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Title: **Double-stranded RNA Isolated from Grapevine Affected by Grapevine Stem-pitting Disease**

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Nucleic acid extracted from dormant cane phloem of grapevines affected by grapevine stem-pitting disease (SP) formed a high molecular weight (MW) band on 1.0% and 0.8% agarose gels. The band was unique to diseased vines. Other bands appeared from samples of both diseased and virus-free vines, but did not associate consistently with either. The nucleic acid species associated with stem-pitting-diseased vines was between 5.4 and 6.2 x 10^6 MW and consisted of at least some double-stranded RNA (dsRNA), as suggested by its resistance to digestion by DNase, and its green fluorescence under acridine-orange staining. Double-stranded RNA species from Geotrichum candidum (hypovirulent strain 223), Helminthosporium maydis (hypovirulent strain HM9), citrus infected with citrus tristeza virus(strain T505), and alfalfa mosaic virus-infected Ajuga reptans were used as size standards and high MW-bracketing markers. Stem-pitting-affected tissue samples were not recognized by grapevine

virus "A" immunoglobulins, but the size of the SP-associated dsRNA suggests that it might represent the replicative form of an 800 nm SP-associated virus. The possibility of using dsRNA electrophoretic patterns as a quarantine screening technique is discussed.

Double-stranded RNA Isolated from Grapevine Affected by Grapevine Stem-pitting Disease

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INTRODUCTION AND LITERATURE REVIEW

Grape Industry Problems

The grape industry in the Northwest began around Jacksonville, Oregon in the 1800s, died out, and experienced a renaissance in 1961. By 1980, 2000 acres had been planted. Most vineyards are located west of the Cascades in the north end of the Willamette Valley or in the southwestern part of the state. The industry in Washington currently consists of 44 wineries, with approximately 9,000 acres planted, mostly east of the Cascades.

The industry relies mostly on Vitis vinifera L. varieties; `Pinot noir', `White riesling',and `Chardonnay'. Most vineyards have been planted with virus-free stock, from the certified collection at the University of California (U.C.) at Davis. Unfortunately, the original selections lacked cold tolerance and winter hardiness. West of the Cascades, vines are needed that can grow vigorously in cooler climes. East of the cascades, the problem is freezing and winter-kill, so late-breaking, or freeze-tolerant varietal clones are

needed. Northwest viticulturalists seek both characteristics in varietal clones from grape-growing areas worldwide. Northern Europe is the most likely clonal source for the Northwest. Desirable import varieties include: `Gewurtztramiener', `White riesling', `Muller thurgau', `Chardonnay', `Pinot gris', `Sauvignon blanc', `Pinot noir', `Cabernet sauvignon' (P. Lombard, professor of horticulture, Oregon State University, Corvallis, Oregon, personal communication).

Presently, some 78 selections of wine grapes and rootstocks have been imported to the Agricultural Experiment Station at Oregon State University. About 40 of these selections were requested by Oregon growers, and about 50 more by Washington growers.

Some clonal candidates in the indexing system are infected with a number of viral diseases, in varying complexes..... "that could easily account for their so-called clonal differences" (Goheen, personnal communication). Graft- transmissable diseases, such as stem-pitting (SP, also known as "legno riccio", "stem-grooving", "rugose wood" and "bois strie'"), corky bark (CB), grapevine leafroll (GLR), grapevine fleck disease (GFL), fanleaf disease (FLD), and grapevine yellow speckle (YS), present a special problem to the industry because propagation of planting material is done exclusively by cuttings. Within the German introductions,

between 1968 and 1978, the United States Department of Agriculture Vitis Quarantine Facility, at Davis, CA, determined that 80% of the candidates indexed positive for virus diseases such as fleck (marbrure), GFL, GLR, and YS. "The Germans are propagating SP-infected varieties almost as fast as they are breeding and releasing them, for world-wide testing. For some reason, the horticulturalists and the pathologists are not cooperating....." (Goheen, personal communication). It is important that these viruses not be released into the relatively virus-free viticulture system in the Northwest.

Quarantines and import permits are required by federal law (United States Department of Agriculture, 1978) and there is general agreement in the Northwest among growers and researchers to keep Oregon grapes virus-free (H. R. Cameron, professor of plant pathology, Oregon State University, Corvallis, Oregon, personal communication). Virus indexing is done in compliance with this agreement. The technique is very sensitive, and theoretically can detect one virus particle in a sample of tissue from a candidate clone. Virus-infected vines can then be heat-treated to rid them of viruses, or rejected from the screening system. Unfortunately the indexing system is limited to indicators of known viral

or graft-transmissable diseases, and is very time-consuming.

The chances are good that a disease like SP could infiltrate the industry or be exported from the Northwest despite quarantine. Even when indexing is correctly performed, SP often takes years to show up on the indicator vine. Some combinations of variety and rootstock do not give symptoms. If varietal material is contaminated and propagated, then previously virus-free rootstock material could be contaminated on a large scale. This could be serious, since SP-affected vines die less often if used as rootstock than if used as scion-wood. Contaminated rootstocks do not pose such a severe problem in Oregon, however, since own-rooted varieties are popular due to the low prevalence of many important grape pests, such as phylloxera and nematodes.

Oregon has developed into a major growing area for `Pinot noir'. Some clones appear to do very well, even when SP-affected (S. Price, horticulturalist, Oregon State University, Corvallis, Oregon, personal communication). The demand to export `Pinot noir' clones such as `Chenin blanc', `Pinot blanc' (`Chardonnay'), `Pinot gris', `Blauer spatburgunder', `Fruhburgunder' and `Mullererebe' has recently increased (H. Becker, viticulturalist, University of Geisenheim, Geisenheim, Federal Republic of Germany, personal communication). The

quarantine-indexing must be reliable in dealing with disease latency, and irregular distribution of the disease agents within and among plants (Abrasheva, 1985) if any varietal export plan is to succeed.

The quarantine period for candidate clones may last two years or more. In the indexing procedure, a bud from the candidate clone (donor) is "chip-budded" onto an indicator vine. The graft is made so that the donor chip can be checked periodically to see that it is alive. The longer the donor tissue lives, the better the chances of transmitting viruses which may be naturally slow in intercellular transmission. It is important to remeber that viruses are obligate parasites and can only "live" in living tissue. If the donor chip dies soon after the graft, the virus may not have an adequate chance to move into the indicator. Under the best of conditions, the bud will graft permanently onto the indicator. Transmission is considered to have occurred if the donor bud is still alive several months after the graft.

Indexing is usually done on three standard indicators which detect the known transmissable diseases that cause yield and quality losses in grapes; *V. rupestris* Scheele. variety `St. George', *V. vinifera* variety `Mission' or `Cabernet franc', and `LN-33'. The full indexing process at OSU and at U.C. Davis uses `LN-33' to detect CB (foliar and stem symptoms) and GLR. *V.*

vinifera variety `Baco blanc' will show FLD and GLR. `Mission' is used to detect YS and GLR. `Cabernet franc' is also used to detect GLR. It is used in Oregon because `Mission' lacks cold hardiness. `St. George' can reveal foliar symptoms of fanleaf disease and fleck. Symptoms of CB and SP are also visible on `St. George' by peeling the bark off the stem (Uyemoto, Cummins, and Abawi, 1977). The indicator vines are typically planted out in the field or in a screenhouse, and are observed for at least two years. The disease, if transmitted, may take years to become symptomatic. Some diseases will show symptoms rather quickly. At Davis, in 1979, `LN-33's budded from Aguascalientes vines showing CB showed CB symptoms in four months (Teliz, Goheen, and Valle, 1980). Potted indicators may be maintained in the greenhouse, but SP and CB are more difficult to spot in the greenhouse than in the field. Foliar virus symptoms may also be masked by foliar symptoms (such as chlorosis due to lighting conditions, or reddening and rolling due to potassium deficiency) associated with pot-bound vines.

Heat treatment is well established as a plant viral curative. It has been effective with SP. In heat-treated *V. vinifera* variety `Servant' clone A94 (affected by SP) no SP was seen on susceptable recipient rootstock *V. riparia* Michx. x *V. berlandieri* Planch. `Kober 5BB'. When `Servant' clone A94 recieved no heat treatment, symptoms

on the recipieant rootstock `Kober 5BB' were clear. . The minimum duration for effective heat-treatment of SP was 146 days. Eighty-two percent of the subjects were cured within 300 to 693 days. GLR was eliminated after 300 days under the same conditions. There was no elimination of vein mosaic or vein necrosis (Legin, Bass, and Vuittenez, 1979).

Meristem culture, and use of various anti-viral compounds, such as (S)-9-(2,3-dihydroxypropyladenine (DHPA) and 9-beta-D-arabinofuranosyladenine (vidarabine) have not been successful at curing GLR-diseased vines. The compound
1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) "looks promising" for GLR, but no data is available for SP (Monnette, 1984).

Enzyme-linked immunosorbant assay (ELISA) for grapevine diseases will doubtless become an important part of the quarantine system, and is now being developed. Antisera production for ELISA requires mechanical transmission and purification of viruses. Such production from viruses of woody perennials is difficult, and has been slow to develop. Part of the problem is in distinguishing one disease from another. Immunodetection of viruses in imported varieties can only be useful for determining if a vine is free of the virus to which the antisera has been prepared. Association of an antisera to

a particle is possible, but associating that particle with a specific disease is more difficult. Details will be discussed later.

Disease Description

Stem-pitting disease has been observed in California only since 1971 (Hewitt, 1975). But it ranks close to leafroll in prevalence, and is distributed throughout grape-growing areas of the world (Eggar et al., 1985). An Italian study showed that among 200 varieties collected from the U.S.A., France, and West Germany, five graft-transmissable diseases were prevalent. They were (in descending order of prevalence) : GLR > SP > FLD = yellow mosaic = vein banding (Egger, Borgo, and Antoniazzi, 1985).

In Aguascalientes, Mexico ,`LN-33' and 49 other varieties were planted in a foundation block in 1968. Corky bark and SP were in 33% of the local vineyards and 21 out of 32 varieties surveyed. Stem-pitting occurred in all local vines that showed CB symptoms (Valle and Teliz, 1983).

Seven thousand ha. of vines support the table grape and brandy industry in Aguascalientes. Stem-pitting and CB are prevalent. Most vineyards must be re-planted every eight to ten years because of the low yield caused by these diseases (Teliz et al., 1980). By 1978, all but two

`LN-33' vines had SP and CB. Of the varieties planted, `Tokay', `Malaga', `Champagne', `Cardinal', `Exotic', and `St. Emilion' were the most severely affected. This amounted to the first evidence for the spread of SP or CB. Although SP has been associated with fanleaf, none was seen in Mexico on the foundation vines, nor had Xiphinema index been associated with the diseased vines (Teliz et al., 1980).

