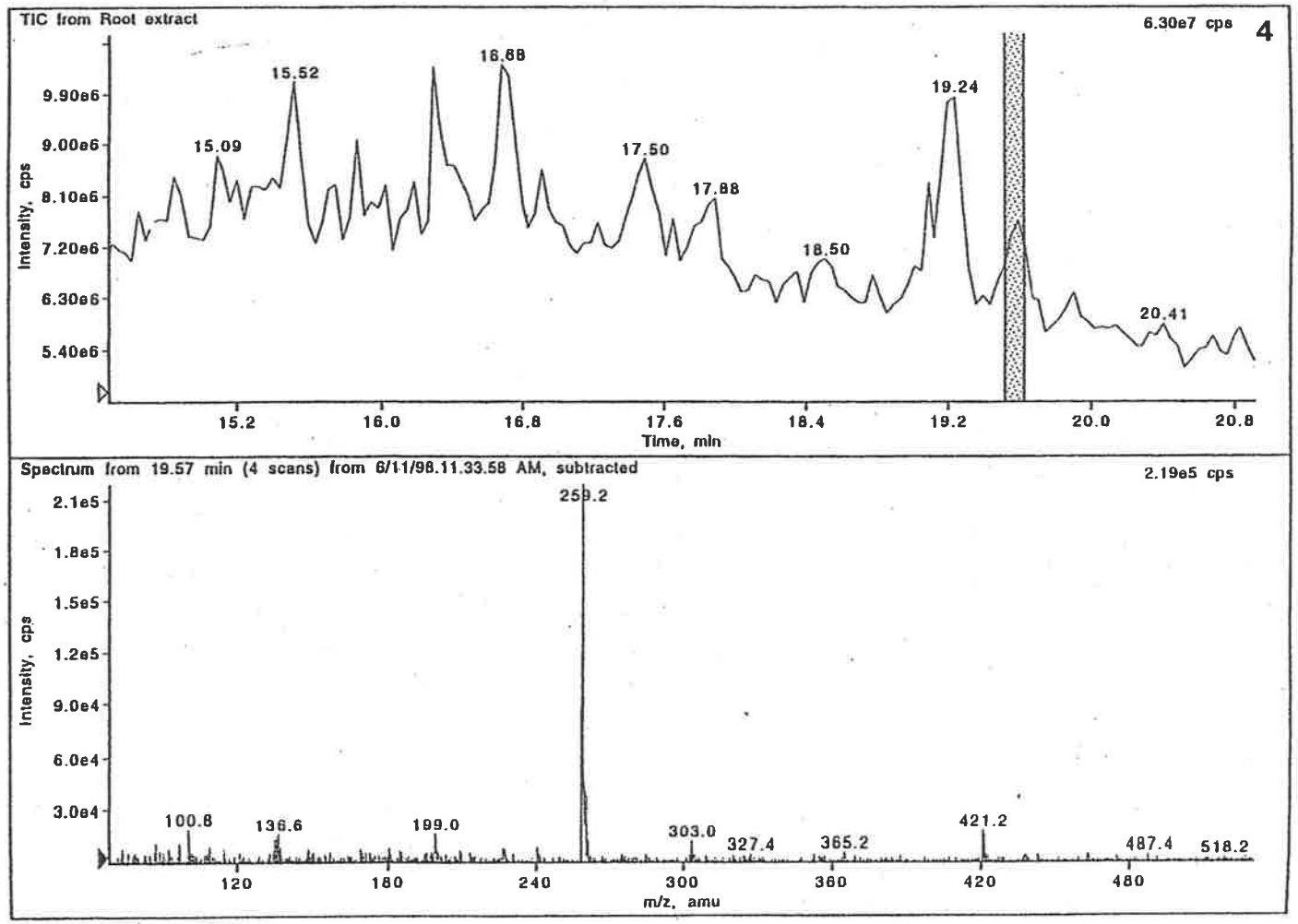
Bio—MultiView 1.2d7 Wednesday, 2 December 1998 12:45 PM page 1 of 1
Root extract original:



Bio—MultiView 1.2β7 Friday, 6 November 1998 12:50 PM page 1 of 1
Root extract et al.



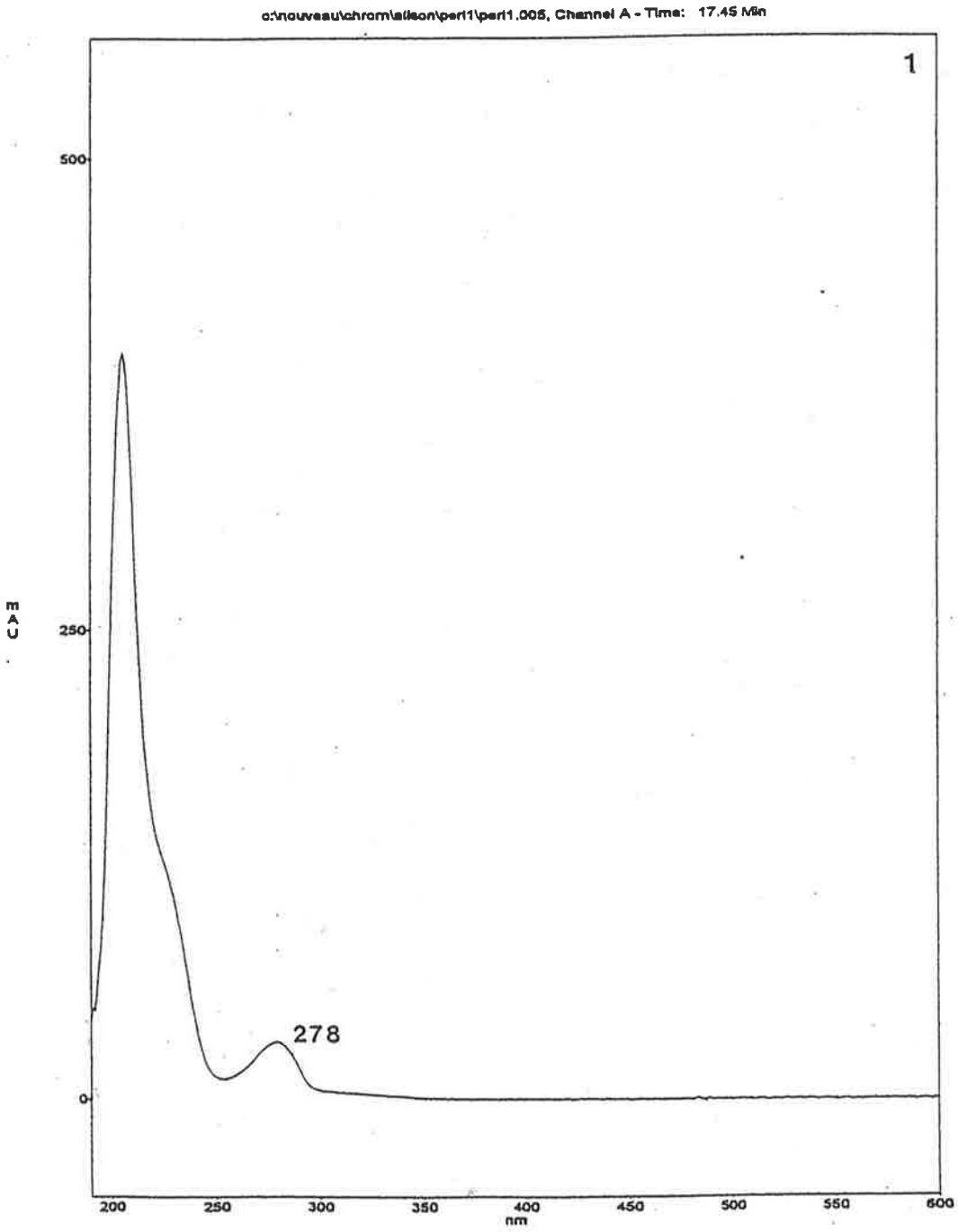
Bio—MultiView 1.2B7 Friday, 6 November 1998 12:51 PM page 1 of 1
Root extract et al.

