Histochemistry and anatomy of phylloxera (*Daktulosphaira vitifoliae*) nodosities on young roots of grapevine (*Vitis* spp).

A. Forneck[1], S. Kleinmann[1], R. Blaich[1] and S. F. Anvari[2]

Universität Hohenheim, Institut für Obst-, Gemüse- und Weinbau, [1] Fachgebiet Weinbau (370a),
[2] Fachgebiet Obstbau (370b), Stuttgart, Deutschland

Summary

Phylloxera (*Daktulosphaira vitifoliae* Fitch) induce galls (nodosities) on young grapevine roots. Histological and histochemical methods were applied to study the gall's morphology and enzyme activities (peroxidases, leucine aminopeptidases and acidic phosphatases). Susceptible *V. vinifera* cv. Cabernet Sauvignon was compared to the resistant rootstock 5 BB (*V. berlandieri* x *V. riparia*) using aseptic dual culture conditions. The gall induction phase was analyzed before visible signs of potential resistance responses were detected. Elevated metabolic activity has been found in nodosities compared to uninfected roots. Starch granule incorporation was detected in young galls and was highest at the feeding site. As galls mature, the starch density decreased at the feeding site and increased towards the periphery of the gall. Peroxidase, acidic phosphatase and leucine aminopeptidase activities were highest at the incision. No differences in enzyme activities could be detected between the two cultivars tested.

K e y w o r d s : *Vitis vinifera*, rootstock, phylloxera, nodosity, resistance, gall formation, host-parasite interaction.

Introduction

Grape phylloxera, *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae), are gall forming aphids native to North America and monophagous on grape (Davidson ans Nougaret 1921). After being introduced into Europe in the middle of the 19th century, *D. vitifoliae* spread and destroyed vineyards planted with *Vitis vinifera* L. (Vitaceae). The breeding of rootstocks based on American *Vitis* species with resistant roots led to the eventual recovery of European viticulture. *D. vitifoliae* forms pocket-like galls on leaves and hooked galls on root tips (nodosities). Feeding and gall development on lignified parts of grape roots cause cracking and enables attack by soil-borne pathogens which can result in host plant death (Granett *et al.* 2001).

Phylloxera depend on specific feeding tissue (galls) during their entire life cycle. First-stage *D. vitifoliae* penetrate the meristematic zones of root tips with their rostrum (stylet bundle) and stay sedentary until adulthood. Probably as a result of salivary secretions, root cells close to the feeding site become polyploid, whereas cells far from the feeding site enlarge and seem to be used as food deposit until completion of phylloxera's life cycle (4-6 weeks later).

Morphological studies of nodosities illustrate the anatomy of the root galls which contain hypertrophic and hyperplastic cells (Millardet 1898, Niklowitz 1955, Kleinmann 2001) and polyploid cell nuclei (Anders 1960). Cells close to the feeding site decrease in size and parenchymal cells on the opposite side of the vascular tubes enlarge and contain starch granules. Other authors studied the biochemical composition of nodosities (*e.g.* Stoev 1966, Rilling 1974, 1975). Schäfer (1985) found increased starch deposits, soluble proteins and phenolic substances in nodosities and Kellow *et al.* (2000) detected higher amounts of amino acids except asparagine in nodosities if compared to uninfected root tips. However the protein composition detected in SDS gels showed no differences between infected and non-infected tissue. Generally the nodosity acts as strong physiological "sink" as postulated by Steffan and Rilling (1981), though their results suggest that transportation of carbohydrates is restricted within the root.

In this study, the anatomy of nodosities on two cultivars differing in their resistance to phylloxera was compared to non-infected root tips in an aseptic culture system, which facilitates histochemical and molecular genetic studies. Such a system excludes interfering factors that may result in artifacts and allows precise inoculation and controlled feeding time. To better understand the events that take place during the early steps of feeding site development and gall induction we applied histochemical techniques to monitor cellular enzymatic expression and morphological changes within phylloxera-induced root galls.

Material and Methods

V i t i s x p h y l l o x e r a i n t e r a c t i o n : *In vitro*-propagated *Vitis vinifera* cv. Cabernet Sauvignon and the rootstock Kober 5 BB (*V. berlandieri* x *V. riparia*) were co-propagated with a laboratory-raised strain of *D. vitifoliae* in an aseptic dual system previously described (Forneck *et al.* 1996). Samples of root galls were taken by dissecting nodosities (2-6 day old) including the associated *D. vitifoliae*, according to the gall's developmental stages. Uninfected root tips were gathered from non-infected, separated *in vitro* plants.