Stem-pitting was first described in Italy in 1959 on Italian rootstocks: V. berlandieri, V. rupestris and V. riparia crosses (Graniti and Ciccarone, 1961; Ciccarone, 1961). Symptoms include delayed spring bud push and pitting and grooving of the trunk. Progressive decline of whole scion follows. Vines decay during dormancy, especially following dry weather. This may be due to a transport insufficiency. Size reduction, low vigor, then die-back to the original graft union in are typical symptoms. The rootstock may survive and develop vigorous shoots. The bud union can be of two different diameters above and below the scion, the scion being narrower. The vine may not set fruit at all or may produce small clusters.

Stem-pitting is mainly graft-transmitted. It transmits well to `LN-33', `Kober 5BB', and `Teleki 5A' (Hewitt, 1975). In the Aguascalientes SP-survey, 11 out of 15 vines showed a high population of Longidorus

macrosistus Micol. and/or Xiphinema diversicaudatum Micol. (Teliz et al., 1980). Data on Plannococcus ficus (Targ.Tozz.) or Pseudococcus longispinus (Targ.Tozz.) transmission of SP is confusing because the source plants in the experiments invariably had more than one suspected virus disease (Caudwell and Dalmasso, 1984; D.J. Engelbrecht, research plant pathologist, Plant Protection Research Institute, Stellenbosch, South Africa, personal communication).

Annual rings can indicate the time of disease progress in the host (Hegedus and Lehoczky, 1978). The disease is usually evident from the second to the fifth ring. The xylem is reduced. Grooves and pits under the bark are seen as deep cracks and disorganized phloem in cross section (Garau, Prota, and Servazzi, 1973). There is an histological disorder in the medullary rays and radial woody sectors, accompanied by an increase in parenchyma. If new wood can compensate with an even annual ring, then no grooving or pitting occurs. In the absence of compensation, deep pits and cracks appear in the outer wood surface (Garau, Prota, and Servazzi, 1974).

The outer peridermal face of the phloem features elongated, furrowed depressions and pits. If the bark is peeled off, it will show elongated protrusions matching the pits and furrows in the outer phloem. These can be to

0.5 cm deep and to 6 cm long, and run parallel to the longitudinal axis of the cane. Near the bud union or roots, the angle becomes more oblique and disorganized. Bark thickening is also common, accompanied by the above-mentioned ray hypertrophy and parenchymatosis. Protrusions are shown to correspond to hypertrophied rays going from bark to xylem. Both CB and SP symptoms are sometimes present (Fortusini and Belli, 1978).

Some researchers believe that SP is a disease distinct from CB, and that stem-grooving also is a different disease reaction from SP (Goheen, personnal communication). Since SP looks like CB in `LN-33' and `St. George' (Hewitt, 1975), it is understandable when observations show a 100% correlation between CB and SP on `LN-33'. Some SP isolates in `LN-33' index negative for CB and results of SP indexing in `St. George' have been too erratic to make comparisons with `LN-33'(Engelbrecht, personal communication).

In the Aguascalientes study, plants with CB always had SP, but not all plants with SP had CB symptoms. Perhaps SP is the initial symptom of a single disease eventually showing both CB and SP. The SP appears first in the woody cylinder, but CB shows first in the cortex, or bark (Teliz et al., 1980). To add to the confusion, leaves can show a light, diffuse mosaic, along with malformation, such as short internodes, double nodes, and

other characteristics suggesting FLD (Graniti and Martelli, 1965; Savino et al., 1975; Azeri, 1980).

Stem-pitting and fanleaf are probably separate diseases. In a study at the University of California at Davis, both `LN-33' and `St. George' produced FLD without showing SP. Stem-pitting was, however produced by 18 FLD-free plants (A.C. Goheen, professor of plant pathology, University of California, Davis, CA., personal communication; Teliz et al., 1980).

A new disease, called "Kerner disease" (The variety V. vinifera variety `Kerner' = `Trollinger' x `White riesling'.) was described in W. Germany (Gartel, 1984), and is quite similar to SP. Grooves and pits appear on the wood at the union point, and the trunk is swollen more on the scion than on the rootstock. The symptoms develop more quickly than SP symptoms. Soil in contact with diseased vines will provide inoculum for non-infected vines. All types of vineyard soils have been affected. Disease incidence is typically from 5 to 20% in a vineyard, and often up to 75%. It is typical to see quick evolution of disease severity, with pitting on the scion, rather than on the rootstock (Bovey, 1984). As the disease progresses, leaves and inflorescences wither and dry and turn red-brown. Since no abscission occurs, leaves and clusters hang on until winter. Diseased vines

always die, either before bloom, or during beginnings of ripening.

Close examination of the cane and trunk shows longitudinal grooves and incursions into the xylem. The rootstock is discolored, while the scion appears normally colored. This is very similar to SP. The rootstock woody cylinder is often split. The rootstock dries slowly (water uptake is restricted). The scion develops superficial, adventitious roots, usually inadequate to support it, then the rootstock dies (Gartel, 1984).

Pleomorphic bacteria have been found in xylem tracheary elements of 2-year-old Kerner vines with Kerner disease (SP-like) symptoms. Rods measure from 0.6 to 3.0 um. There has not yet been any success at culturing this bacteria (Gartel, 1984).

"Jacquez" rootstock (a natural hybrid of V. vinifera L. x V. aestivalis Michaux x V. cinarea Engelman) demonstrated resistance to SP by showing an average of only 14% reduction in growth during the first three years on a `St. George' scion. This is very low for typically non-tolerant scion wood (Engelbrecht ,1973). In studies done in Israel, the variety `Zeini' shows no SP symptoms, while the variety `Dabouki' shows mild symptoms and only 40% disease incidence (Tanne and Arenstein, 1976). Combinations of SP and GFL met with varying resistance in varietal studies of `Monica' and `Italia'. `Monica' loss

went from 35% (SP alone), to 55% when GFL and SP occurred together. 'Italia' showed no difference in loss between having only SP and having SP and GFL (Garau, Cugusi, Dore, and Prota, 1984).

In rootstock graft comparisons for resistance, using Italian scion wood, disease-free rootstock gave better yields consistently. 'Kober 5BB' and '1103-P' gave the best yields, regardless of the origin. However, all trials showed erratic transmission of the disease. Sixty percent of the vines, regardless of donor symptom localization (scion or rootstock), were dead or fruitless six years after the graft. When the symptoms were on both scion and rootstock, the yields were lowest (Savino et al., 1984).

Virus Particle Research

Viral isolation from woody perennials is difficult because of phenolic browning (Morris, 1982). Strategies to overcome this problem include: transferring the virus to an herbaceous host, then proceeding to the antisera production without the browning problem; and purifying the virus (from the woody host) with the aid of low temperatures, anti-oxidants, and divalent cations. The isolate can then be used directly as an antigen in antisera production. The first technique lacks the specificity of working directly with the host plant. The

second technique is limited by browning, even at very low temperatures and in the best of conditions.

Leafroll-associated viruses are isoluble in sufficient quantities for electron micrography (Gugerli, Brugger, and Boveny, 1984; Mossop, 1985), but the length of the particle, combined with the browning problem, make handling the particulate isolate very difficult. Micrographs of leaf dips and thin sections are much more common than micrographs of purified viral preparations.

In Italy, a closterovirus-like particle was isolated from Nicotiana clevelandii (not from grape) following mechanical inoculation from a stem-pitting affected Italian Vitis vinifera `Pigato'. The "grapevine stem-pitting associated virus" (GSP-AV) was only isolated once from the above N. clevelandii A. Gray. All other attempts, by any other method, have failed. Symptoms in N. clevelandii included vein clearing and leaf crinkle.

The virus lost sap infectivity after storage at 50C for ten minutes, storage at 22C for six days, or storage at 5C for 15 days. The dilution limit for infectivity was 10^{-5}. The disease was not found to be seed transmissable, nor was it transmitted by Myzus persicae (Sulzer) or Macrosiphus euphorbia (Thomas).

The particle purification was done at pH 7.8 (using STE buffer: 0.1 M NaCl, 0.05 M Tris-HCl, and 1.0 mM EDTA). Particles were 11-12 nm in diameter, with a modal

length of approximately 800 nm. The pitch of the capsid was 3.6-4.0 nm.

The GSP-AV isolate was not serologically related to closteroviruses apple clorotic leafspot, beet yellows, carnation yellow fleck, heracleum latent, lilac clorotic leafspot, apple stem grooving, potato T, or citrus tristeza virus (Conti et al., 1980).

Later studies revealed that the coat protein MW was 2.2×10^4, with an A280/260 = 1.06. The nucleic acid core exhibited zero infectivity after RNAse A digestion, and was denatured to 50% at 52C in a solution of 0.15M sodium cloride, 0.015M sodium citrate. Full denaturation was reached at 75C. The RNA was calculated to be 2.55×10^6 MW (in a 2.4% polyacrylamide gel), and was composed of: U, 22.3; C, 24.9; A, 24.4; G, 28.4 mol%. The nucleocapsid gave a UV absorbance profile typical of a nucleoprotein with a low nucleic acid content: Amax/Amin = 1.12. Amax = 260. Amin = 243. A260/280 = 1.52, with no tryptophan shoulder at 290nm, also typical of a closterovirus (Boccardo and D' Aquilio, 1981).

Closterovirus-like particles associated with CB, SP, GLR, Merlot disease, Yellow speckle, GFL, fleck, and enation have been observed in electron micrographs. The particles (sometimes accompanied by spherical particles of 27 nm diameter) have also been observed in "clean vines" and in vines with disease complexes of SP, CB, and

GLR. The above brings into serious question the closterovirus etiology for any one disease (Corbett and Wiid, 1984).

Stem-pitting-associated particles, as well as isolates associated with GLR and CB, have been tested immunologically against antisera to known viruses, but results are still unclear.

Vines affected by CB and SP have yielded an isometric virus of 30 nm diameter, which was identified as a strain of arabis mosaic virus (Belli, Fortusini, and Vegetti, 1982). Leaf extracts from GLR-symptomatic vines did not react to antibodies to arabis mosaic virus, or potato virus Y (Gugerli et al., 1984). This suggests that the GLR and SP/CB diseases may not share the isometric particle as a causitive agent.

Grapevine stem-pitting associated virus antisera (Conti et al., 1980) was used, along with ISEM and EM to reveal that <u>two serologically distinct</u> closterovirus-like particles are widespread and frequent in grapevines. One or both particles were often, but not always, found associated with GLR. Some vines affected with SP had both, or just one particle. It has been suggested that GSP-AV be called "A" (grapevine virus "A", GVA), and the other "B" (GVB) (Milne et al., 1984).

Recently, a closterovirus was isolated from a `Waltham Cross' vine with severe GLR symptoms by

transmission with Plannococcus ficus (Targ. Tozz.) to N. clevelandii A. Gray. The particle (isolated from N. clevelandii) decorated in ISEM tests with antisera to GSPAV (Conti et al., 1980) otherwise known as "Grapevine virus A" (GVA) (Engelbrecht, personal communication). But ISEM work in New Zealand showed that antisera to GVA did not recognize GLR-associated particles isolated directly from GLR affected grapevines (Mossop, 1985).