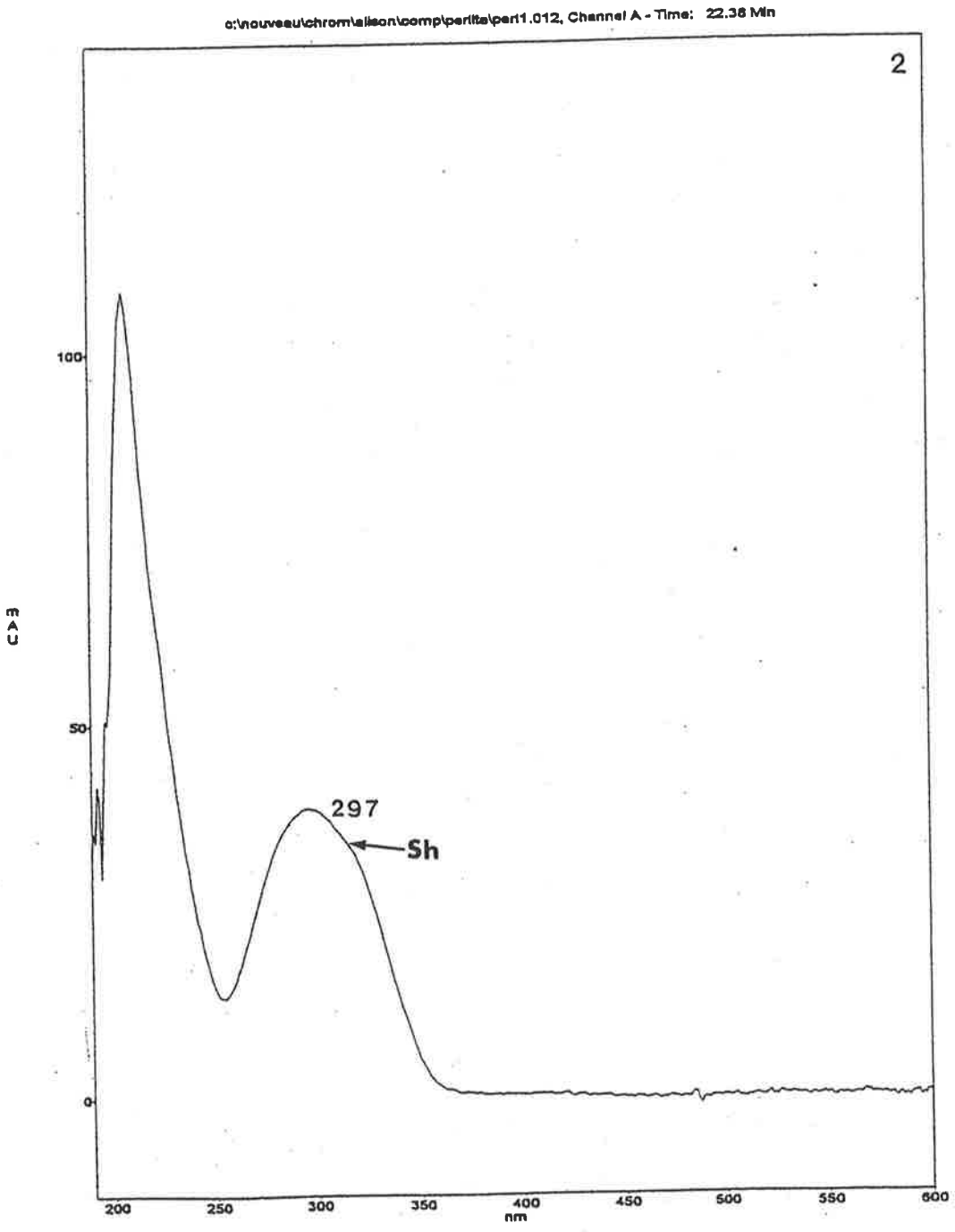


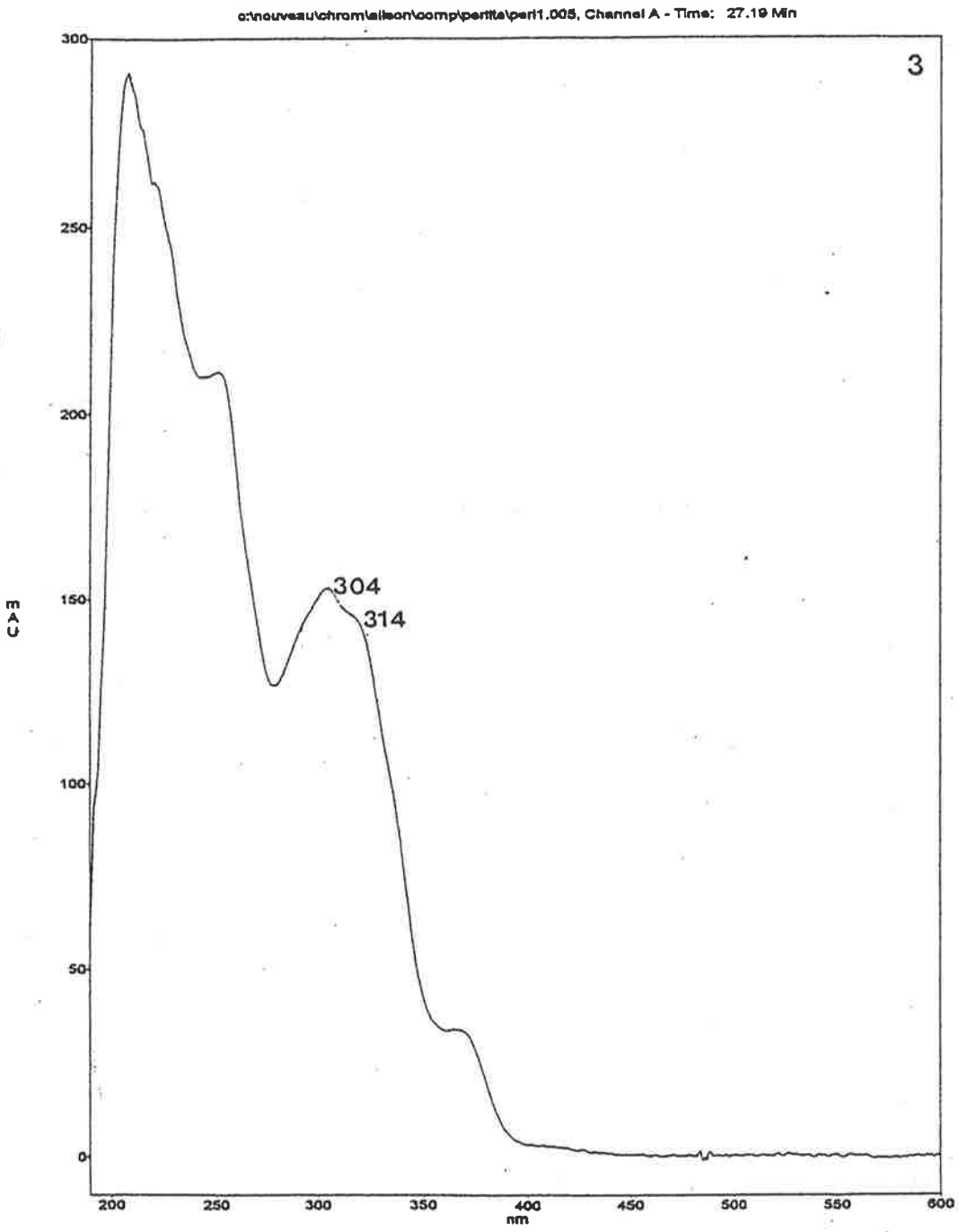
APPENDIX SEVEN - ABSORPTION SPECTRA OF HPLC PEAKS 1-11