Histological studies of the anatomy of nodosities: The specimen were fixed in FPA (formaldehyd-propionic acid-ethanol), dehydrated and paraffin-embedded, following the conventional procedures. 8 µm sections were cut with a microtome, stained with toluidine blue and iod-iodacetamide respectively and inspected with a Zeiss Axioplan microscope (Kleinmann 2001).

Histochemical localization of enzyme activity: Root galls and root tips were sampled, immediately frozen in liquid nitrogen and embedded in Tissue Tek (O.C.T. Compounds, Miles Inc. USA): H_2O (50:50) solution. Frozen embedded material was sectioned at 8-10 µm at -20 °C in a cryomicrotome (Frigocut 2700) and stained immediately.

Activity of peroxidases (PER) was assayed according to Heintz and Blaich (1990) with minor modifications described by Kleinmann (2001). Acidic phosphatases (APT) and leucine-aminopeptidases (LAP) were assayed according to Lodja et al. (1976). Sections incubated without substrate were used as controls. Other controls were heated on a hot plate for 3 min until the sections were dry. Frozen sections from nodosities and uninjured root tips were evaluated for the pattern of staining, localization and intensity of enzyme activity.

Results and Discussion

Phylloxera induce root galls (nodosities) of the histoid type (Fig. 1). The active presence of the gall inducer is essential for the development of the gall during all stages of cecidogenesis. A cessation of larval activity brings about a rapid change in the nutritive cells (Bronner 1992). Nutritive cells and tissues are defined as all kinds of tissues found within the enlarged gall, radially surrounding the feeding site. The outer part of the gall tissue is referred to as storage nutritive tissue because it contains starch (Bronner 1992).

Fig. 1: In vitro-generated phylloxera nodosity on Cabernet Sauvignon (three days old).

Cecidogenesis involves complex metabolic interactions between phylloxera and grape roots and may be divided into three phases: cell conditioning, gall induction and gall maturation (Hori 1992). Biochemical or molecular genetic evidence has rarely been documented for any of the phases. To obtain relevant significant data, fragmentation of the different metabolic states of the gall is required. This differentiation seems to be essential for further studies utilizing molecular genetic approaches.

Here we describe nodosities in the gall induction phase within the first 2-6 days after aphid begins feeding.

Anatomy of nodosities: D. vitifoliae inserted the stylet along the meristematic zone of young in vitro root tips (Figs. 1, 2). The expansion of the gall associated with phylloxera was completed with the aphid reaching adulthood and starting of oviposition.
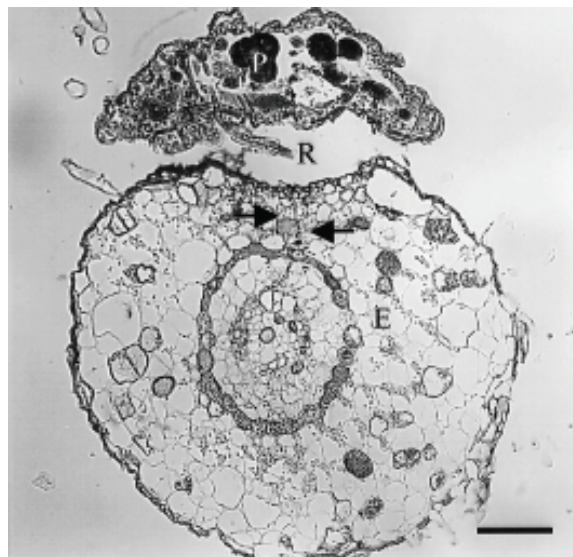

Fig. 2: Phylloxera (P) feeding on a 5-day-old nodosity of Cabernet Sauvignon (cross section, Toluidine blue staining). (R = Rostrum; E= Endodermis). Bar: 500 µm.

The site of penetration was marked by browning of epidermal and cortical tissue in the resistant 5 BB host. Much less browning of cortical tissue was detected in the susceptible V. vinifera host Cabernet Sauvignon. Initiation and development of nodosities involves a combination of hypertrophy of cortical cells distal to the feeding site and lack of radial expansion of cortical cells proximal to the feeding site. The endodermis showed no marked signs of degeneration in any of the specimen investigated. The gall's structure can be divided into two parts: the metabolically active feeding site and the parts of the gall where starch was incorporated (later referred to as storage part).

The active feeding site is located in the upper layers of the root cortex. In most cases, cavities under the exodermis were observed (Figs. 3, 4 a), which probably resulted from the interference of phylloxera salivary enzymes with parenchymal tissue. No data on the enzyme composition of phylloxera saliva is available, however studies on other parenchymal feeding aphids suggest that peroxidases, pectinases and amylases are present (Miles 1999).

