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Anatomical and tissue culture studies of Rupestris stem pitting-affected grapevines

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Abstract. Young shoots and leaf veins of Rupestris stem pitting affected grapevines were examined by light and electron microscopes. The disease infection in vines were verified by chip-bud grafting field indexing technique on healthy indicator plants at Davis, California. Degeneration of xylem vessel of young shoots was evident in some varieties of Rupestris stem pitting affected vines in light microscopic studies. In electron microscopic examination, virus particles were detected from phloem parenchyma cells of young shoots affected by Rupestris stem pitting. In infected cells, the virus appeared to exist as aggregates which were morphologically very similar to the closterovirus we found previously in shoot tissue of leafroll or corky bark affected vines. Diameter of these particles was about 7-8 nm. Virus concentration in the Rupestris stem pitting affected vines was low; and the virus associated small vesicles commonly found in leafroll or corky bark infection, were not detected. Tissue culture studies indicated that callus tissues that were derived from young shoots of Rupestris stem pitting or corky bark affected grapevines required less exogenous auxin for root differentiation as compared to those derived from healthy control vines. The role of auxin during symptom development of these disease needs to be further explored.

Key words: Auxin; Closterovirus; Electron microscopy; Grapevine; Pathological effects; Rupestris stem pitting; Tissue culture studies.

Introduction

Rupestris stem pitting is an important disease of potential threat to the grapevine cultivation industry worldwide (Prudencio, 1985). The disease was first recognized by A. C. Goheen in Davis, California in 1976. In the Foundation Plant Materials Service of the University of California at Davis, the disease incidence was first found to be high among cultivar selections introduced from Western Europe and Australia (Goheen, 1988). It was later noticed that the disease was fairly widespread in French-American hybrids growing in commercial vineyards in the northern and eastern United States and Canada. In field, wood symptoms of Rupestris stem pitting (Fig. 1) had certain similarities as compared to that of leafroll or corky bark affected plants. The diseased grapevines generally have reduced growth and vigor (Fig. 2); and both fruit quality and vine yield were greatly affected. The leaves on Rupestris stem pitting-affected vines might become small and slightly chlorotic, but would not become yellow or red like that on leafroll or corky bark affected vines. In some cases, unique stem pitting symptoms similary to that of corky bark infection (Goheen, 1988) were detected on the root stocks of affected vines. The impact could be as devastating as leafroll or corky bark infection; stringent quarantine measurement has

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thus been imposed by most grapevine growing countries to safeguard their grape cultivation from the plaguing of this disease (Goheen, 1980; Schöffling and Goheen, 1988). The similarity of disease symptoms among leafroll, corky bark, and Rupestris stem pitting, however, used to be a problem for related workers while doing disease identification. Up to now, the chip-bud grafting field indexing technique in which St. George (*Vitis rupestris*), Cabernet Franc (*Vitis vinifera*), Mission (*Vitis vinifera*), and LN-33 (Couderc 1613 × Thompson Seedless) were used as indicator plants was the main way to distinguish the three diseases (Table 1).

Fig. 1. Typical wood pitting symptom of Rupestris stem pitting on St. George (*Vitis rupestris*) on the right as compared to the healthy control on its left.

The viral nature of Rupestris stem pitting was well accepted by the related workers for long time mainly based on the evidence that it was transmissible by grafting. Among the three diseases mentioned, associa-

Fig. 2. Effect of Rupestris stem pitting on vigor of growth of Pinot Noir grapevine. Plants on the left line were affected by the disease; those on the right line were the comparable healthy control.

Table 1. *Symptom expression of Rupestris stem pitting (RST), leafroll (LR), and corky bark (CB) on grapevine virus indicator plants*

Indicator plants	Diseases		
	RST	LR	CB
St. George (*Vitis rupestris*)	Wood pitting below inoculation bud	—	Corky bark, wood grooving, rough bark
Cabernet Franc (*Vitis vinifera*)	—	Red leaves	Red leaves
Mission (*Vitis vinifera*)	—	Red leaves	Red leaves
LN-33 (Couderc 1613 × Thompson Seedless)	—	Red leaves	Red leaves, vine dwarfing, wood grooving

tion of closterovirus has been demonstrated in both leafroll and corky bark diseases (Tzeng and Goheen, 1984; Zee *et al.*, 1987). The etiological aspect of Rupestris stem pitting, however, has never been explored. In this study, the pathological effect and the viral association of this disease was investigated by light microscopy and transmittance electron microscopy respectively. Also, because symptom development of corky bark and Rupestris stem pitting diseases, such as late defoliation, dwarf growth, abnormal cell division and poorly differentiated conducting-tissue system suggested that hormonal metabolism in the infected vines was upset by the virus infection, part of the study was directed toward a possible role of auxin in disease development.