Currently, Italian workers are still conducting research using vines affected by both GLR and SP, instead of just one disease at a time. Most of the 25 vines examined in a recent experiment contained GSP-AV "A" and "B", or just "B". Healthy vines yielded neither virus. Virus "A" was not mechanically transmitted back to grape from N. clevelandii, nor was "B" transmitted to any herbaceous host from the grapevine host (Conti and Milne, 1984). The very first step necessary to end the confusion in immunology, even without the isolation of associated particles, is to study one disease at a time, whenever possible.

Double-Stranded RNA Research

Early in the infection of the host, the replicative form (RF) of the viral genome is present as a double strand of ribonucleic acid (RNA, in the case of most viruses), separate from the coat protein (Stevens, 1983).

The dsRNA is more soluble in aqueous buffer than are coat protein subunits, or any plant protein or membrane constituent. The advantage of a dsRNA isolation technique is that it allows nucleic acid separation from all components of the browning process, very early in the isolation. If the correct conditions are maintained throughout the isolation, high molecular weight (MW) bands of dsRNA are visible following gel electrophoresis. The bands can be regarded as an in-situ characterization (a sign of disease) of early infection by the virus. They are direct, in being from the primary host (not from an assay species, such as N. clevelandii), and should not occur in virus-free plants of the same species (Dodds, Morris, and Jordan, 1984).

High MW dsRNA is not usually found in healthy plants it has been found in several varieties of bean P. vulgaris variety `Black Turtle Soup' (`BTS'). Some varieties are free of dsRNA. Partial characterization shows some homology to the host genome, as well as to the genomes of other varieties (Wakarchuk and Hamilton, 1985).

An efficient method for identification of low MW circular dsRNA in viroid-infected plants was developed at U.C. Berkeley. Samples are separated electrophoretically as to size in tube gels, then they are run in a second dimension on denaturing slab gels for analysis as to

circularity. If a healthy and a control are used, the patterns produced are mirror images of each other. Any spots appearing above the 45 degree line of RNA species are species with a structure different from that of the linear RNAs (Morris, 1983).

Some improvements have been made on the Morris procedure. A rapid procedure for small samples of herbaceous host dsRNA, has been developed, along with a scaled-up procedure for difficult hosts. This would include rosaceous hosts with low-titre viruses and tissue with high polyphenoloxidase (PPO) activity. The procedure consists of a basic phenol-chloroform extraction, using sodium laural sulfate (SDS), mercapto ethanol (MeETOH), polyvinylpyrolidone (PVP), Dieca, bentonite and TJM buffer (0.1M Tris, 0.5M NaCl, 0.01M MgAcetate, pH 8.5). This system was also used to detect dsRNA in Ribes sp., Rubus sp., and Fragaria sp. (Jones et al., 1984).

The current methods for isolating dsRNA segments of less than four million MW, use a column chromatography washing procedure twice, with DNAse digestion in between. Product yield is sufficient for detection of as little as 5 to 10 ng dsRNA on agarose gels, stained with ethidium bromide. For larger segments, CTAB is used in an isolation slightly different from the low MW dsRNA procedure.The method proved successful for detecting raspberry latent ringspot virus in Rubus spp. previously

only detectable by indexing. "Five prime" 32p labeling of gel-purified dsRNA was then used to produce a hybridization probe to detect common sequences in some other viruses (Kurppa and Martin, 1984).

Double-stranded RNA of roughly 12 and 11.5 million MW was isolated from `Corum' and `Napolean' cherries affected with necrotic rusty mottle disease (NRM). The 11.5 million MW dsRNA was also found in non-NRM-symptomatic trees (Moore and Cameron, 1985).

The most useful denaturation and length calculation procedure for dsRNA consists of electrophoresis of glyoxalated RNA in agarose gels and is preferred to other electrophoretic methods for determining the molecular weight of RNA molecules larger than about 1.7×10^6 (about 5000 nucleotides). It appears to be reliable for MW up to at least about 3.5×10^6 (or about 10,000 nucleotides)(Murant et al., 1981).

The dsRNA of stem-pitting-associated "grapevine virus A" in N. Clevelandii (Conti et al., 1980) was expected to be about 5.2×10^6 MW. It was observed to migrate faster than the dsRNA isolated from GLR-affected vines (found to be about 8×10^6 MW). Grapevine CB is also associated with dsRNA and closterovirus-like particles, but there was so much intervarietal variation in the lower MW dsRNA, that no solid conclusions could be

reached. Although affected vines did not always show high MW dsRNA, unaffected vines never did.

Research Purpose

The nature of this research is both applied and basic. The Oregon grape industry needs to import, certify and distribute new varieties more suitable to Northwest climes. The quarantine/certification step is the slowest part of the process. This research attempts to develop a quick and efficient SP-screening system that is useful against stem-pitting disease, for as many varieties of grapevine as possible. Since the causal agent of SP disease (believed to be a virus) has not yet been isolated, a relatively new technology (dsRNA isolation) will be used. This technique allows study of the disease without a having to isolate a virus particle.

The causal agent of SP in grapevine has not been isolated from grapevine hosts. This reflects a problem common to the study of low-titre, phloem-limited viruses in woody or perennial hosts. The host tissue presents a browning problem that makes research progress in this virus group very slow. This study also attempts to add to what little is known about stem-pitting disease in grapevines. Recent studies suggest a MW of about 5.2 x 10^6 for the replicative form of a virus associated with

SP. The dsRNA isolation technique will allow confirmation of this datum.

INTRODUCTION TO SECTION

The grape industry in the Northwest is limited by the lack of cold tolerance and winter hardiness in its most widely used varieties. Cold-tolerant and winter-hardy varieties, from grape-growing areas worldwide, are sought by Northwest viticulturalists in an effort to increase vineyard profits. Quarantine and virus-indexing for certification of candidate clones may require two years or longer before propagation and field trials can begin. The virus indexing system commonly used is very sensitive, but is limited to indicators of known viruses, and is very time consuming. Indexing for grapevine stem-pitting requires considerably more time than for diseases with readily visible foliar symptoms.

Enzyme-linked immunosorbant assay (ELISA) for grapevine diseases is now being developed. Antisera production for ELISA requires purification and mechanical transmission of viruses. Such production from viruses of woody perennials is difficult because of phenolic browning. Strategies to overcome this problem include: transferring the virus to an herbaceous host, then proceeding to the antisera production without the browning problem, and purifying the virus (from the woody host) under very low temperatures and non-oxidative conditions to use directly as an antigen in antisera

production. The first technique lacks the specificity of working directly with the host plant. The purification technique is limited by browning, even at very low temperatures and under the best conditions.

Grapevine stem-pitting ("legno riccio") disease was initially described in Italy in 1959, and has been observed in California only since 1971 (Hewitt, 1975). The disease causes delayed spring bud push and pitting and grooving of the trunk. Progressive decline of whole scion follows. Vines decay during dormancy, especially following dry weather. The vine may not set fruit at all or may produce small clusters. The disease is graft-transmissable and presents a special problem to the Northwest grape industry because propagation of planting material is done exclusively by cuttings.

Stem-pitting-associated, closterovirus-like virus particles have been isolated from a transfer-host (Nicotianna clevelandii A. Gray.) in sufficient quantities for electron micrography and physical analysis (Conti et al., 1980). The length of the virus was approximately 800 nm and it consisted of about 5% nucleic acid, 95% protein, typical of a closterovirus. The "grapevine stem-pitting associated virus" (GSP-AV: now called GVA) was only isolated once by the Italian researchers from the above host. Engelbrecht, in 1984, was able to develop antisera to GVA by isolating virus

from a 'Waltham cross' grapevine (showing leafroll symptoms) by transfer to N. clevelandii using Plannococcus ficus (Targ. Tozz.).

Vines affected by corky-bark and SP have yielded an isometric virus of 30 nm diameter, which was identified as a strain of arabis mosaic virus (Belli, Fortusini, and Vegetti, 1982). The data on particulate isolates of SP, as well as its relation to other viruses and grapevine diseases, are as yet unconfirmed.

Early in the infection of the host, the replicative form of the viral genome is present as a double strand of nucleic acid (dsRNA, in this case), separate from the coat protein (Stevens, 1983). The advantage of a dsRNA isolation technique is that it allows nucleic acid separation from most components of the browning process (such as polyphenol oxidase and vacuolar phenolic monomers) very early in the isolation. If the correct conditions are maintained throughout the isolation, high MW bands of dsRNA are visible following gel electrophoresis (Morris, 1982). The bands can be regarded as an in-situ characterization (a sign of disease) of early infection by stem-pitting virus. DsRNA bands are direct, in being from the primary host, and do not occur in healthy plants of the same species.

This study may be able to support earlier claims that a closterovirus-like virus with a ssRNA nucleic acid core of 2.55 x 10^6 MW is associated with stem-pitting disease in grapevine (Boccardo and d'Aquilio, 1981) by documenting the presence of high MW dsRNA found in grapevines affected by stem-pitting disease.

MATERIALS AND METHODS

Source-plants and indexing. Vitis vinifera L. varieties `Pinot noir' and `Pinot gris' were used as source-plants for dsRNA studies. Clones, originating from the University of California at Davis Foundation Seed and Plant Materials Service (F.P.M.S.), were selected from the Oregon State University Lewis Brown Horticultural Farm Mother Block Vinifera Plot 20 (LB). One `Pinot noir' clone (UCD 2AD) was taken directly from F.P.M.S., at Davis, where all clones had been virus indexed. Five of the clones (`Pinot noir' UCD 17, UCD 10, UCD 4, UCD 12, and `Pinot gris' UCD 3) were affected by SP disease, according to the U.C. Davis indexing. Four clones (`Pinot noir' UCD 2AD, UCD 2ALB [a duplicate of UCD 2AD], UCD1A, UCD 29, and UCD 13) were registered as virus-free. Two more clones (Colmar numbers; `Pinot gris' 142 and `Pinot gris' 156), also from the Lewis Brown Farm, were indexed at O.S.U. These were also included in the dsRNA analysis.

DsRNA extraction and electrophoresis. The double-stranded RNA (dsRNA) extraction was based on a method designed for rosaceous hosts (Morris, 1983), and modified for use on grapevines (D. Mossop, research plant pathologist, Department of Scientific and Industrial

Research, Auckland, New Zealand, personal communication), cherry, and pear trees (Moore and Cameron, 1985).

New leaves (taken one month after "bud break") served as the tissue source for UCD 17. All other clones were represented by phloem scrapings from two-year-old, dormant canes (frozen immediatly after cutting). Fifteen-gram samples were powdered in liquid nitrogen. The powder was transferred to a 200-ml centrifuge bottle, containing 45 ml (3 volumes) of double-strength (2X) salt-tris-EDTA buffer (STE: 0.1 M NaCl, 50 mM Tris-HCl, 1.0 mM EDTA, pH 8.0), 1.5% SDS, 30 ml buffer-saturated phenol (pH 8.0), and 1.0 ml 2-Mercapto-ethanol. Two volumes (30 ml) of chloroform-pentanol (25:1) were added, and the mixture shaken for 1 hour at 22 C. After 20 minutes of centrifugation at 10,000 g (22 C), the supernatent was recovered.