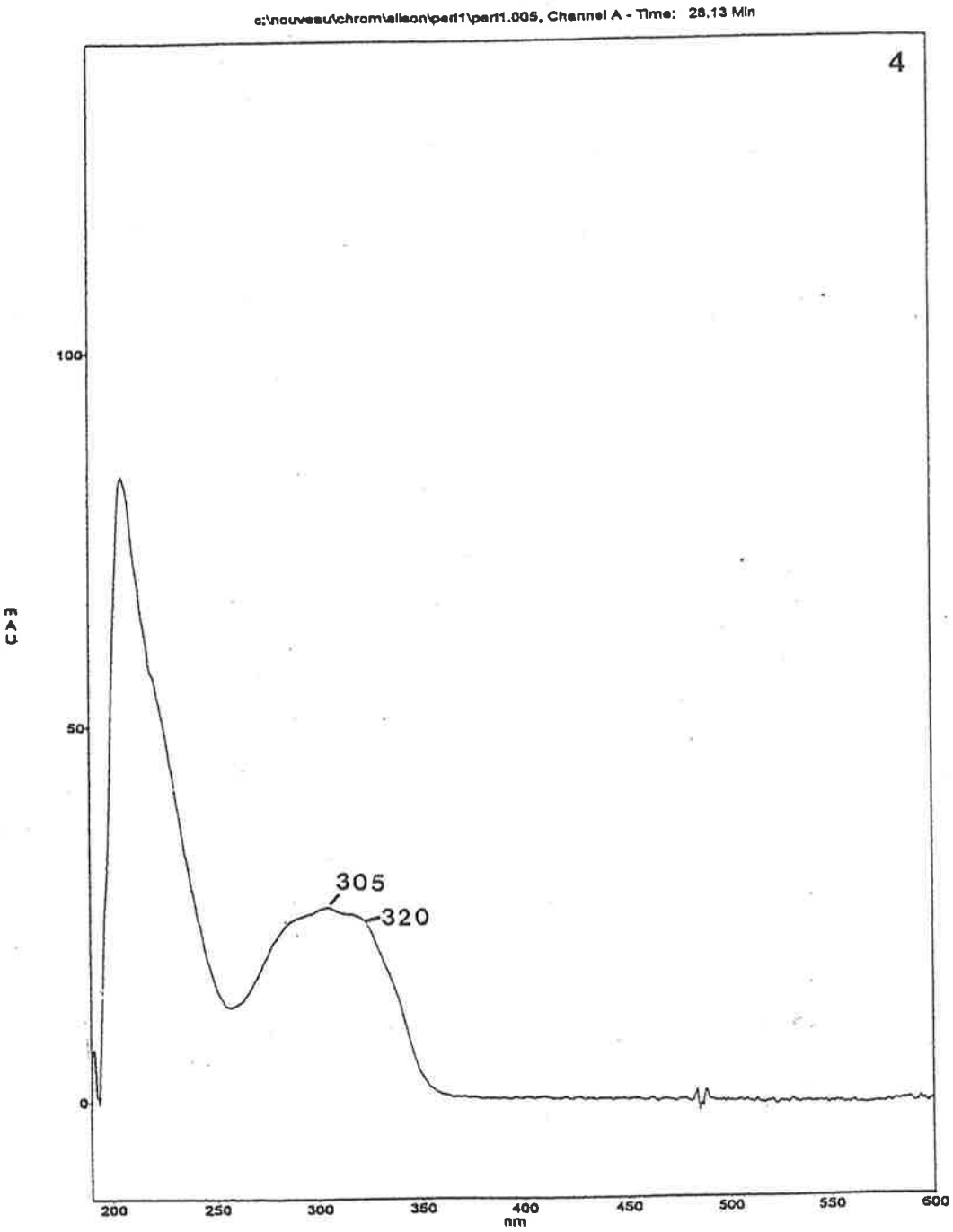
Each absorption spectrum is presented on a separate page and numbered according to peak number as listed in Chapter five, table 5.4.