Materials and Methods

Light Microscopy

Healthy and diseased grapevine cuttings were collected from a virus disease indexing field at UC Davis during the winter of 1982. In a greenhouse at Davis, California, these cuttings were rooted in river sand and then transferred to UC mix (Baker, 1957) in 5-inch pots during the spring of 1983. Temperatures and relative humidity of the greenhouse were kept at optimum levels for plant growth throughout the experimental period. Plants that were approximately two months old were used for the experiment. The fourth internodes (counted from the shoot apex) of the young shoots were collected and fixed in formaldehyde-glacial acetic acid-alcohol (FAA). The materials were then thin-sectioned with a freeze-microtome to 18-20 μm thickness and the anatomical differences between the healthy and diseased vines were compared using a Zeiss light microscope. Plant materials examined in this experiment included Rupestris stem pitting-affected Cabernet Franc, Dattier, Gewurztraminer, Malbec, Pinot Noir, Sylvaner, White Riesling, and LN-33 (latent infection). Five plants per variety, three shoots per vine, and about 30 sections per plant were examined.

Electron Microscopy

Plant materials used for electron microscopic examination included all the above mentioned greenhouse materials used for light microscopic study as well as the Rupestris stem pitting affected Pinot Noir and the corky bark affected Cabernet Franc, LN-33, and St. George, collected directly from the 1982 grapevine virus disease indexing field and the grapevine virus collection plot at UC Davis. Midveins of grape leaves and bark tissues of young shoots were collected from either healthy or diseased vines during the summer of 1983. These tissues were cut into approximately 2 × 5 mm pieces, fixed in 2% glutaraldehyde (in 0.1 M phosphate buffer at pH 7.0) at 4°C, post fixed in 2% osmium tetraoxide (in the same buffer), dehydrated in a graded acetone series and finally embedded in epoxy resin mixtures (Shalla and Shepherd, 1981). Before ultrathin sectioning, all the embedded materials were trimmed under a dissecting microscope in order to position the vascular tissues. These tissues were then ultrathin-sectioned in a direction perpendicular to their long axis. The ultrathin sections were stained with uranyl acetate and lead citrate and examined by an AE-1 transmittance electron microscope.

Tissue Culture

Field-infected grapevines with Rupestris stem pitting (cv. Pinot Noir) and corky bark (cvs. Semillon and LN-33) were collected from a commercial planting or from research plots at UC Davis in the summer of 1983. Ten vines per variety, fifteen shoots per vine were used in each experiment. The 4th and 5th internodes of the young shoots (counted from the apex) were collected from the plants and cut into pieces approximately 1 cm in length. These materials were surface sterilized with 1.75% Na-hypochlorite for 15 min and rinsed 3 times with sterile distilled water. The basic components of the culture medium contained the inorganic salts of Murashige and Skoog (1962), d-biotin 0.05 mg/l, folic acid 0.5 mg/l, pyridoxine HCl 0.5 mg/l, nicotinic acid 5.0 mg/l, glycine 2.0 mg/l, thiamine HCl 0.5 mg/l, sucrose 30 g/l, and Difco Bacto agar 9.0 g/l. Different amouts of NAA (0.5, 1.0, 2.0 and 4.0 mg/l) and kinetin (0.5, 1.0, 2.0, and 4.0 mg/l) were then added to the basal medium for comparison. The pH of the culture medium was adjusted to 5.8 before autoclaving. All culture media were autoclaved at 15 lbs, 121°C for 15 min and then dispensed equally (32 ml/dish) to 9 cm Petri dishes. Six pieces of surface-sterilized internode tissues were then evenly inoculated onto the agar plates. The cultures were kept at 25°C under Sylvania "Gro-lux" fluorescent lights (approximately 1 W/m^2) on a daily cycle of 16 h light and 8 h dark.

Results

Light Microscopic Studies

From most plant materials examined, no obvious pathological changes were observed. Detectable tissue abnormality was found only from Rupestris stem pitting-affected Pinot Noir (Fig. 3), White Riesling and Sylvaner grapevines (Fig. 4). On these varieties, xylem vessels, especially those close to vascular cambium tissues, appeared to be much smaller as compared to those in control healthy vines.