Supernatents from bottles representing 15 grams of tissue each were combined by-twos (about 90 ml supernatent, representing 30g tissue) and ethanol added to a concentration of approximately 16%. The dsRNA (representing 30 g tissue) was bound to 2.5 g Whatman CF-11 cellulose by gentle stirring of the supernatent/ethanol/cellulose mixture at 4 C for 1 hour. The cellulose was then washed twice by centrifugation at 5,000 g, using 15% ethanol in single strength (1X) STE buffer. The cellulose was then loaded into a column, and

washed with 15% ethanol-1X STE until the wash solution was clear. The dsRNA was eluted with three 3-ml volumes of 1X STE buffer, care being taken to rid the system of ethanol by air-purging the column after the wash and between the 3-ml elutions. The 9-ml eluate was then subjected to DNAse (10ug/ml bovine pancreatic deoxyribonuclease 1, Sigma Chemical Co., St. Louis, MO.) digestion, in the presence of 0.3 ml 1.0M MgCl, at 37 C, for 1 hour.

RNAse-free DNAse was prepared by calcium protection of the DNAse, followed by protein digestion (protease-K, from Tritirachium, Sigma Chemical Co., St. Louis, MO.). The DNAse-digestion was stopped by addition of 0.3 ml 0.5M EDTA. Ethanol was then added to 16%, followed by addition of 0.5 g of Biorad N-1 cellulose powder to each 9-ml volume. The dsRNA was bound to the cellulose by rotary shaking at 22 C for 20 minutes, then washed on columns, with at least 80 ml 15% ethanol-1X STE. The dsRNA was eluted with aliquots of 0.5, 1.0, and 1.0 ml 1X STE, again air-purging after the wash and between elutions. The eluate was spun at 10,000 g for 1 minute to pellet any cellulose. The supernatent was drawn off, mixed with 0.1 volume of 0.2 M sodium acetate and 2.5 volumes of ethanol, stored at -20 C, for at least 8 hours, pelleted, and resuspended in 20% sucrose-.001%bromophenol blue loading buffer. Usually, isolate

representing only 8 to 15 g of tissue was loaded into the gels.

Isolate (from `Pinot gris' c146), representing 45g of tissue, was suspended in 1.0 ml of 2X STE buffer and analyzed on a UV spectrophotometer at wavelengths of 260 and 280 nm. An absorbance of 21 at 260 nm is assumed for a 1 cm pathlength of a 1 mg/ml dsRNA solution (Billeter, Weissmann, and Warner, 1966). An OD_{260}/OD_{280} between 0.8 and 2.0 indicates a pure sample.

Two gel systems were used to analyze the dsRNA. One percent agarose gels gave good resolution of dsRNA bands and were run at 50V, 26mA and 22 C for eight hours. Gels of 0.8% agarose concentration displayed slightly less distinct bands, but were useful for establishing the presence or absence of a signal. These were run at 80V, 35mA, and 22 C for about four hours. Gels were of either 30 or 60ml total volume. The thicker gels provided more well volume and allowed more sample to be loaded.

Gels were stained with ethidium bromide (1.0 ug/ml) for 20 minutes in single-strength (1X) TBE (70mM tris-HCl, 70mM boric acid, 10mM EDTA, pH 8.0), then destained up to eight hours, with several washes of deionized water. Destaining continued until the dsRNA bands could be distinguished from the background. Bands were visualized on a UV transilluminator (TM-36, 302nm, UVP Inc., Fullerton, CA). Photographic exposure depended on

the intensity of the bands and the amount of background in the lanes of interest. Exposures were from 3 to 6 minutes, at f 5.6, using a yellow Kodak #15 Wratten filter and Polaroid Type 55 (ASA 50), Kodak Tri-X Pan (ASA 320), or Kodak Plus-X Pan (ASA 125) film.

The size of any dsRNA bands was determined by comparison to several dsRNA reference markers. Citrus tristeza virus (CTV) dsRNA isolate T505 (MW=13.3x10^6) was kindly supplied by Rodrigo Valverde of Allen Dodds lab at U.C. Riverside. Steve Garnsey, of the U.S.D.A. Horticultural Research Laboratory, Orlando, Florida, supplied leaf and stem tissue from Citrus aurantium L. `Madam Vinous' (Sweet Orange) infected with CTV isolate T4 (MW=13.3x$10^6$). Inoculation of our own Citrus medica (`citron') and Citrus aurantifolia (Christm.) Swingle (`mexican lime') trees was by patch graft. Both isolates were used as references. The T4 dsRNA was prepared by the above procedure from the sample sent by Garnsey (less than 4 grams). Helminthosporium maydis American Type Culture Collection [ATCC] #32450 HM9 (HM9), containing dsRNA (MW=6.3 x 10^6) associated with a fungal virus (Bozarth, 1977) was purchased from ATCC, and grown on V-8 juice agar (solid or liquid shaker). Geotrichum candidum strain 223 (Geo 223), containing dsRNA (MW=3.6 x 10^6) also associated with a fungal virus (Mor et al., 1984), was kindly supplied by E. E. Butler of U.C. Davis,

California. This fungus was maintained on solid or liquid potato dextrose agar.

Alfalfa mosaic virus (AMV) occurs locally and was maintained in Ajuga reptans. The dsRNA available from AMV-infected Ajuga sp was of 2.1, 1.4, and 1.2 x 10^6 MW (Jaspars and Bos, 1980).

DsRNA size estimation. Photographic negatives, and some gels, were scanned with a `Soft-Laser' scanning densitometer with graphic analysis software by Biomed Instruments Inc.(1020 S. Raymond Avenue #8, Fullerton, CA. 92531). This analysis enabled us to use negatiives or gels to detect bands, locate peaks, and measure their migration more precisely than would have been possible using only measurements from photographs.

The migration data obtained from the laser densitometer analysis, along with the published MW of our four markers was used to fit a reference curve for estimation of the MW of dsRNA bands from SP-diseased clones. A simple regression curve fitting program on `Stat-Graphics' statistical analysis software was used to generate the best fitting curve for our data.

To confirm the absence of dsRNA bands in the control clones, one set of samples from the virus-free clones (UCD 2AD, UCD 1A, UCD 13, UCD 29, and UCD 2ALB) was analyzed on a 0.8% agarose (Ultrapure DNA Grade, Biorad

Chemical Company, Richmond, CA.) gel loaded with extract from one and three times the amount of tissue in the standard analysis. For UCD 2A, UCD 1A, UCD 29, UCD 2ALB, 45 and 15g were loaded. For UCD 13, only 5 and 15g were loaded, because of a shortage of tissue. The electrophoresis was run, and the gels were stained, and analyzed as above.

Denaturation of dsRNA. Samples of dsRNA from **UCD 3** and **UCD** 4 (considered typical of clones with SP), AMV, Geo 223, and HM9 were denatured (McMaster and Carmichael, 1977) and compared, by agarose gel electrophoresis, to non-denatured samples of the same origin. Less than 20 ug dsRNA was suspended in 5.7 ul of 0.1M NaH2P04 , pH 7.0, and combined with 8.0 ul dimethylsulfoxide (DMSO) and 2.7 ul of deionized 6M glyoxal. This mixture was incubated at 50 C for 60 minutes, brought quickly to 20 C, combined with 4 ul of sterile loading buffer (50% glycerol, 0.01M NaH2P04 pH 7.0, 0.4% bromophenol blue) and loaded onto a 1% agarose gel. Gels were made up and run with 0.01M NaH_2PO_4 buffer, pH 7.0 (without ethidium bromide). Electrophoresis lasted eight hours at 50V in a recirculating buffer system. Acridine orange (30ug/ml, in 0.01M NaH_2PO_4, pH 7.0) was used to stain gels for 30 minutes. In this system, single-stranded nucleic acid fluoresces red while double-stranded nucleic acid

fluoresces green. When single-stranded bands are detected on gels, their MW must be estimated in reference to other single-stranded markers of known MW. Destaining was done in enamel trays, in the same buffer, for 1 hour at 22 C. Gels were photographed on the UV transilluminator and migration distances observed with the laser densitometer system.

RNase treatment. Stained gel lanes were treated with RNase (bovine pancreatic, 10ug/ml, Sigma Chemical Co. St. Louis, MO.) by incubating them in 100 ml sterile, distilled H_2O or 0.3M NaCl, at 22 C, for 2-3 hours (Dodds and Bar-Joseph, 1983). The gel segments were photographed and analyzed as above.

ELISA. David Engelbrecht donated antiserum, prepared in goat, against grapevine virus "A" (Milne et al., 1984). The grapevine virus "A" (GVA) had originated in a 'Waltham Cross' grapevine. Engelbrecht transferred it to Nicotianna clevelandii A. Gray. using Plannococcus ficus (Targ. Tozz.). The N. clevelandii isolate was the final antigen and the antiserum had a slide precipitin titre of 1/128 (Engelbrecht, personal communication). Edna Tanne contributed antiserum prepared in rabbit against grapevine potyvirus-like virus (GPV) (Tanne, Sela, Klein, and Harpaz, 1977).

Gamma globulins from both GVA and GPV antiserum were prepared by saturated ammonium sulfate precipitation, followed by dialysis against half-strength phosphate buffered saline (PBS). Half of each preparation was conjugated with alkaline phosphatase enzyme using 0.06% gluteraldehyde, followed by eight hours of dialysis in PBS. The conjugate was cross absorbed with a 1/20 dilution of frozen cane and fresh leaf extract in PBS, with Tween-20 detergent (Nutritional Biochemicals Corporation, Cleveland, OH), and polyvinylpyrrolidone-40 (Sigma Chemical Co., St. Louis, MO.). Gamma globulin well-coat and cross-absorbed conjugate were used in dilutions of 1/500 and 1/1000 for incubations with cane and leaf extracts. Initial incubation of extracts with the coated wells was 24 hrs, at 4 C. Incubation of the conjugate lasted 1 hr at 37 C. Thirty minutes after addition of alkaline phosphate (1mg/ml), the plates were read on a Gifford diagnostic microtitre plate reader and scores were recorded automatically.

RESULTS

All of the SP-affected `Pinot noir' and `Pinot gris' clones contained a dsRNA segment of similar size (Figures 1 and 2). Stem-pitting-affected V. vinifera varieties `Zinfandel', `Cabernet franc', `LN-33', and V. rupestris variety `St. George' were also processed, but did not yield detectable levels of dsRNA. The relative mobility of the SP-associated dsRNA band was close to that of the HM9 dsRNA marker (Figure 5). Stem-pitting-associated bands were not detected with the virus-free clones, even when three times the normal amount of tissue was represented in agarose gels (Figure 3). Laser densitometer analysis of the photographic negatives confirm this finding (Figure 4).