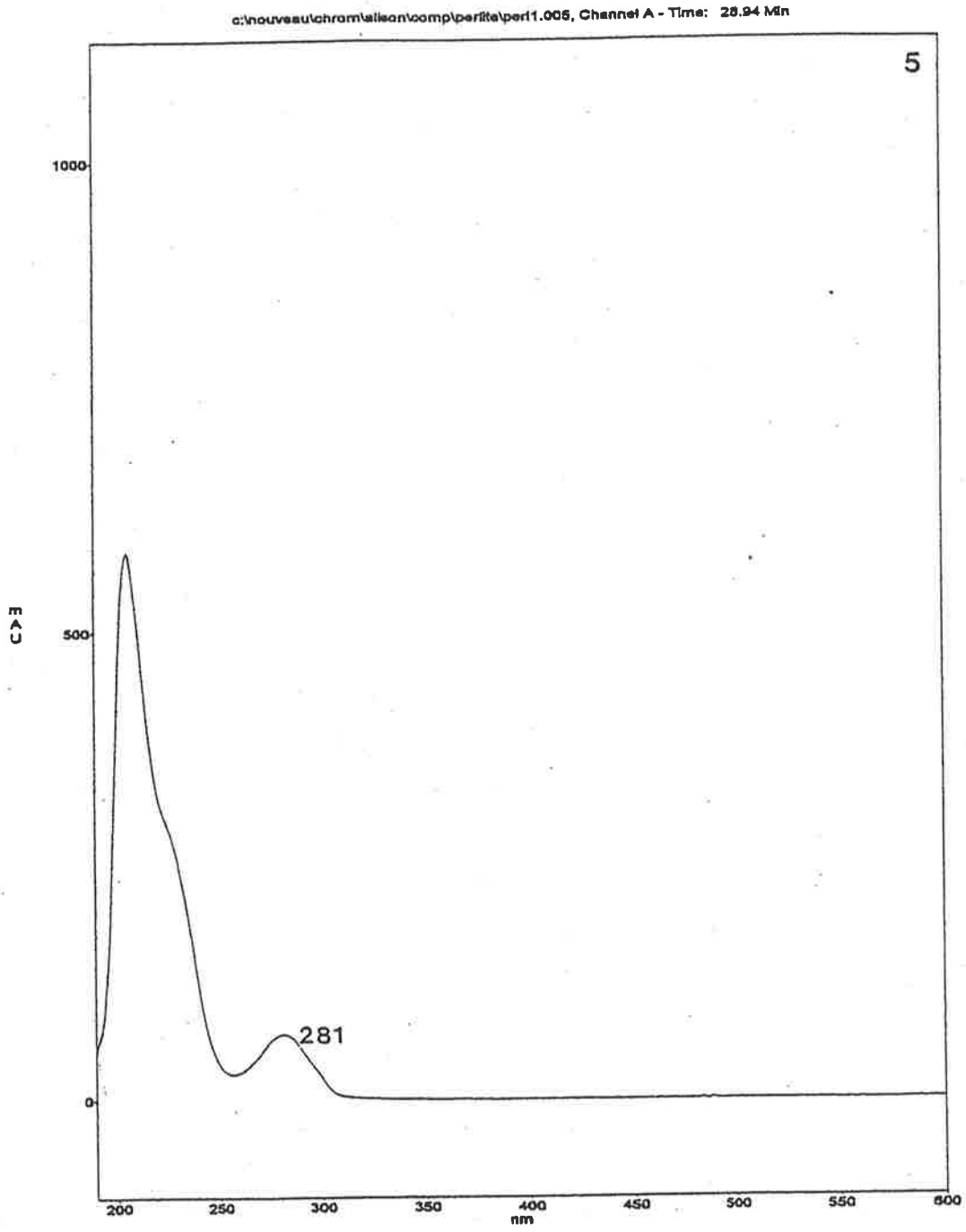
Spectra of peaks 1, 3-11 were obtained after HPLC analysis of Schwarzmann tissue cultured vine root extract, peak 2 is from analysis of *V. rotundifolia* tissue cultured vine root extract. A slight shoulder on peak two is marked (Sh) (see discussion of putative identity of this peak, Chapter 5, section 5.3.3). Extracts were prepared by free phenolic extraction method one (Chapter 3, section 3.2.1), and separated using HPLC chromatography method nine (Chapter 3, section 3.2.2).