Electron Microscopic Studies

Among the samples examined, no virus was detected from Rupestris stem pitting affected leaf tissues. Long-flexuous rod shaped virus particles were detected from phloem parenchyma of young shoot of a Rupestris stem pitting affected Sylvaner (Fig. 5). Virus particles found in these infected tissues appeared to have an arrangement in parallel with the long axis of the vascular bundles (Figs. 6 and 7). Diameter of the virions was about 7–8 nm which was identical to the viral agents detected from corky bark affected Cabernet Franc (Tzeng *et al.*, 1989). Virus concentration in infected cells was very low. Generally, only about 3 cells in one vascular bundle contained virus. In each infected cell, usually only one virus aggregate could be detected.

An outer membrane which enclosed the virus aggregates was observed occasionally (Fig. 5). The virus particles seemed to be confined in a small area in the infected cell and spreading into the entire cell was not observed. Arrangement of virions in the aggregate resembled that of those found in leafroll and corky

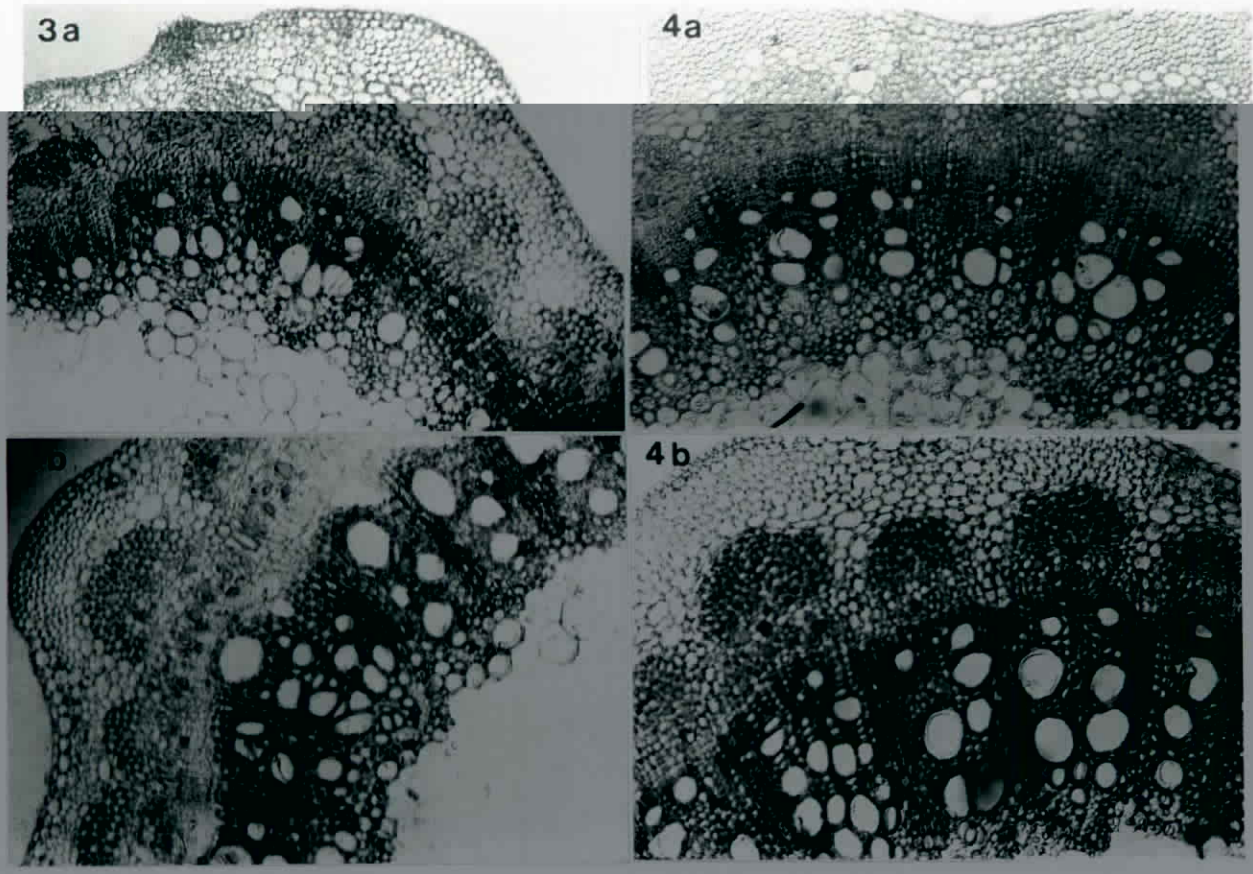

Fig. 3. Cross section of young shoot (4th node) of (a) Rupestris stem pitting affected and (b) compared healthy control Pinot Noir grapevine. (100 ×)