DsRNA isolation. The OD_{260} for the `Pinot gris' Colmar 146 dsRNA (isolated from cane tissue) was 0.034, indicating a concentration of approximatly 1.04×10^{-6} mg/ml, or 23 ng dsRNA/g tissue. The $OD_{260/280}$ was 1.01, well within the 0.8 to 2.0 range indicating purity. The brightness of this band (Figure 5) is about average for the dsRNA samples from the `Pinot noir' clones (except for UCD 17), indicating that the above yield of dsRNA/g tissue is typical of almost all of the SP-positive clones.

Size estimation of isolate. A 1.0% agarose gel, containing all reference dsRNAs, SP-positive `Pinot noir' UCD 12, virus-free `Pinot noir' UCD 1A, and `Pinot gris' clone Colmar 146, is shown in Figure 5. Migration percentages of the marker dsRNAs (measured from densitometer readings [Appendix A]), along with their reported molecular weights, are shown in Table 1. When all the dsRNA markers are included, the curve for this 1.0% agarose gel is described by the reciprocal function:

$$1/Y = a + bX \qquad (1)$$

where Y = MW, X = migration percentage, a = intercept = -0.270677, and b = slope = 0.311345. The R^2 value for this curve is 0.947. The MW of the HM9 marker dsRNA estimated by using this formula is 8.43×10^6, as compared with the reported 6.3×10^6 MW. The function does not fit the reported point in this region of the curve (Figure 6). This may be due to some inaccuracy in the reported value for the MW of the HM9 marker. It is also possible that the electrophoretic migration vs. MW is described by a complex, non-linear function.

When the CTV dsRNA marker migration distance is excluded, the curve for the 1.0% agarose gel is described by the simple linear function:

$$Y = mX + b \qquad (2)$$

where Y = MW, X = migration percentage, b = intercept = 9.53878, and m = slope = - 0.267318. The R^2 value for

this model is 0.995. The MW of the HM9 marker dsRNA estimated by equation 2 is 6.2 x 10^6, and is very close to the reported value of 6.3 x 10^6. Equation 2 fits well in this region of the curve (Figure 7). This is important because the SP-positive dsRNA band migrates faster, but very close to the HM9 marker dsRNA in the 1.0% and 0.8% agarose gels (Figures 5 and 1, Table 1). A linear model (excluding the CTV marker) of migration vs. MW for the 0.8% agarose gel (Figure 8) also fits the HM9 dsRNA migration percentage very well. The migration curve for the 0.8% agarose gel is described by a linear function, with slope = -0.199037 and intercept = 10.4157. This model has an R^2 value of 0.996. The molecular weight of the HM9 marker dsRNA, estimated by equation 2 (with slope and intercept from the 0.8% agarose gel migration) is 5.4 x 10^6. If both gel types are considered, the MW of the SP-associated dsRNA appears to be between 5.4 and 6.2 x 10^6 MW.

Denaturation of dsRNA. Geotrichum 223 marker dsRNA was less visible after glyoxal denaturation, as was the AMV dsRNA marker (Figure 9). The dsRNA bands from a `Pinot noir' clone (PN123), affected by grapevine leafroll and vein-banding diseases (indexed at O.S.U.), showed no reduction in visibility following glyoxal denaturation (Figure 9). The effect of denaturation on samples of SP-positive UCD 3 and UCD 4, could not be determined by

photographs, and a densitometer scan (Figure 10) showed no peaks for either denatured or native samples. Such was also the case for the HM9 marker dsRNA, which usually gives a very bright band. All bands fluoresced green, regardless of treatment, when stained with acridine orange, and fluoresced orange when stained with ethidium bromide. Staining with acridine orange never produced both green and orange fluorescence. Staining with ethidium bromide always produced orange fluorescence from all bands. The background produced by acridine orange staining of agarose gels made it very difficult to see if the denaturation process altered the migration distance of the dsRNA bands of interest. In some cases, with native and denatured samples (Figure 9 and 10, Geo 223), the fluorescence of the denatured sample dissappeared and no red-fluorescing bands were detected at greater migration distances, as would be expected for single-stranded RNA produced by denaturation. This indicates that the denatured material either migrated off the end of the gel,or was digested by residual RNase while in the ssRNA form.

RNase digestion. The results of RNase digestion are shown in Figure 11. Bands representing 15 g of tissue (about .35 ug dsRNA) from UCD 12 or UCD 3, were digested completely by RNase in water. Partial or complete loss of the band was also evident when lanes were exposed to 0.3

M NaCl, with or without RNase. This indicates that residual RNase may have digested the samples during some step of the procedure other than that in which the lanes were exposed to controlled amounts of RNase.

ELISA. ELISA tests using both GPV and GVA gamma-globulins indicted no recognition of stem-pitting disease by either antibody. There were no significant differences in A_{405} between wells representing clones which had "indexed positive" for SP, and wells representing virus-free clones (Appendix B). Only at a dilution of 1/500, did GVA gamma-globulin show a stronger average A_{405} (1.212 +/- .073 ODU) than the virus-free sample (1.04 +/- .097 ODU)(Appendix B).

TABLE 1. Electrophoretic migration percentages, curve-estimated MWs, published MWs, for both 1.0 and 0.8% agarose gels. All SP+ grapevine clones and dsRNA markers included

dsRNA source	% of Total Migration in 1.0% agarose gel. Figure 5.	Est' MW 1.0% ($x10^6$)	% of Total Migration in 0.8% agarose gel. Figure 1.	Est' MW 0.8% ($x10^6$)	Published Molecular Weight ($x10^6$)
CTV	09.8	—	16.8	—	13.3[a]
HM9	12.5	6.2	21.1	6.2	6.3[b]
GE0223	21.8	3.7	33.4	3.7	3.6[c]
AMV					
1	27.7	2.1	42.5	1.9	2.1[d]
2	29.5	1.6	44.3	1.6	1.4[d]
3	32.3	0.9	47.0	1.1	1.2[d]
UCD17	—	—	25.3	5.4	—
UCD10	—	—	25.3	5.4	—
UCD4	—	—	25.3	5.4	—
UCD12	12.5	6.2	25.3	5.4	—
UCD3	—	—	25.3	5.4	—

[a] Dodds and Bar-Joseph, 1983.
[b] Bozarth, 1977.
[c] Mor, Steinlauf, and Barash, 1984.
[d] Jaspars and Bos, 1980.

Figure 1. Agarose gel electrophoresis (0.8%) of dsRNAs from `Pinot noir' and `Pinot gris' clones affected by stem-pitting disease (SP+), (B-G), or clones certified to be virus-free (SP-), (H-L), compared with the dsRNA molecular-weight standards: AMV (2.1, 1.4, and 1.2 x 10^6 MW), GEO 223 (3.6 x 10^6 MW), HM9 (6.3 x 10^6 MW), and CTV strain T505 (13.3 x 10^6 MW). Lanes: **GEO/AMV**, GEO and AMV; UCD17, SP+ UCD17, cane; UCD17, SP+ UCD17, leaves; **UCD10**, SP+ UCD10, cane; **UCD12E**, SP+ UCD12, cane; UCD4, SP+ UCD4, cane; **UCD3**, SP+ UCD3, cane; **UCD2A**, SP- UCD2A, cane; UCD1A, SP- UCD1A, cane; UCD13, SP- UCD13, cane; UCD29, SP- UCD29, cane; UCD2ALB, SP- UCD2ALB, cane; CTV, CTV; HM9/GEO, HM9 and GEO. Electrophoresis was at 80V for 4 hrs. Gels were stained with ethidium bromide (1ug/ml), and destained with sdH_2O. DsRNA (approximately 0.16 to 0.92 ug) loaded into wells represented 7 to 40 g of host tissue. Migration is from top to bottom.

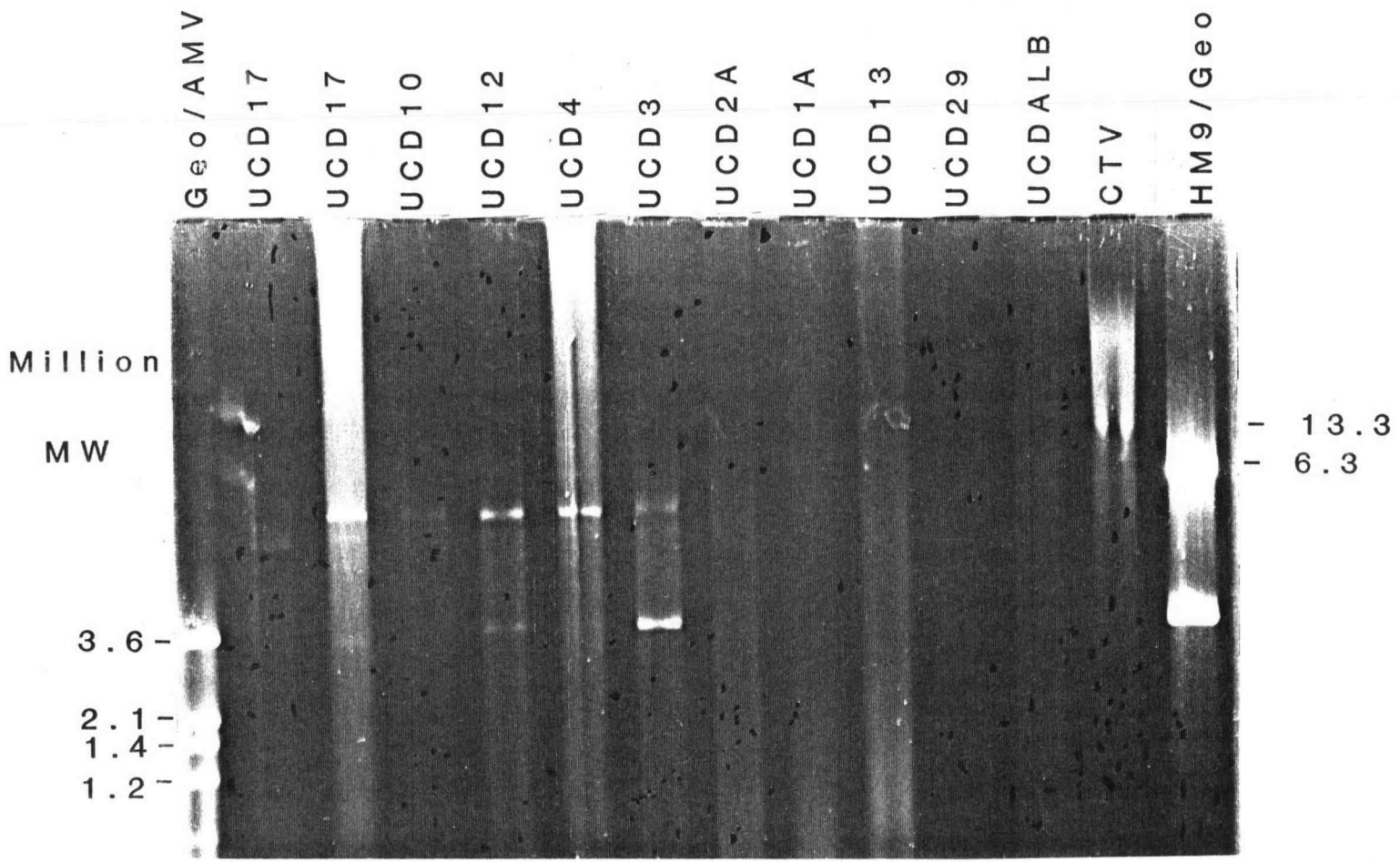
Geo/AMV
UCD17
UCD17
UCD10
UCD12
UCD4
UCD3
UCD2A
UCD1A
UCD13
UCD29
UCDALB
CTV
HM9/Geo
Million
MW
3.6
2.1
1.4
1.2
13.3
6.3

Figure 1.