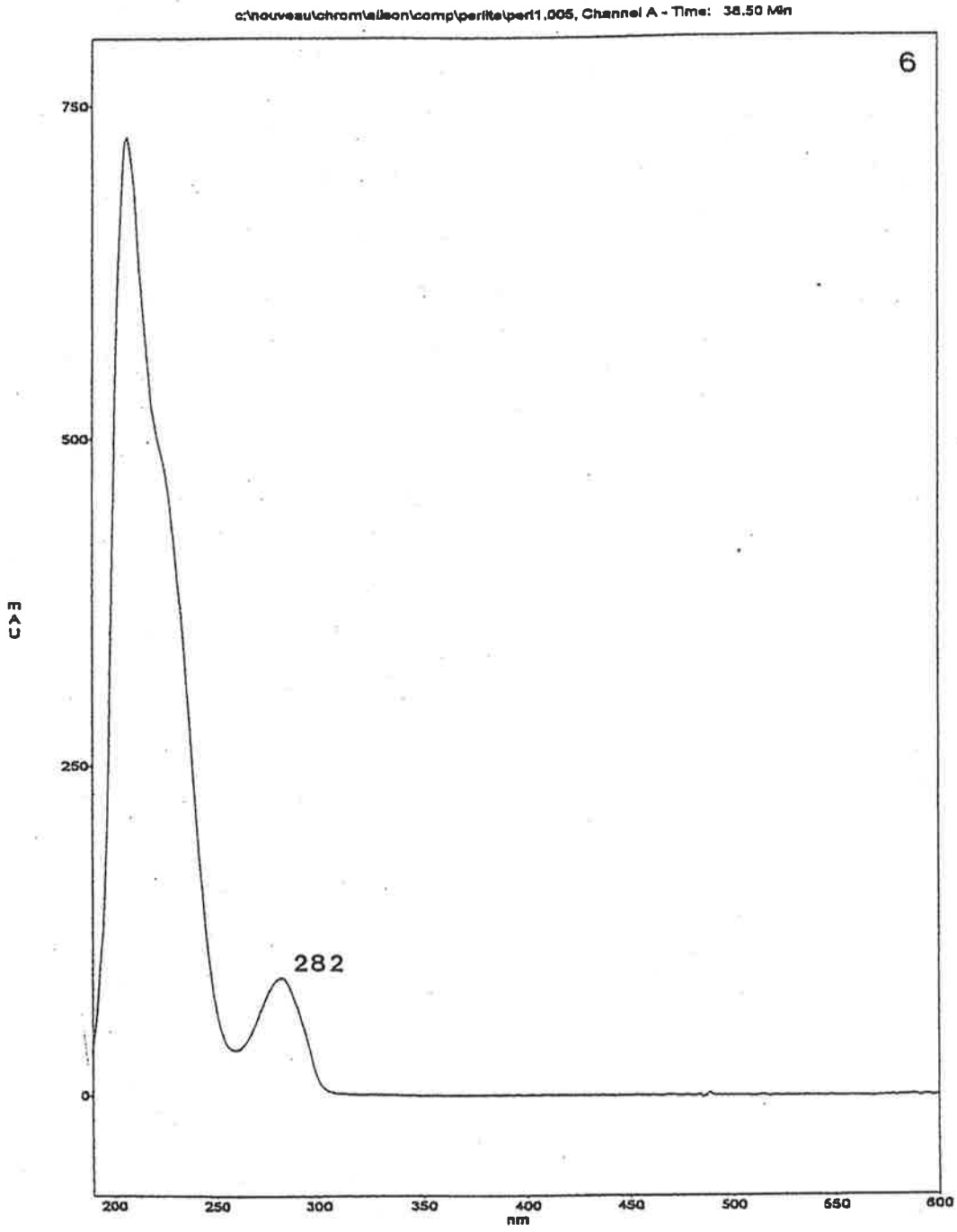

c:\nouveau\chrom\alison\per11\per11.005, Channel A - Time: 17.45 Min
1
500
250
0
CAm
278
200
250
300
350
400
450
500
550
600
nm

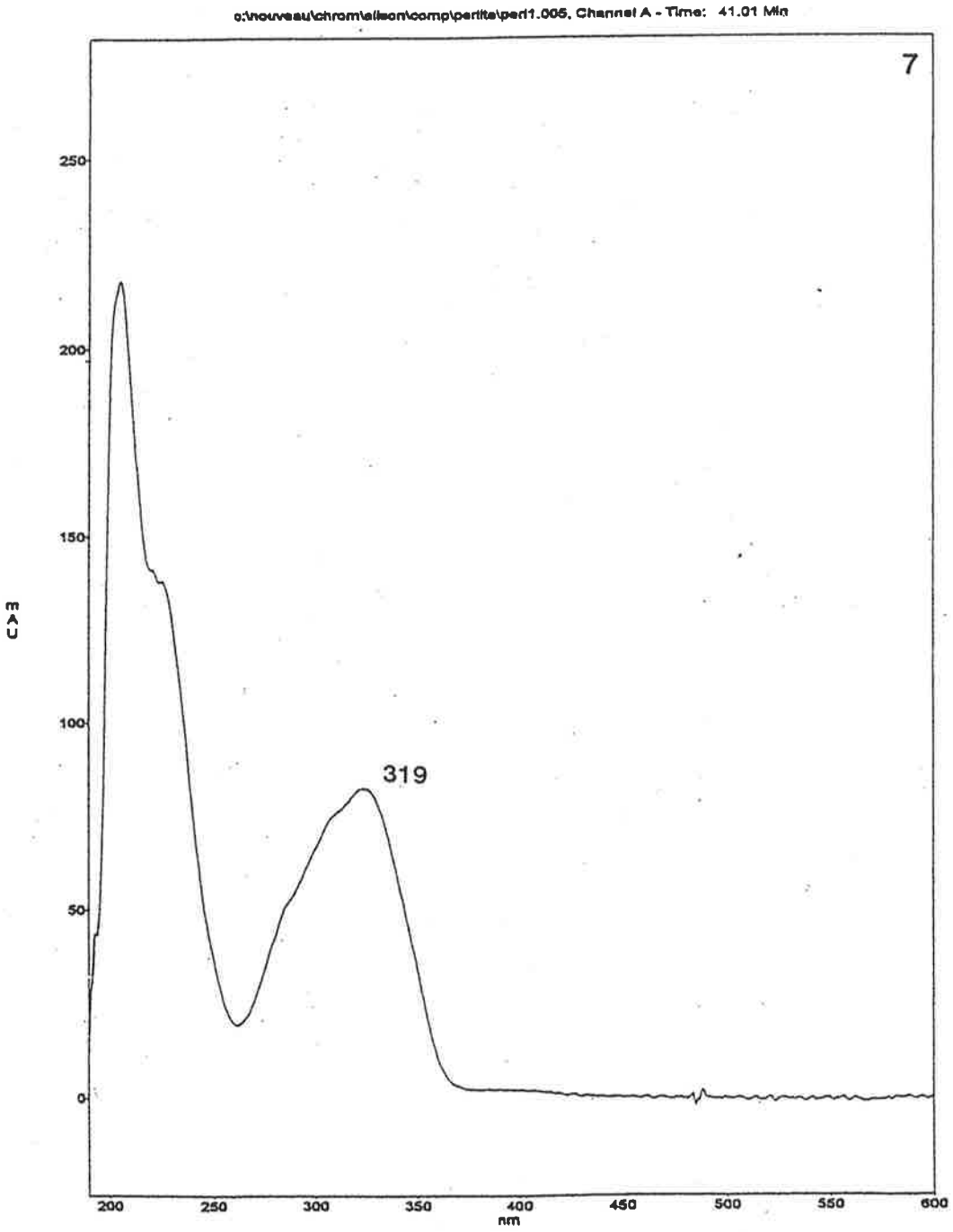

c:\nouveau\chrom\alison\comp\perlite\perl1.012, Channel A - Time: 22.36 Min
2
100
50
0
mAU
297
Sh
200
250
300
350
400
450
500
550
600
nm