Fig. 4. Cross section of young shoot (4th node) of (a) Rupestris stem pitting affected, and (b) compared healthy control Sylvaner grapevine. (100 ×)

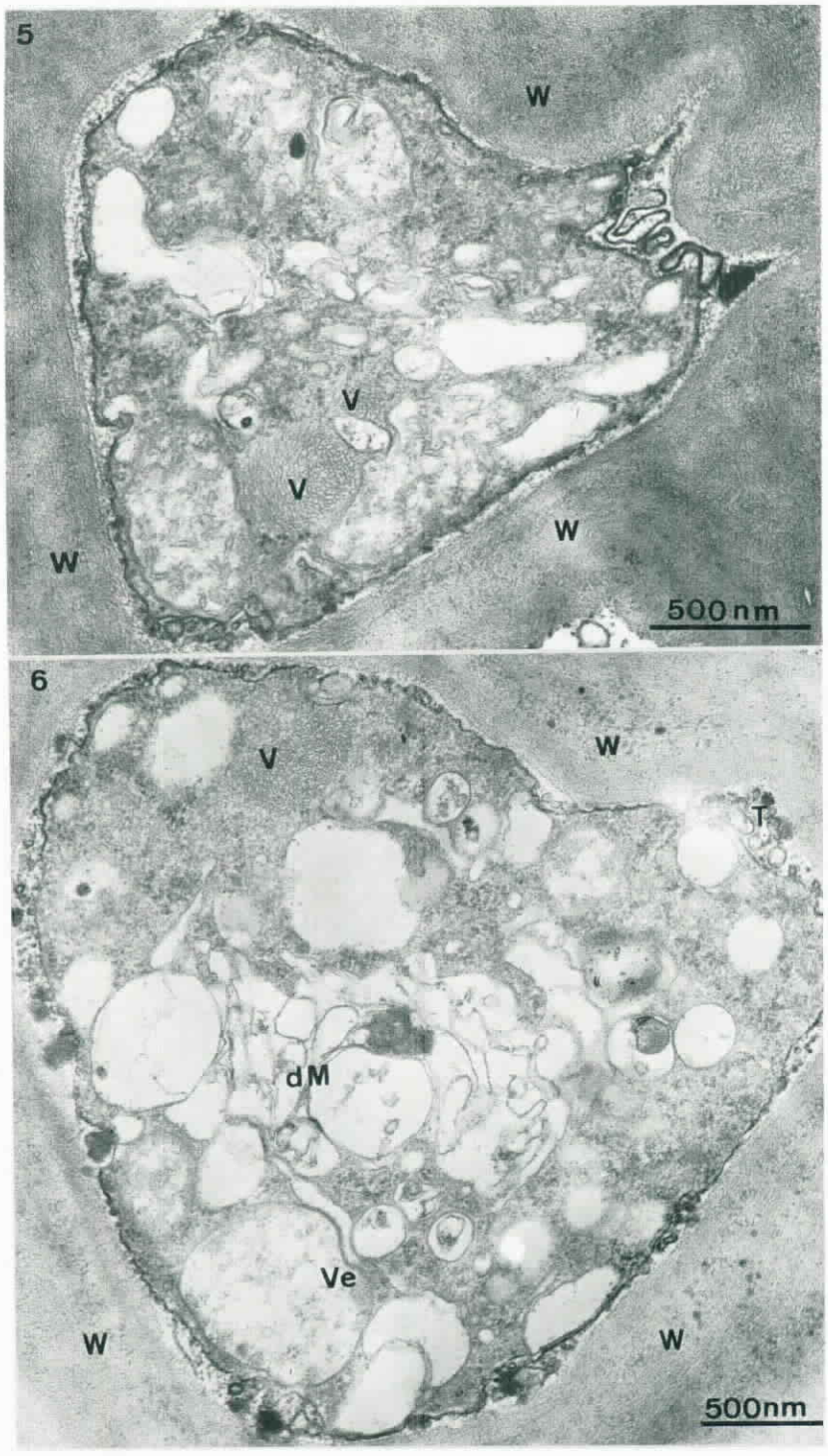

Fig. 5. Phloem parenchyma cell of Rupestris stem pitting affected Sylvaner grapevine. Virus particle lie either in parallel or perpendicular to the axis of the vascular bundle.

Fig. 6. Degeneration of membrane system and appearance of abnormal vesicles in Rupestris stem pitting affected phloem parenchyma cell. Tubules were found between the plasmalemma and cell wall.
(dM, degenerated membranes; T, tubules; Ve, vesicles; V, virus; W, cell wall.)