Figure 2. Laser scanning densitometer readings of a photographic negative of some lanes of 0.8% agarose gel electrophoresis shown in Figure 1. Migration is from left to right (0 to 100% migration). Arrows in scans of UCD17, 10, 12, 3, and 4 point out signal from SP-associated dsRNA band. Small variation in length of some scans is due to changes in scan-speed.

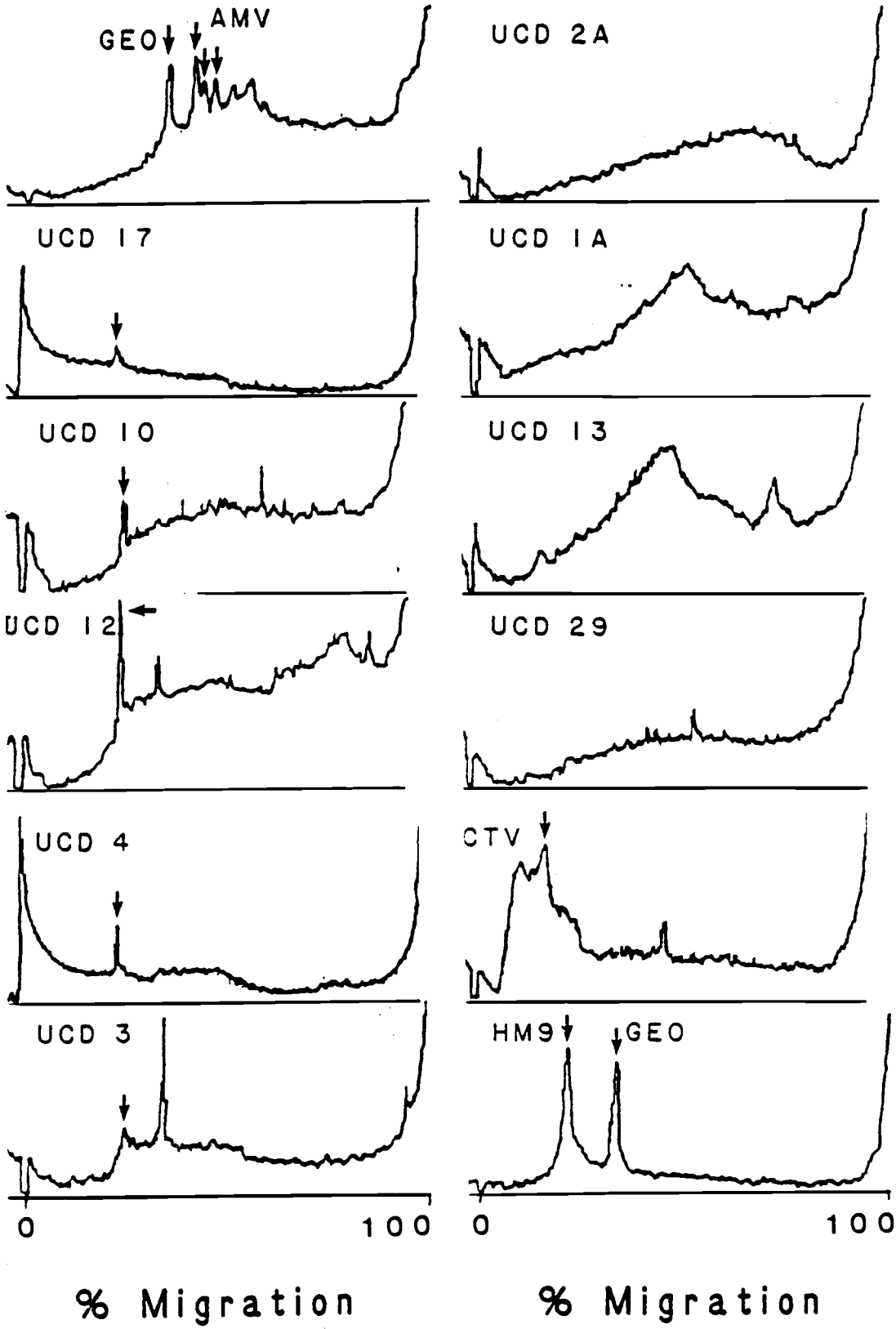
GEO
AMV
UCD 2A
UCD 17
UCD 1A
UCD 10
UCD 13
UCD 12
UCD 29
UCD 4
CTV
UCD 3
HM9
GEO
0
100
0
100
% Migration
% Migration

Figure 2.

Figure 3. Agarose gel electrophoresis (0.8%) of extracts from SP- 'Pinot noir' clones, compared with the dsRNA molecular-weight standards; AMV (2.1, 1.4, and 1.2 x 10^6 MW), GEO 223 (3.6 x 10^6 MW), and with SP+ UCD12. Lanes representing non- SP+ clones were loaded with extract representing one and three times the normal amount of tissue required to show a SP+ -associated dsRNA band. Lanes: **GEO**, GEO; **UCD12**, SP+ UCD12, 6 g cane; **UCD2A**, SP- UCD2A, 45 g cane and; **2A-15**, 15 g cane; **UCD13**, SP- UCD13, 15 g cane, and; **13-5**, 5 g cane; **UCD29**, SP- UCD29, 45 g cane, and; **29-15**, 15 g cane; **UCD1A**, SP- UCD1A, 45 g cane, and; **1A-15**, 15 g cane; **UCD2A**, SP- UCD2A, 45 g cane, and; **2A-15**, 15 g cane; **AMV**, AMV; **GEO**, GEO. Electrophoresis was at 50V for 12 hrs. Gels were stained with ethidium bromide (1ug/ml), and destained with sdH_2O. Migration is from top to bottom.

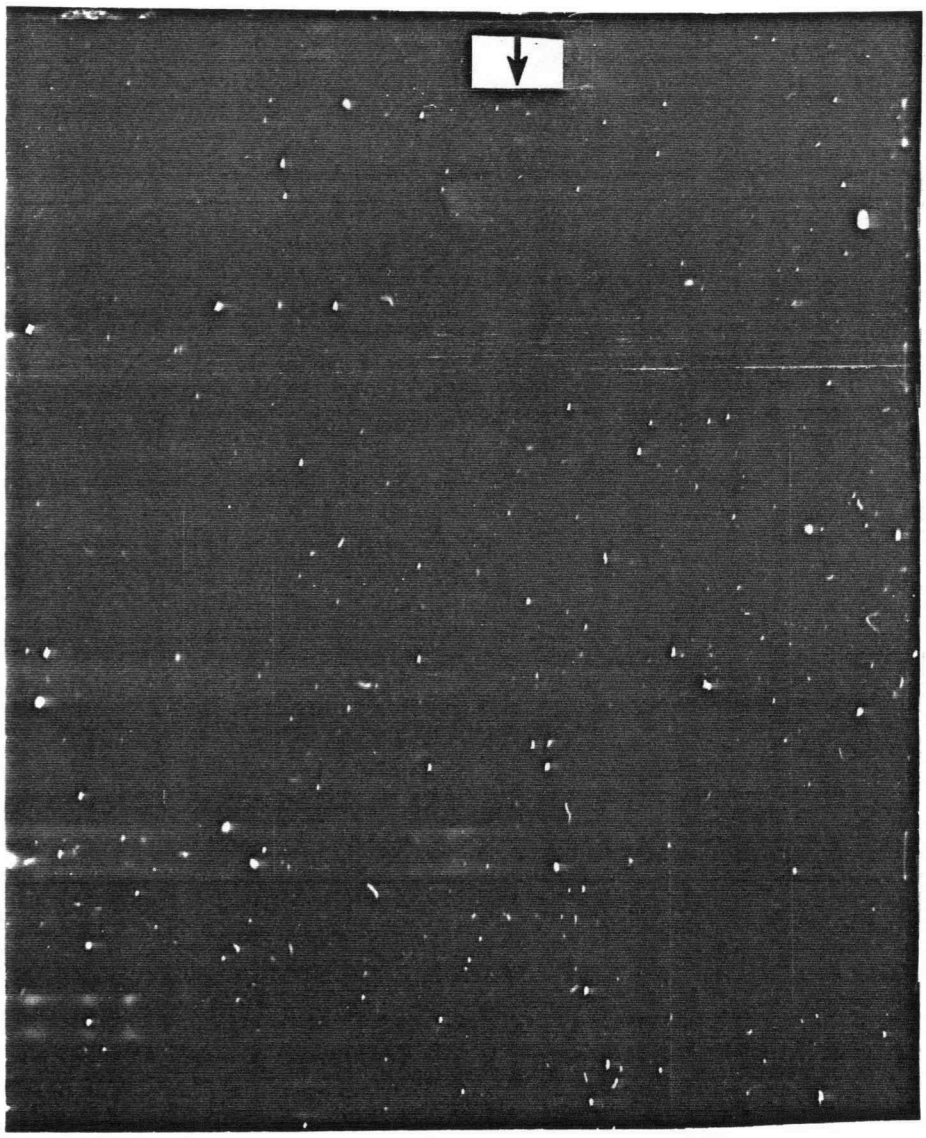
GEO
UCD12
UCD2A
2A-15
UCD13
13-5
UCD29
29-15
UCD1A
1A-15
UCD2A
2A-15
AMV
GEO

Figure 3.

Figure 4. Laser scanning densitometer readings of a photographic negative of triple-loaded lanes of 0.8% agarose gel electrophoresis shown in Figure 3. SP+ UCD12 is included as a reference. Migration is from left to right (0 to 100% migration). Small variation in length of some scans is due to changes in scan-speed.