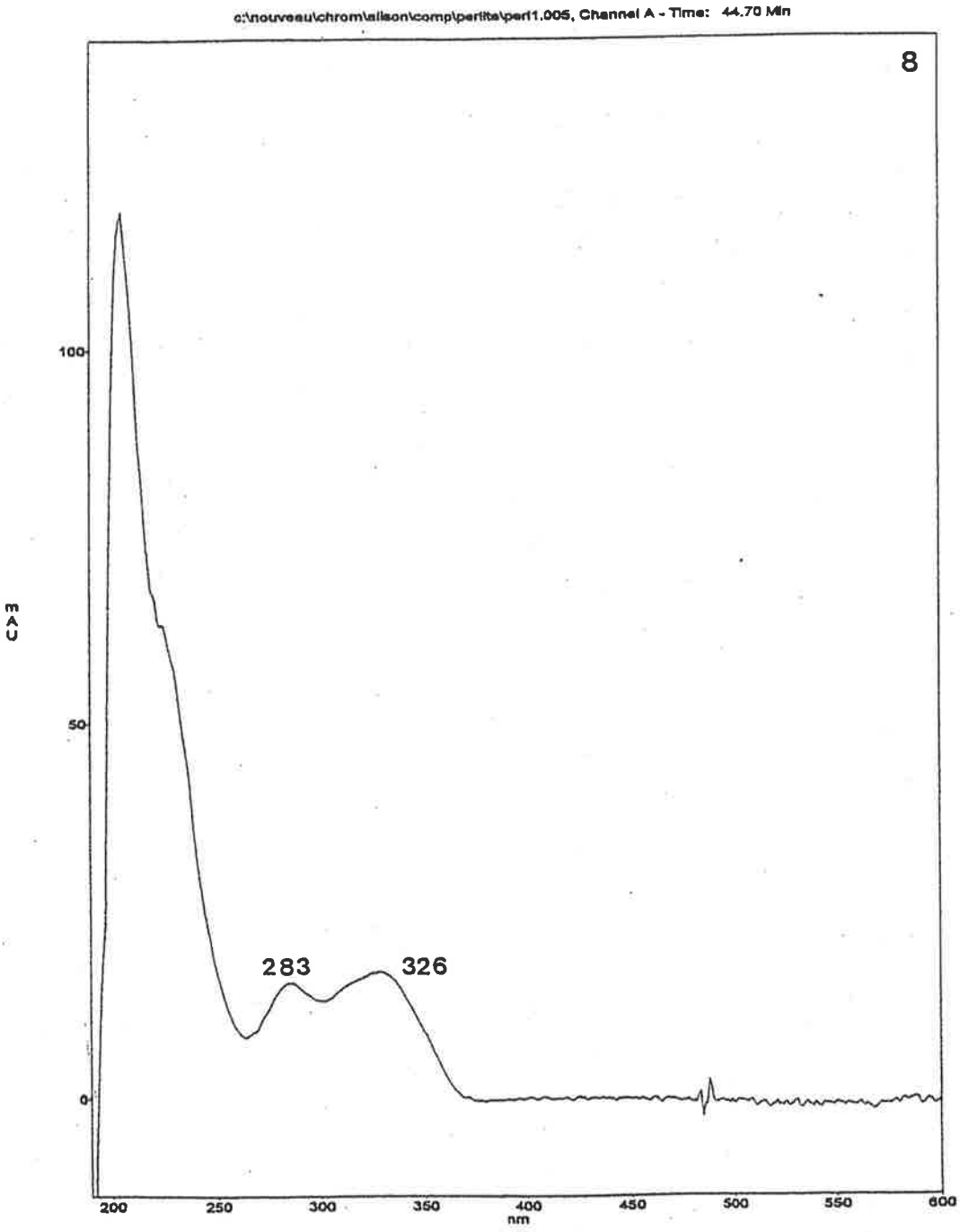

c:\nouveau\chrom\alison\comp\perlite\perl1.005, Channel A - Time: 27.19 Min
3
304
314
mAU
nm
300
250
200
150
100
50
0
200
250
300
350
400
450
500
550
600

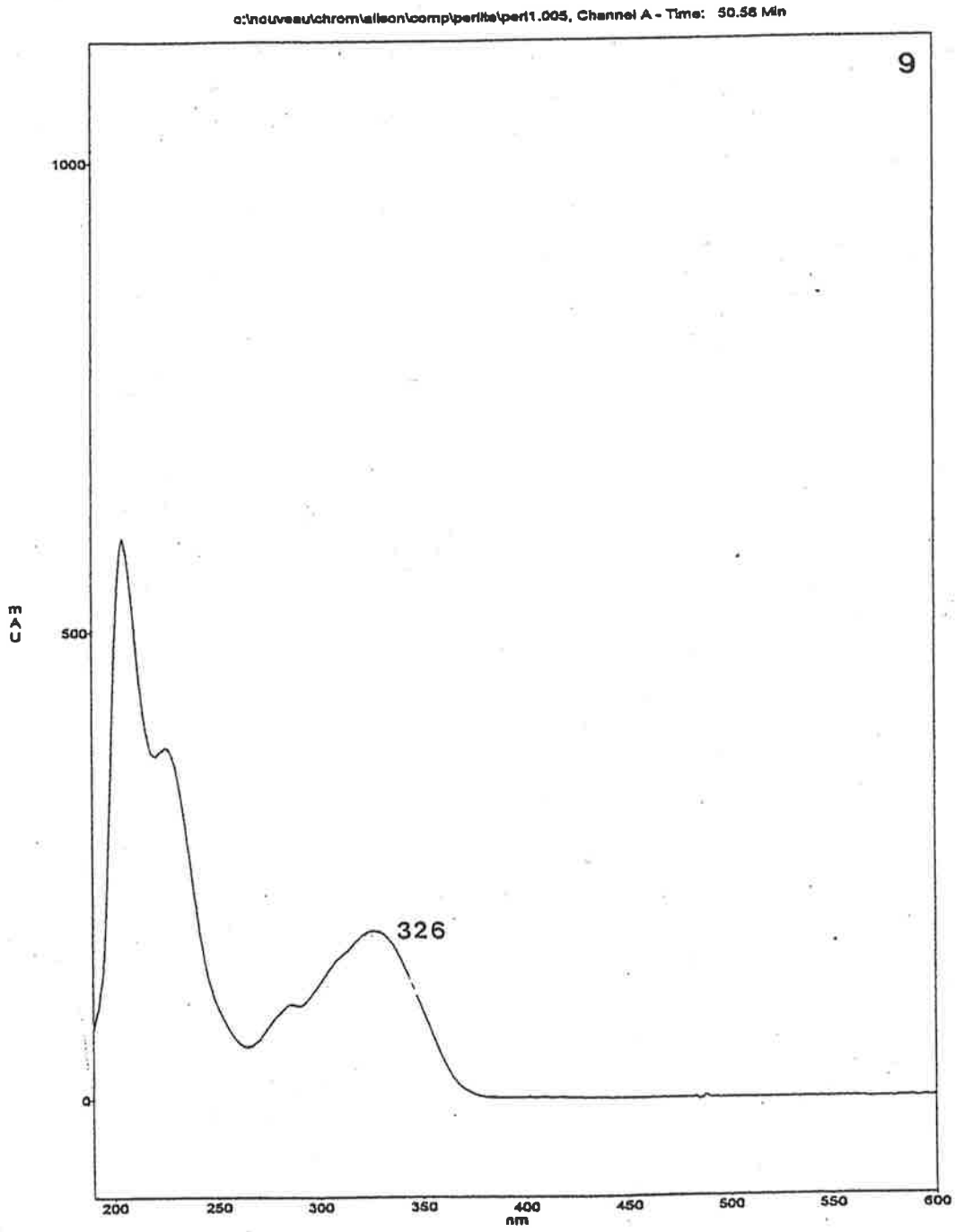

c:\nouveau\chrom\alison\per11\per11.005, Channel A - Time: 28.13 Min
4
100
50
0
mAU
305
320
200
250
300
350
400
450
500
550
600
nm