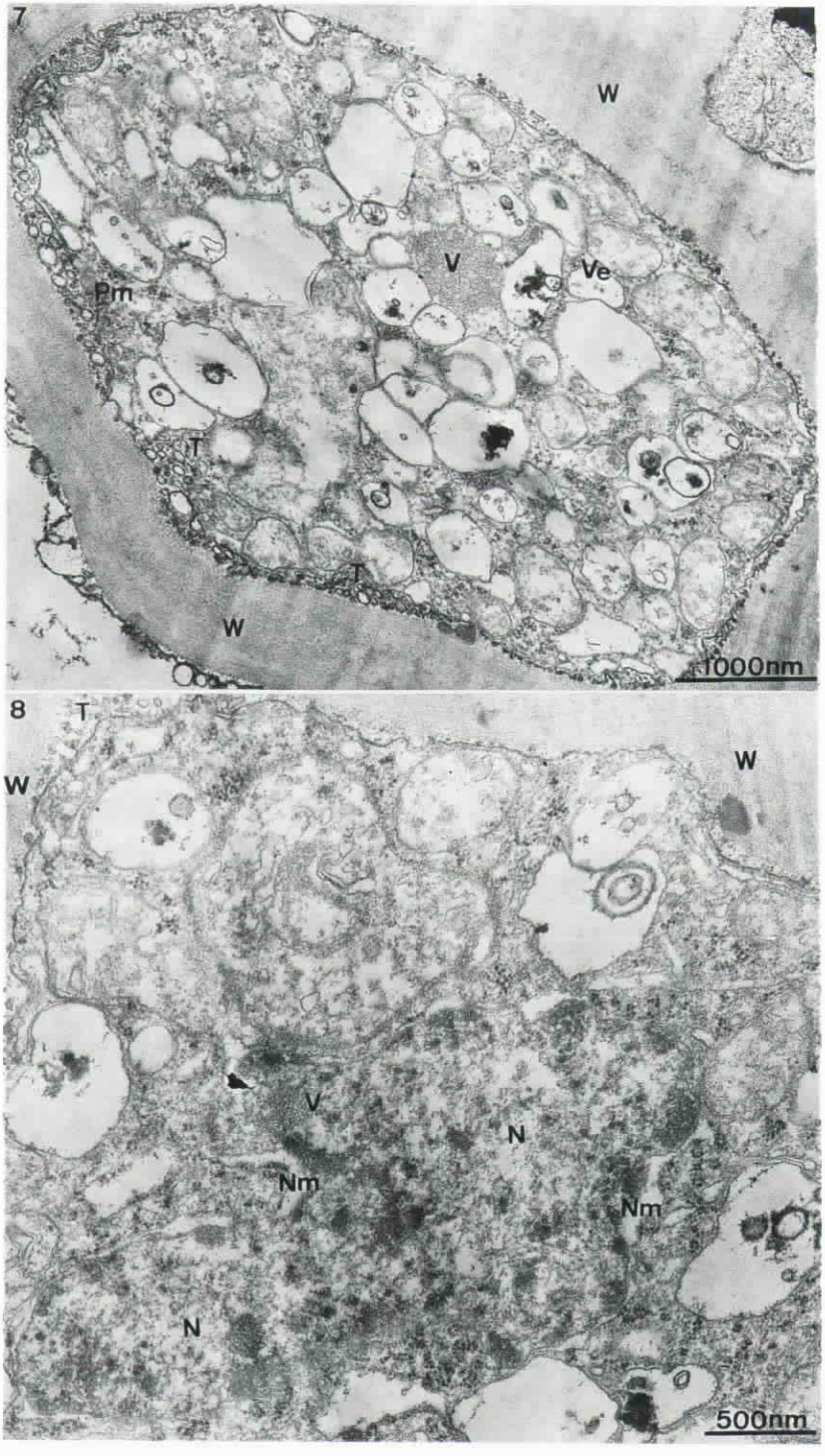

Fig. 7. Abundant tubules reside between cell wall and plasmalemma of Rupestris stem pitting affected phloem parenchyma cell. A virus aggregate was found residing among abundant abnormal vesicles.

Fig. 8. Virus particles detected from nucleus of a Rupestris stem pitting affected cell. The nucleus was deformed into a lobe-like structure and was densely stained.
(N, nucleus; Nm, nuclear membrane; Pm, plasmalemma; T, tubules; V, virus particles; Ve, vesicles; W, cell wall.)