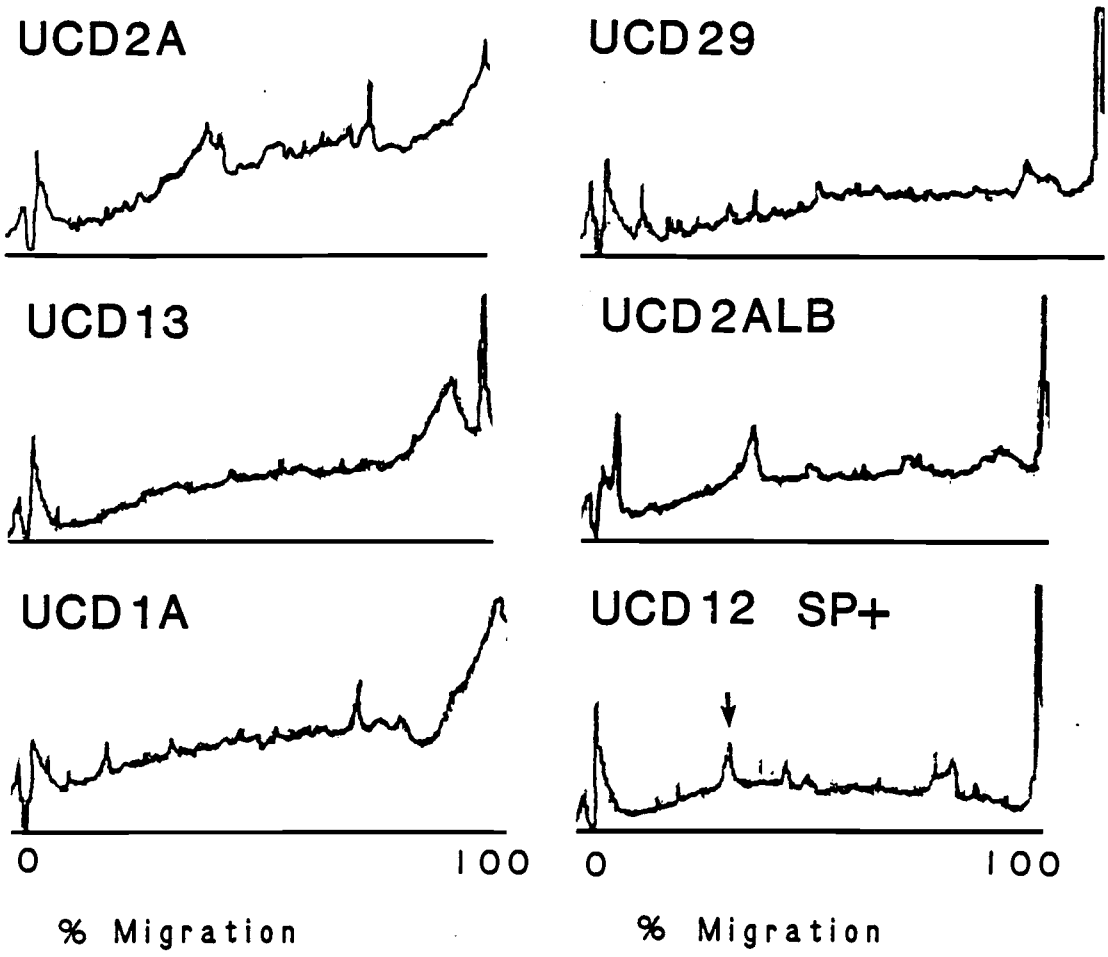
UCD2A
UCD29
UCD13
UCD2ALB
UCD1A
UCD12 SP+
0
100
% Migration
0
100
% Migration

Figure 4.

Figure 5. Agarose gel electrophoresis (1.0%) of dsRNAs from SP+ UCD12, and suspected SP+ `Pinot gris' C146 clones, compared with the dsRNA molecular-weight standards; AMV (2.1, 1.4, and 1.2 x 10^6 MW), GEO 223 (3.6 x 10^6 MW), HM9 (6.3 x 10^6 MW), and CTV strain T505 (13.3 x 10^6 MW). Lanes: **GEO**, GEO; **HM9**, HM9; **AMV**, AMV; **CTVT4**, CTV (T4 strain, not used as a MW standard) ; **CTVT505**, CTV (strain T505); **UCD12+**, SP+ UCD12 cane; **UCD1A-**, SP- UCD1A cane; **C146**, suspected SP+ `Pinot gris' C146 cane; **NRM**, DsRNA from necrotic rusty-mottle-affected `Napolean' cherry; **GEO**, GEO. Electrophoresis was at 50V for 18 hrs. Gels were stained with ethidium bromide (1ug/ml), and destained with sdH_2O. DsRNA (approximately 0.32 ug) loaded into `Pinot noir' and `Pinot gris' wells represented approximately 14 g tissue. Migration is from top to bottom.

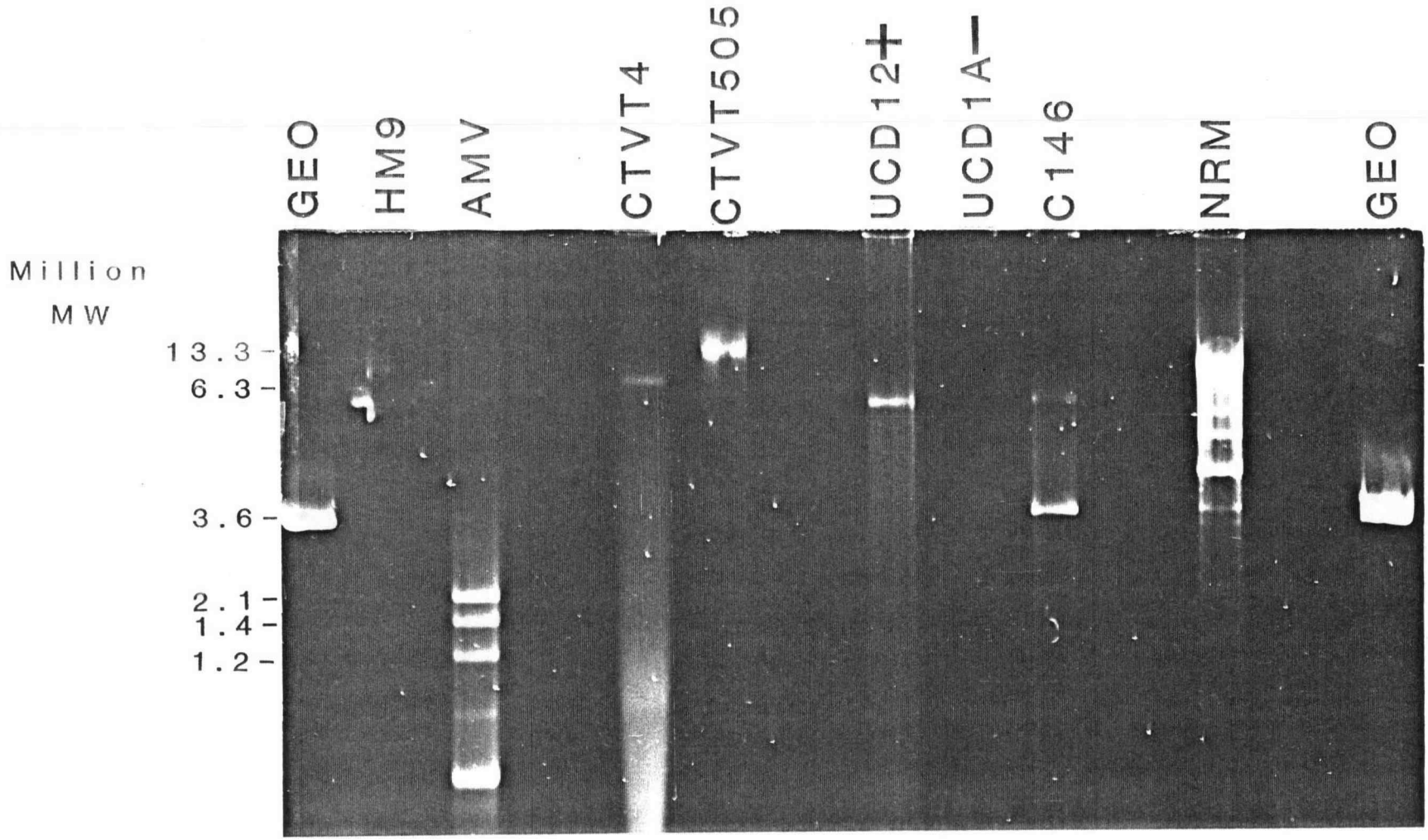
GEO
HM9
AMV
CTVT4
CTVT505
UCD12+
UCD1A−
C146
NRM
GEO
Million
MW
13.3
6.3
3.6
2.1
1.4
1.2

Figure 5.

Figure 6. Regression curve of molecular weight on percent migration on 1.0% agarose gel (Figure 5), using dsRNA molecular weight markers: AMV (2.1, 1.4, and 1.2 x 10^6 MW), GEO 223 (3.6 x 10^6 MW), HM9 (6.3 x 10^6 MW), and CTV strain T505 (13.3 x 10^6 MW). The curve is described by the reciprocal function: $1/Y = a + bX$ where Y = MW, X = migration percentage, a = intercept = -0.270677 and b = slope = 0.311345. The R^2 value for this curve is 0.947.

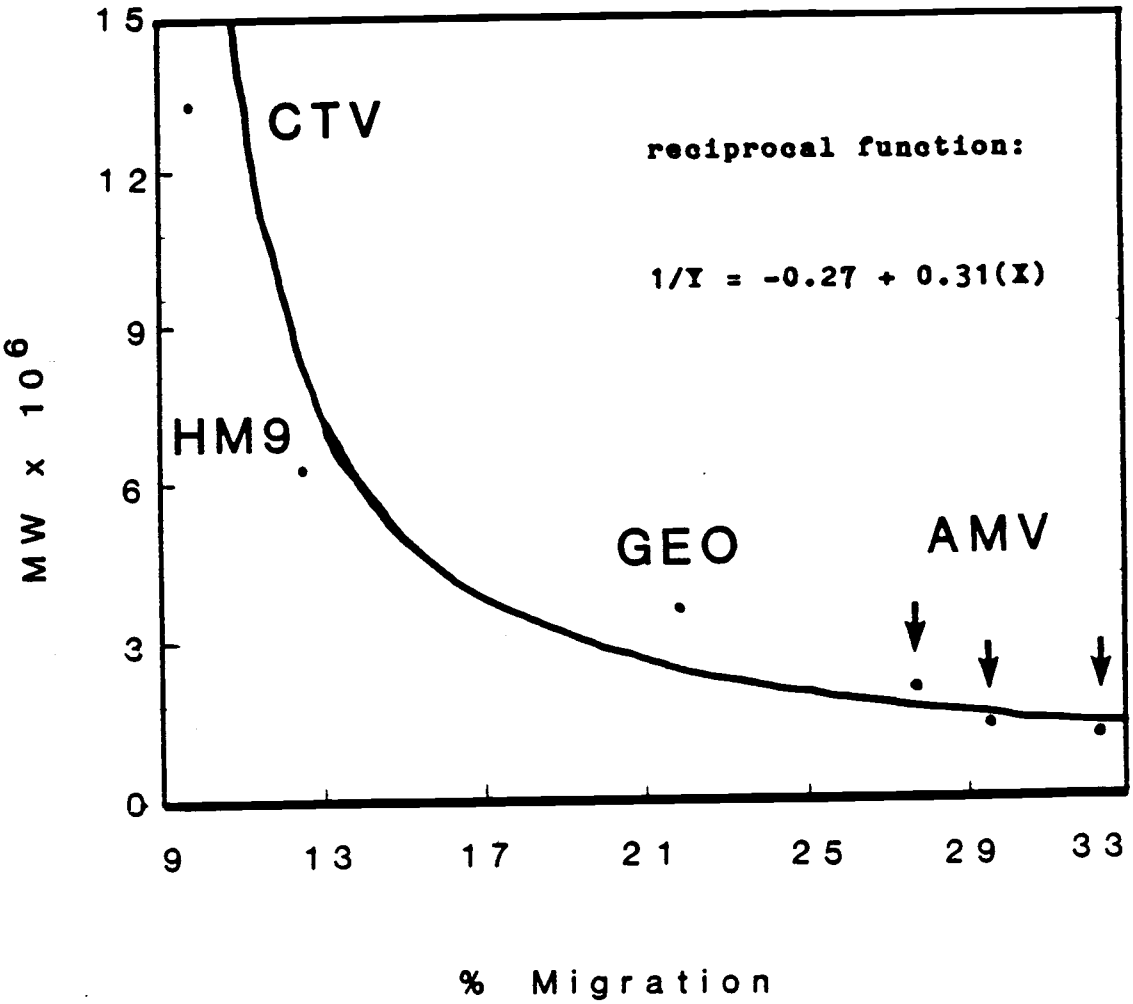
15
12
9
6
3
0
MW x 10^6
CTV
reciprocal function:
1/Y = -0.27 + 0.31(X)
HM9
GEO
AMV
9
13
17
21
25
29
33
% Migration