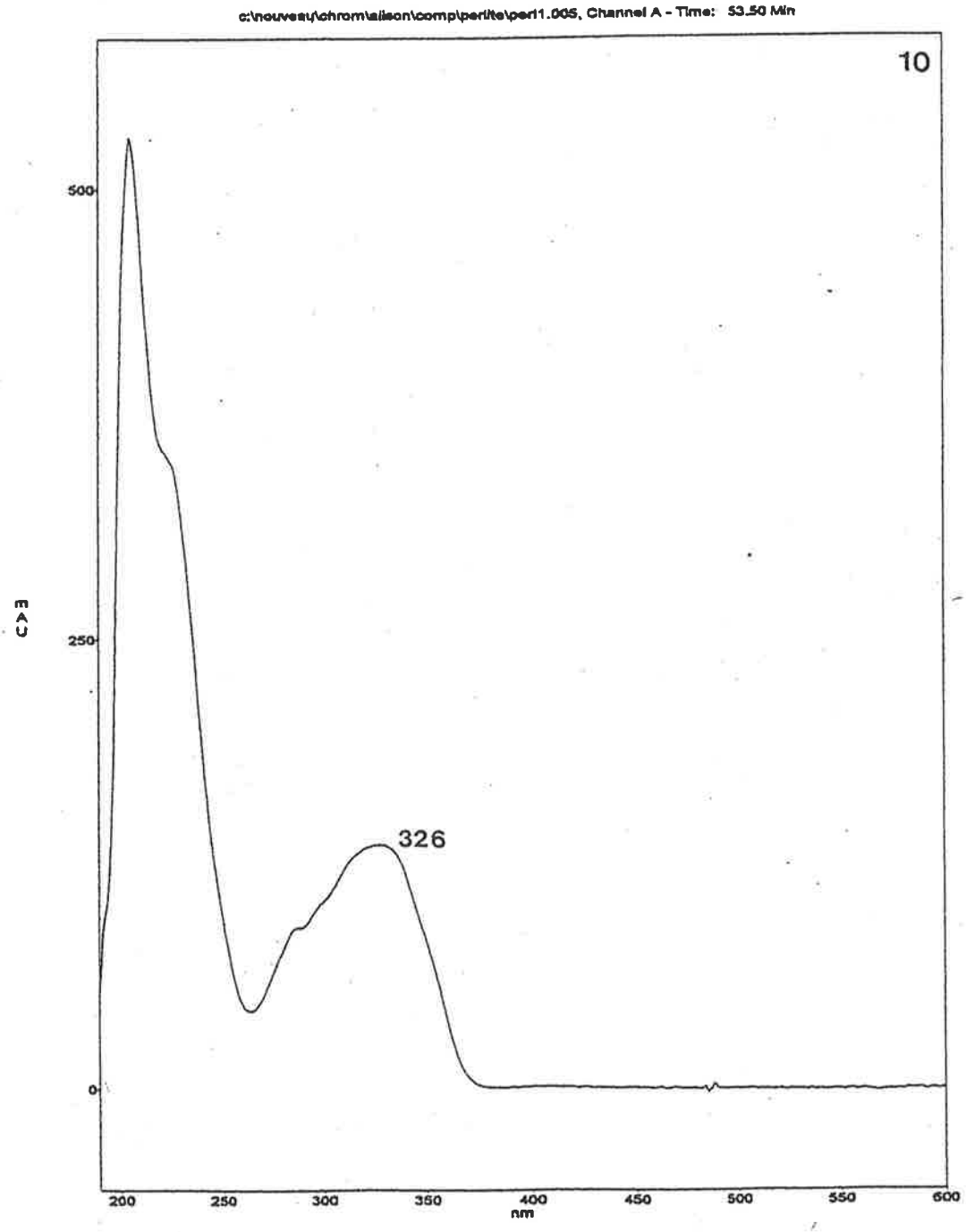

c:\nouveau\chrom\alison\comp\perlite\perl1.005, Channel A - Time: 28.94 Min
5
1000
500
0
mAU
281
200
250
300
350
400
450
500
550
600
nm