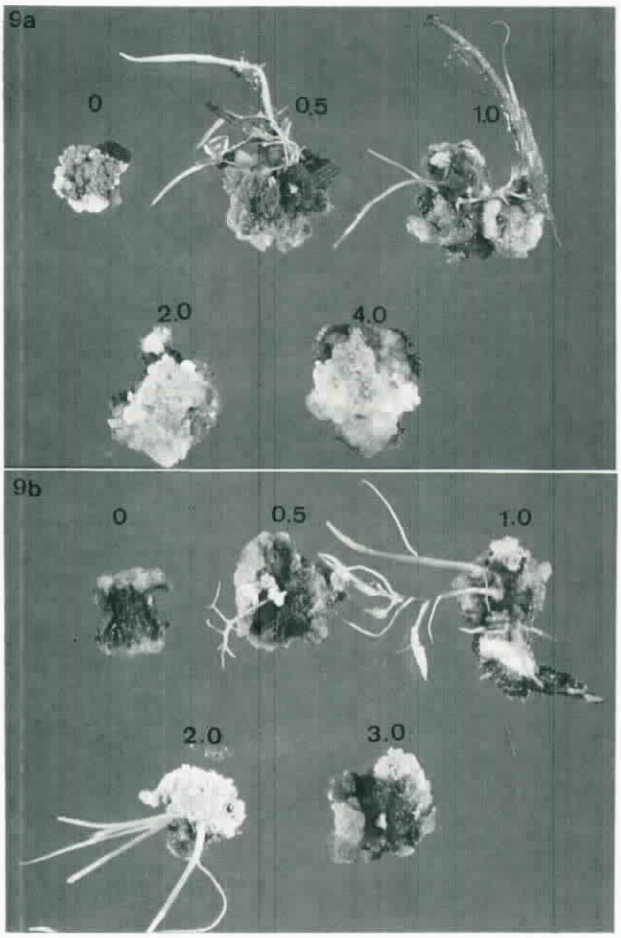

Fig. 9. Effect of exogenous NAA (mg/l) on root differentiation of callus tissue derived from (a) Rupestris stem pitting affected, or (b) compared healthy Pinot Noir grapevine.

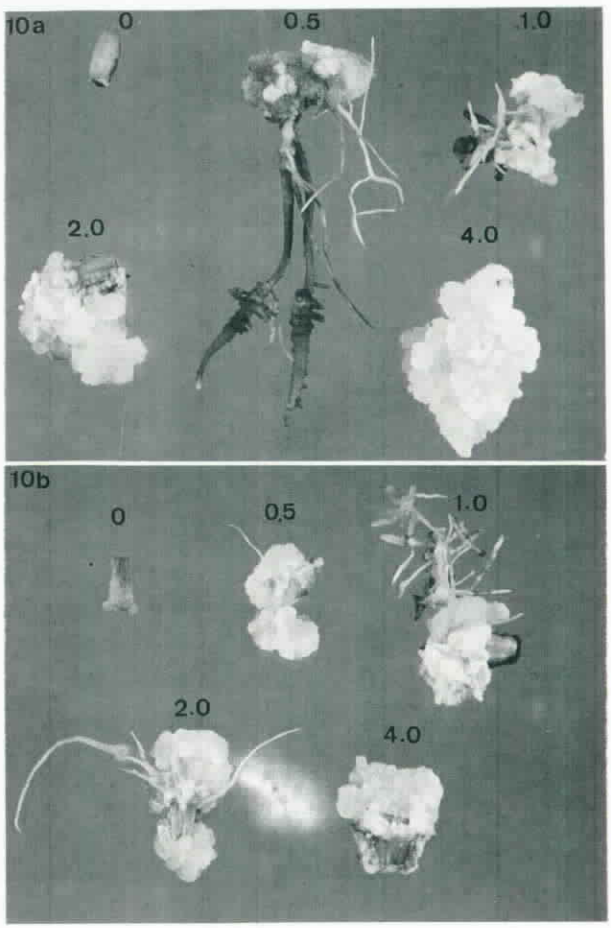

Fig. 10. Effect of exogenous NAA (mg/l) on the root differentiation of callus tissues derived from (a) corky bark affected, or (b) compared healthy Semillon grapevines.

bark affected vines. Unlike leafroll or corky bark diseases, circular tubule bodies were found abundantly between cell wall and plasmalemma of the Rupestris stem pitting-affected Sylvaner grapevine tissues (Fig. 7). The tubules embedded in between the cell wall and plasmalemma were sometimes detectable in cells without evidence of virus accumulation. However, the number of tubules in such cells was much less than those in virus accumulated ones. Degeneration of cell organelles in infected tissues, especially the membranous structures, was similar to either leafroll or corky bark diseases studied (Fig. 6). The virus particles resided mostly in the cytoplasm, while in some cases, virus-like particles could also be found in the nucleus of infected cells in which other cell organelles in the cytoplasm remained without recognizable damage (Fig. 8). Nuclei that contained virus substances usually became amorphous, lobed and densely stained. In addition to Sylvaner grapevines, similar cellular alterations were also observed in Rupestris stem pitting-affected White Riesling and Dattier grapevines although an attempt to find virus particles in these varieties was not successful.