The stylet sheath was clearly visible by the various staining techniques and ended intracellularly in most cases (Figs 4 a, b). Branching of the stylet sheath was observed frequently as was reported earlier (e.g. Rilling 1966).

Starch was incorporated in cells near the active feeding center and in parenchyma cells opposite to the feeding site. We observed the starch granules located in gradients to-
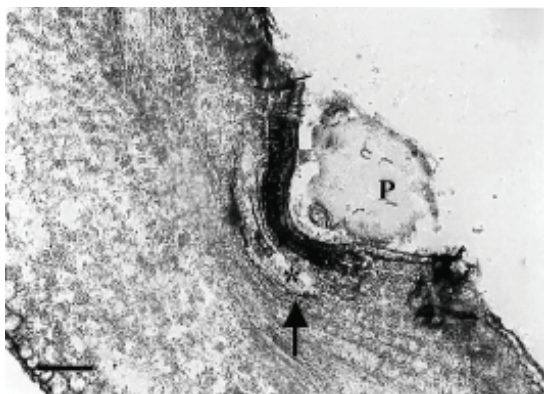
Fig. 3: Phylloxera (P) feeding on a Kober 5 BB-derived nodosity (longitudinal section). Peroxidase activity at the upper cell layers of exodermis. Hollow space underneath the feeding site marked by asterisk (*). Bar: 200 μm.
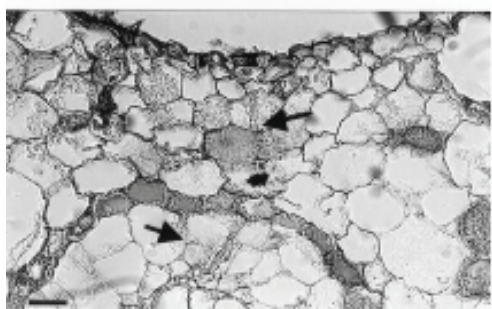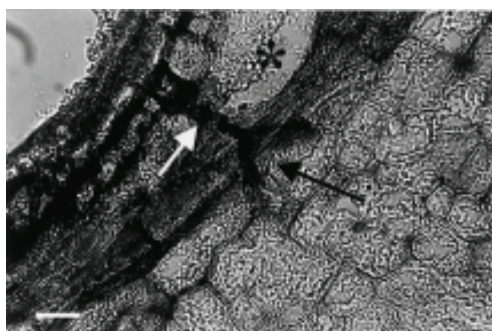

Fig.4: **a**: Phylloxera stylet sheath on a 6-day-old nodosity (Kober 5 BB) with peroxidase staining (longitudinal section). Branching of the stylet pathway indicated by arrow. Bar: 10 μm. **b**: Phylloxera stylet pathway on a 4-day-old nodosity (Cabernet Sauvignon) stained with toluidine blue (cross section). Bar: 50 μm.
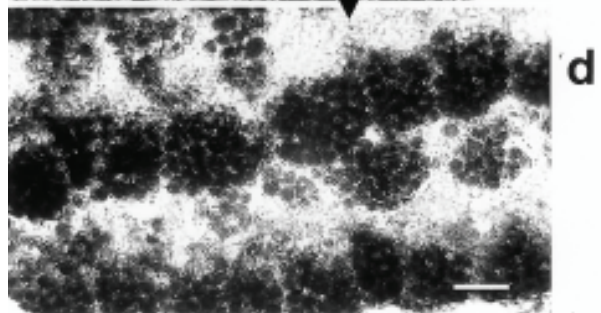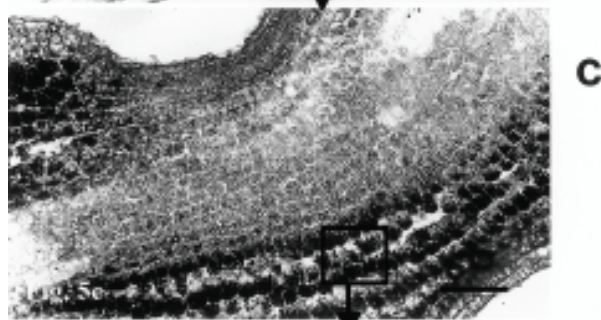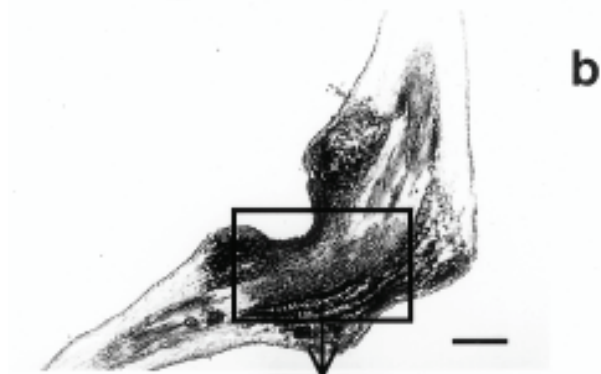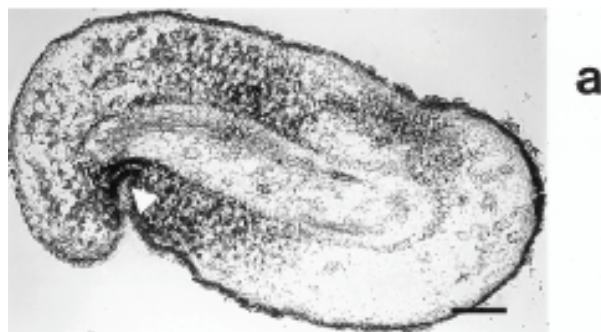