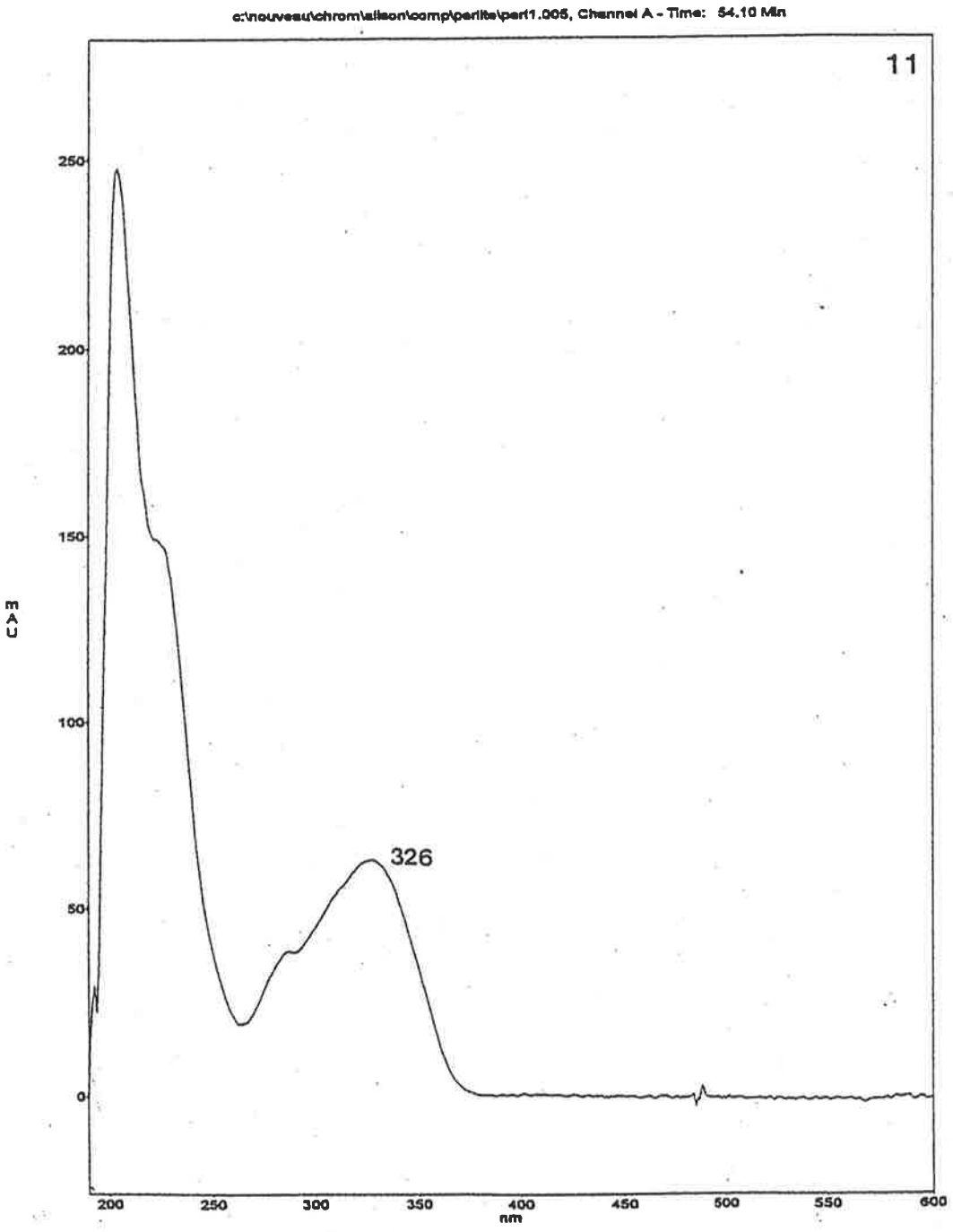

c:\nouveau\chrom\alison\comp\perlite\per11.005, Channel A - Time: 38.50 Min
6
750
500
250
0
mAU
282
200
250
300
350
400
450
500
550
600
nm


c:\nouveau\chrom\alison\comp\perlite\perl1.005, Channel A - Time: 41.01 Min
7
319
250
200
150
100
50
0
mAU
200
250
300
350
400
450
500
550
600
nm


c:\nouveau\chrom\allson\comp\perlite\perl1.005, Channel A - Time: 44.70 Min
8
100
50
0
283
326
200
250
300
350
400
450
500
550
600
nm

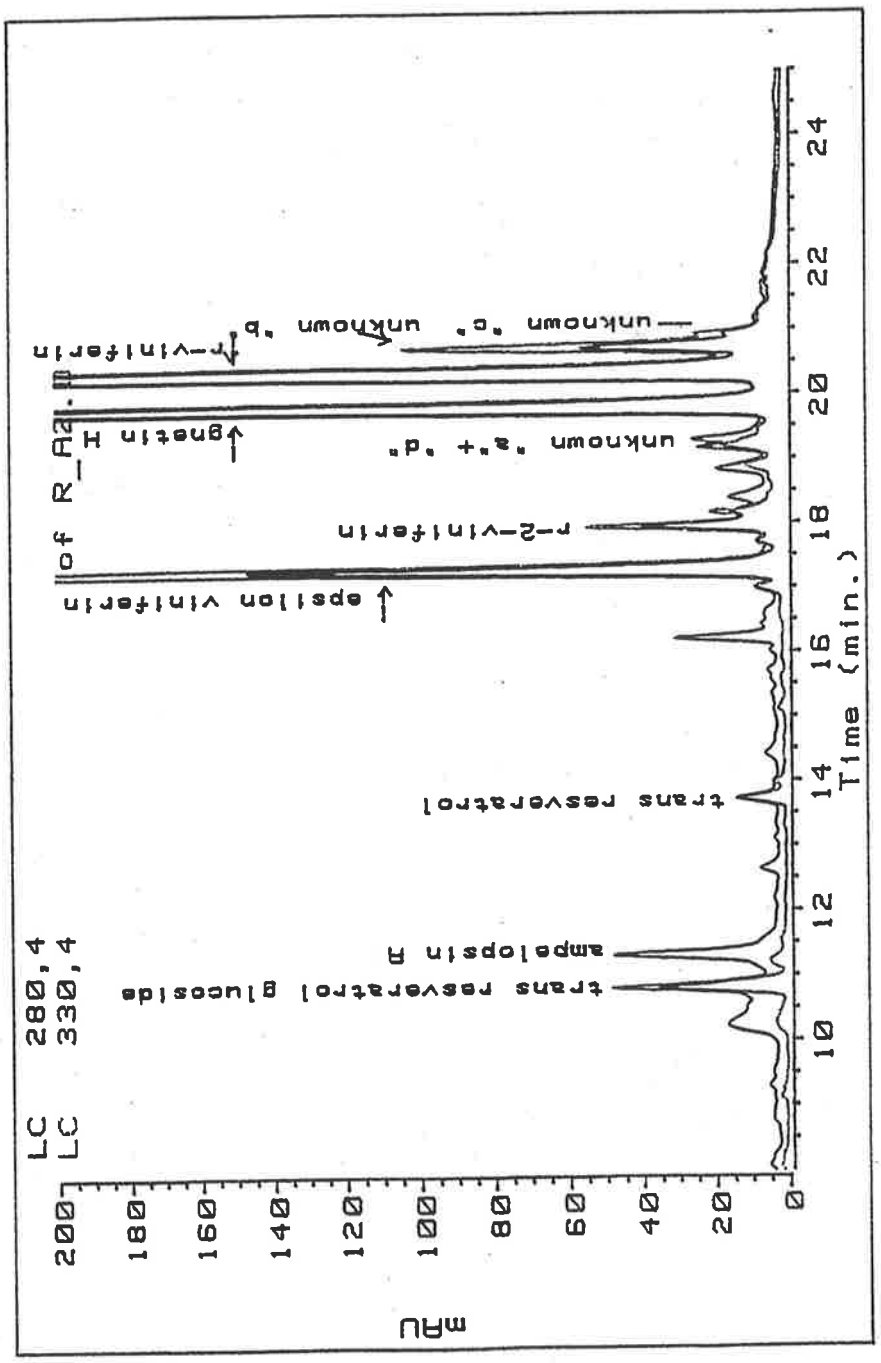

c:\nouveau\chrom\alison\comp\perlite\perl1.005, Channel A - Time: 50.58 Min
9
1000
500
0
mAU
326
200
250
300
350
400
450
500
550
600
nm


c:\nouvest\chrom\alison\comp\perlite\perl1.005, Channel A - Time: 53.50 Min
10
500
250
0
326
200
250
300
350
400
450
500
550
600
nm


c:\nouveau\chrom\alison\comp\perlite\per11.005, Channel A - Time: 54.10 Min
11
326
mAU
250
200
150
100
50
0
200
250
300
350
400
450
500
550
600
nm

APPENDIX EIGHT - IDENTIFICATION OF MAJOR COMPOUNDS IN GRAPEVINE ROOT EXTRACTS

This HPLC chromatogram of an ethanol extract of Schwarzmann fibrous roots was obtained by Dr F. Mattivi, Instituto Agrario, San Michele all'Adige, Italy, using methods described in (Mattivi *et al.* 1996). These results were used to assign putative identities to HPLC peaks 1,3 5-11, Chapter five, table 5.4. The extract was prepared from 2g fibrous roots of Schwarzmann accession WA/R317A using free phenolic extraction method one (Chapter 3, section 3.2.1).



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