Responses of Healthy and Diseased Tissues to Exogenous Naphthalene Acetic Acid (NAA) in Tissue Culture Studies

Callus that developed from Rupestris stem pitting-affected Pinot Noir tissues in response to different concentration of NAA showed no significant difference as

compared to that from healthy tissues. Both healthy and diseased tissues had most callus development in the medium with 4 mg/l NAA. However, it was noticed that 0.5 mg/l of NAA was the optimum concentration for root differentiation from diseased tissues, which occurred about 3-4 weeks after incubation (Fig. 9a). In contrast, 1.0 mg/l of NAA appeared to be optimal for root differentiation from healthy tissues (Fig. 9b). Similar responses were observed on corky bark-affected Semillon grapevine tissues in which optimal concentration of NAA required for root differentiation from diseased tissues (0.5 mg/l) was lower than that for healthy tissues (1.0 mg/l)(Figs. 10a & b). On the culture medium with 2 mg/l NAA, callus tissues developed from healthy vines still had fairly good root differentiation, however, no root growth was observed from callus tissues originating from diseased vines when the content of NAA in the medium was raised to this level.

Discussion

In the electron microscopic study proceeded, the long flexuous rod virus particles were detected strictly from Rupestris stem pitting-affected Sylvner grapevine. In comparable healthy plants, these virus particles were not found. Because the identity of individual disease in this experiment was verified by paralleled field-indexing tests, the risk of confusing several viruses from complex infection was avoided. Association of virus particles with the studied disease was quite evident because no virus particles were detected from the comparable healthy plants. In Rupestris stem pitting-affected Sylvaner grapevine, virus particles were detected only from young shoots of Sylvaner that showed no obvious leaf symptoms. The diamenter of these particles appeared to be fairly close to that detected in corky bark-affected grapevines, but it was substantially smaller as compared to that of leafroll virus (Hu *et al.*, 1990; Tzeng *et al.*, 1989). Whether or not the morphological distinction have any connection to the differentiation of the studied viral taxon remained to be further explored. Morphology of virus particle aggregates resembled those found in shoot tissues of leafroll-affected Cabernet Franc grapevines (Tzeng *et al.*, 1990). Also like grapevine leafroll virus cles of Rupestris stem pitting in the infected cell mostly had an arrangement in parallel with the long axis of the vascular system (Figs. 6 and 7). This might indicate that these particles were ready to be moved upward or downward in the vascular system. The tubules embedded in the cell wall of Rupestris stem pitting-affected Sylvaner were similar to those reported from bean pod mottle virus (BPMV) infected plants. Kim and Fulton (1971) noticed that some tubules in BPMV infected cells contain virus particles. However, in this study, virus particle was not observed in tubules detected from Rupestris stem pitting-affected grapevine tissues. Tubules in BPMV-infected cells, were considered to represent an active membrane system that associated closely with virus replication. Because no virus particles were detected from tubular structures in Rupestris stem pitting-affected grapevines, their possible involvement in disease development remains to be understood.

Up to now, field indexing is the main test for diagnosis of Rupestris stem pitting of grapevines. Disease symptoms developed on chip bud-grafted St. George and LN-33 is the major characteristic depended to distinguish corky bark from Rupestris stem pitting. It is unfortunately that most previous works did not include the examination of symptoms on stems of St. George for infection of Rupestris stem pitting; thus it is often uncertain whether the disease was present in the material examined (Hewitt, 1975; Martelli, 1980). Whether or not the leafroll or corky bark affected vine materials, which earlier workers investigated, were contaminated with Rupestris stem pitting should be reexamined. In this EM study, we have revealed the morphological characteristics of the virus particle and the plant parts where virus infection can be detected. The results should provide useful information for a further investigation on the purification of the virus involved.