Fig. 5: **a**: Starch granules accumulation and gradient towards the feeding site on a 5-day-old nodosity derived of Cabernet Sauvignon (longitudinal section). Bar: 500 μm. **b**: Starch granules accumulation and gradient towards the periphery of the gall (14-day-old; Kober 5BB) in a longitudinal section. Bar: 1000 μm. **c**: Bar: 500 μm. **d**: Starch granules within cells. Bar: 50 μm.

wards the feeding site (Fig. 5 a) in young nodosities of the gall induction stage, as was described for aphids (Bronner 1977). A gradient of starch granules could also be observed within the cells (Fig. 5 d). As galls mature, the density of starch granules increased towards the periphery of galls (Figs 5 b, c). Galls of different phases may show differences in starch distribution especially in the starch formation and deposition as was noted by Rohfritsch and Anthony (1992).

Starch accumulation seems essential for providing adequate food resources and reserves for phylloxera. These food sources require balanced C/N supplies since phylloxera do not contain siphones to discharge excess carbohydrates.

Localizaton of enzyme activity: All three enzymes studied stained noticeably near the stylet sheath (Fig. 4 a). Enzymes have been detected in the stylet sheaths of aphids, among them polyphenol oxidase (Miles (1965)

and peroxidase (Miles and Peng 1989). At this point it is questionable, if the stylet sheath-associated enzymes derived from the phylloxerids' saliva, from the plant's wounding response, or if they were the result of an interaction between phylloxera saliva and the responding root tissue. Since enzymes are dominant in aphids' saliva, the way they may interact with enzyme systems in the plant's root is of essential interest for further host-plant interaction studies.

The proexodermis and endodermis as well as the provascular strands showed marked staining for all the enzymes tested, whereas the parenchymal cells of the cortex

and pericycle cells of the central cylinder showed no enzyme activity (Table). Cell walls of mature xylem stained also for acidic phosphatase and leucine aminopeptidase. However, the latter reaction was also present in heat-inactivated tissue and may be an artifact.

The cells situated around the incision and the endodermis stained for all the enzymes tested, throughout the phase of gall induction. The parenchymal cells of the cortex and central cylinder had no enzyme activity except limited activity of peroxidase (Table).

Leucine aminopeptidases (LAP) are exopeptidases that catalyse the hydrolysis of amino acid residues from the amino terminus of proteins and peptides. Plant aminopeptidase activity seems to be in response to abscisic acid and jasmonic acid, wounding and pathogen infection and suggests a role of LAP in defence reaction (Walling 2000). The cell layers adjacent to the active feeding site showed activity of LAP within the cytoplasm. Staining of LAP was absent in the parenchymal cells far from the feeding site and in pericycle cells (Fig. 6 a).

Peroxidases (PER) of plants participate in many processes of growth and defense and plants often contain several forms (isoenzymes). PER require hydrogen peroxide as an electron acceptor. H_2O_2 is generated in all aerobic plant tissues, although it is rapidly destroyed by endogenous catalases. PER activity was dominant at the feeding site and in the surrounding parenchymal root tissue. Staining became visible in the cell walls, xylem vessels, exodermis and endodermis. Enzyme activity decreased progressively from the feeding site towards the uninjured part of the root (Fig. 6 b).

Acid phosphatases (APT) are located in cell walls and vacuoles; they are involved in most metabolic processes (e.g. wounding and callus development) without having substrate specificity. According to Schäfer (1982) phosphatases in combination with phosphorylases take part in starch degradation in grape roots. APT could be localized at the active feeding site, the xylem vessels, the exodermis and the endodermis but not in parenchymal and pericycle cells.

Unlike Niklowitz (1955) and Kellow et al. (2000) who found differences in nodosity-based plant responses between susceptible and resistant plants we could detect no significant alteration in plant response. This may be due to the limited range of test plants used and the particular phylloxera strain utilized in this study. Significant influence of the phylloxera strain on gall induction, both in aseptical and greenhouse studies, have been reported in earlier studies. The tissue investigated in this study derived from galls in the gall induction phase showing no visible signs of degradation. It may occur, that in later stages nodosities become metabolically different by activating specific resistance response. It is noteworthy, however, that in the earlier stages of gall induction neither the physiological nor the morphological parameters studied differed significantly among the two cultivars tested (Forneck et al. 2001).
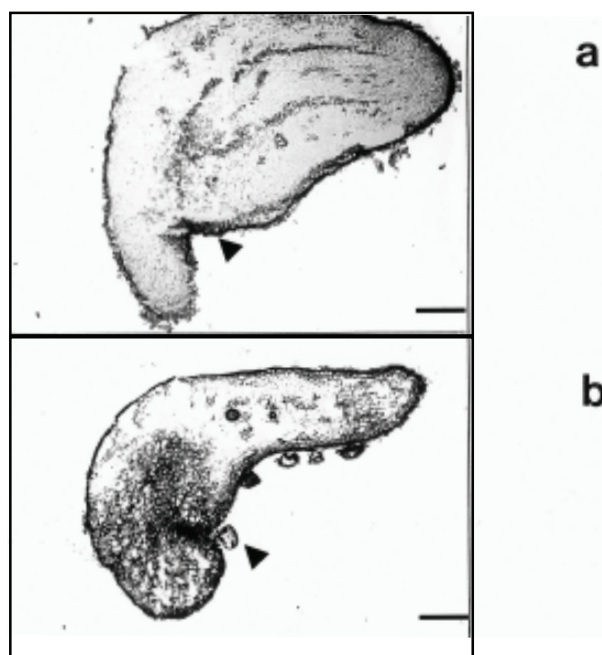

Fig. 6: **a**: Activity of leucine-aminopeptidase (longitudinal section). Bar: 500μm. Arrowhead points to the incision. **b**: Activity of peroxidases (longitudinal section). Bar: 500 μm. Arrowhead points to the incision.

Table

	Root gall (nodosity)			Non-infected root tip		
	Acid phosphatase	Peroxidase	Leucine-aminopeptidase	Acid phosphatase	Peroxidase	Leucine-aminopeptidase
Active feeding center	+++	+++	++	0	0	0
Stylet sheath	++	+++	+++	0	0	0
Root cap	+++	+++	+++	+++	+++	+++
Provascular strands	+++	+++	+++	+++	+++	+++
Endodermis	+++	+++	+++	+++	+++	+++
Parenchymal cells						
Central cylinder	0	0	0	0	0	0
Cortex	0	+/++	0	0	0	0

High activity: +++, moderate activity: ++, low activity: +, no activity: 0
Note: Each measurement is made relative to the activity of a particular enzyme in different tissues.

Conclusion

The phase of nodosity gall induction involves an increased metabolitic activity of peroxidases, acidic phosphatases and leucine aminopeptidases as well as the development of a storage tissue with starch granule incorporation. The feeding site around the stylet sheath is the center of metabolic activity involved in gall induction and maturation.

No significant differences, other than brownish cell layers at the feeding site, could be determined among resistant and susceptible cultivars. Possible resistance mechanisms such as hypersensitive reactions or the expression of PI (protease inhibitors) proteins involving anatomical and histochemical changes, may be triggered in the later stages of gall formation. The timing of these events should be in the focus of future studies. Research that aims to find genes for gall induction must study the early induction phases, whereas those focusing on possible resistance mechanisms should elucidate the gall maturation processes. These however may be influenced by many soil-borne biotic and abiotic factors under field conditions. Artificial conditions as applied in our aseptic dual culture system may hinder the development of a gene-based strategy applicable under commercial field conditions. The short gall induction phase however might be less affected by soil conditions. Thus, further studies that intend to investigate the gall induction process that initiates the phylloxera-grape interaction should consider the nodosity development, both with regard to developmental stages and fragmentation.

Acknowledgements

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