In a Rupestris stem pitting affected grapevine, the considerably deteriorated development of xylem vessel and less well defined cambium tissue were the only microscopical symptoms detected from the tender meristematic tissues. The symptom expression of corky bark and Rupestris stem pitting suggests an upset of the hormonal metabolism of the infected tissues

considered to be one of the principal factors related to the symptom development. Duran-Vila and Semanick (1982) noticed that callus tissues that were derived from citrus excortis viroid infected tomato plants could not respond normally to exogenous auxins. Lack of recognition of auxin molecules by the infected cells, inactivation of auxin, or impaired synthesis of auxin was suggested by these authors as the plausible reason of the inability of the infected tissue to respond to normal auxin supply. The above shown data obtained from corky bark and Rupestris stem pitting-affected plant parts seems to suggest another story. Root differentiation seems to require less exogenous auxin as we found in the callus tissues derived from diseased plants. In contrast to this, no differential responses to exogenous kinetins was observed between the callus tissues developing from healthy and diseased plants (data not shown). The possible involvement of auxin in disease development of corky bark and Rupestris stem pitting needs to be further explored.

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Literature Cited

Baker, K. F. 1957. The U. C. system for producing healthy container grown plants. Univ. Calif., Agric. Sci., Div. Agric. Expt. Sta. Extension Service Manual 23. 322pp.

Duran-Vila, N. and J. S. Semanick. 1982. Effect of exogenous auxin on tomato tissue infected with the citrus exocortis viroid. Phytopathology **72**: 777-781.

Goheen, A. C. 1980. The California clean grape stock program. Calif. Agric. **34**: 15-16.

Goheen, A. C. 1988. Rupestris stem pitting. p. 53. Compendium of Grape Diseases. *In* R. C. Pearson and A. C. Goheen (eds.), American Phytopathological Society, St. Paul, MN. 93pp.

Hewitt, Wm. B. 1975. Graft transmission of grapevine wood pitting and flat trunk diseases. Plant Dis. Reporter. **59**: 845-848.

Hu, J. S., D. Gonsalves, and D. Teliz. 1990. Characterization of closterovirus-like particles associated with grapevine leafroll disease. J. Phytopathol. **128:** 1-14.

Kim, K. S. and J. P. Fulton. 1971. Tubules with virus like parti cles in leaf cells infected with bean pod mottle virus. Virology **43:** 329-337.

Martelli, G. P. 1980. Recent studies on virus diseases of grapevine in Europe with special reference to Italy. Internat. Centenn. Symp. Vitic. Enol., Davis, 1980.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15:** 473-497.

Prudencio, S. 1985. Comparative effects of corky bark and Rupestris stem pitting diseases on selected germplasm lines of grapes. M. S. Thesis. Department of Plant Pathology, University of California, Davis. 36 pp.

Shalla, T. A. and R. J. Shepherd. 1981. Laboratory Outlines in Plant Virology. University of California, Davis. 115 pp.

Schoffling, H. and A. C. Goheen. 1988. Selection of Grapevine. p. 74. *In* R. C. Pearson and A. C. Goheen (eds.), Compedium of grape Diseases. American Phytopathological Society, St. Paul. MN. 93 pp.

Tzeng, H. L. and A. C. Goheen. 1984. Electron microscopic studies on the corky bark and leafroll virus diseases of grapevine. Phytopathology **74:** 1142. (Abstract)

Tzeng, H. L., D. D. Tzeng, and A. C. Goheen. 1989. Electron microscopical studies on the leafroll and corky bark affected grapevines. Bot. Bull. Acad. Sin. **30:** 251-262.

Zee, F., D. Gonsalves, A. C. Goheen, K. S. Kim, R. Pool, and R. F. Lee. 1987. Cytopathology of leafroll diseased grapevines and the purification and serology of associated closterovirus like particles. Phytopathology **77:** 1427-1434.

葡萄莖孔病之解剖學與組織培養研究

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以顯微鏡檢視經加州大學芽接檢定確定罹患莖孔病之葡萄嫩莖與葉脈組織切片，光學顯微鏡檢視結果顯示，爲莖孔病感染之部份葡萄植株，其嫩莖木質部導管發育有明顯退化之現象，利用電子顯微鏡檢視罹病組織超薄切片進而發現，罹病之 Sylvaner 葡萄材料，其嫩莖韌皮部柔膜組織中有病毒顆粒存在，於被感染細胞中，此些病毒係以集結狀態存在，其形態與葡萄捲葉病及栓皮病所發現之長絲狀 Closterovirus 極爲近似，病毒顆粒直徑約在 7-8 nm，病組織中病毒濃度相當低，且未見有如葡萄捲葉病與栓皮病病毒感染常見的小空泡狀構造存在。除病組織切片檢視外，本研究並以組織培養法測試葡萄莖孔病感染對寄主植株之生理影響，試驗結果顯示由莖孔病或栓皮病罹病材料誘導產生的癒傷組織，其根的分化誘導較