Figure 6.

Figure 7. Regression curve of molecular weight on percent migration of 1.0% agarose gel (Figure 5), using dsRNA molecular weight markers: AMV (2.1, 1.4, and 1.2 x 10^6 MW), GEO 223 (3.6 x 10^6 MW), HM9 (6.3 x 10^6 MW), but excuding CTV strain T505 (13.3 x 10^6 MW). The curve is described by the simple linear function: $Y = mX + b$; where Y = MW, X = migration percentage, b = intercept = 9.53878, and m = slope = -0.267318. The R^2 value for this curve is 0.995.

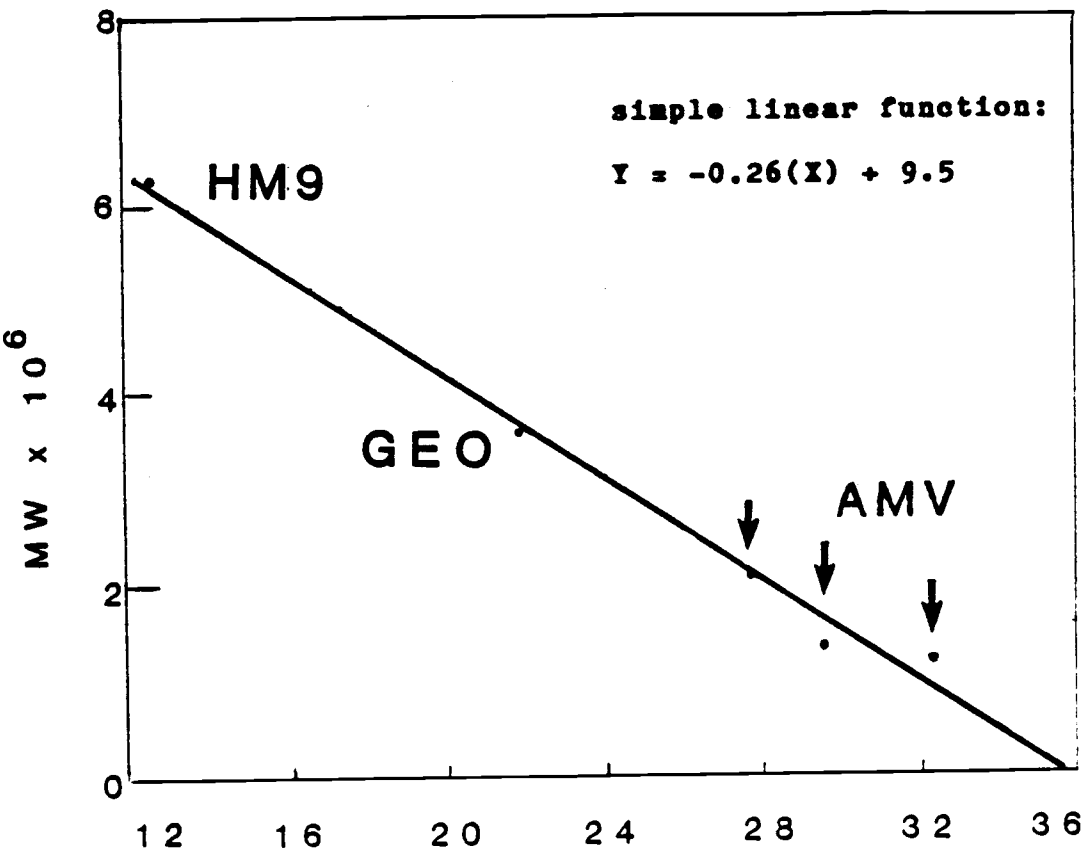
simple linear function:
Y = -0.26(X) + 9.5
HM9
GEO
AMV
MW x 10^6
8
6
4
2
0
12
16
20
24
28
32
36
% Migration

Figure 7.

Figure 8. Regression curve of molecular weight on percent migration of 0.8% agarose gel (Figure 1), using dsRNA molecular weight markers: AMV (2.1, 1.4, and 1.2 x 10^6 MW), GEO 223 (3.6 x 10^6 MW), HM9 (6.3 x 10^6 MW), but excluding CTV strain T505 (13.3 x 10^6 MW). The curve is described by the simple linear function: $Y = mX + b$; where Y = MW, X = migration percentage, b = intercept = 10.4157, slope = - 0.199037. This curve has an R^2 value of 0.996.

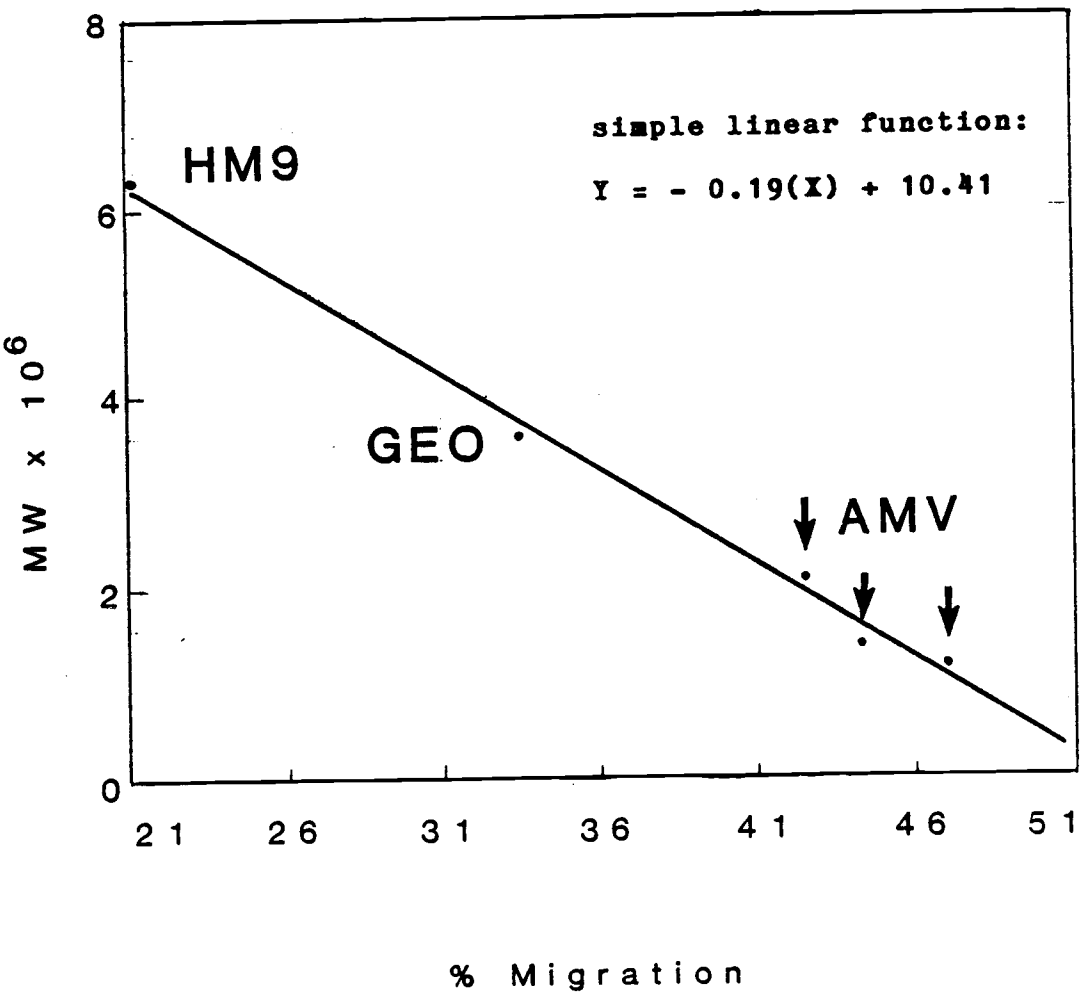
8
6
4
2
0
MW x 10^6
HM9
simple linear function:
Y = - 0.19(X) + 10.41
GEO
AMV
21
26
31
36
41
46
51
% Migration

Figure 8.

Figure 9. Agarose gel electrophoresis (1.0%) of native and glyoxal-denatured dsRNAs from MW markers: AMV (2.1, 1.4, and 1.2 x 10^6 MW), GEO 223 (3.6 x 10^6 MW) and from leaves of 'Pinot noir' clone PN123 showing GLR and FLD symptoms. Lanes: **PN123 D**, denatured dsRNA from 15 g leaves of 'Pinot noir' PN123 with GLR and FLD symptoms; N, Native dsRNA from PN123; **AMV D**, Denatured dsRNA from AMV; N, Native dsRNA from AMV; **GEO D**, Denatured dsRNA from GEO; N, Native dsRNA from GEO; **GEO D**, Denatured dsRNA from GEO; N, Native dsRNA from GEO. Denaturation was performed with less than 20 ug dsRNA, suspended in 5.7 ul of 0.1M NaH_2PO_4, pH 7.0, and combined with 8.0 ul dimethylsulfoxide (DMSO) and 2.7 ul of deionized 6M glyoxal. Electrophoresis was for 12 hrs at 30V. Gels were stained with acridine orange (30ug/ml), in 10mM phosphate buffer, for 30 minutes. Destain was in sdH_2O, for 2 hours.

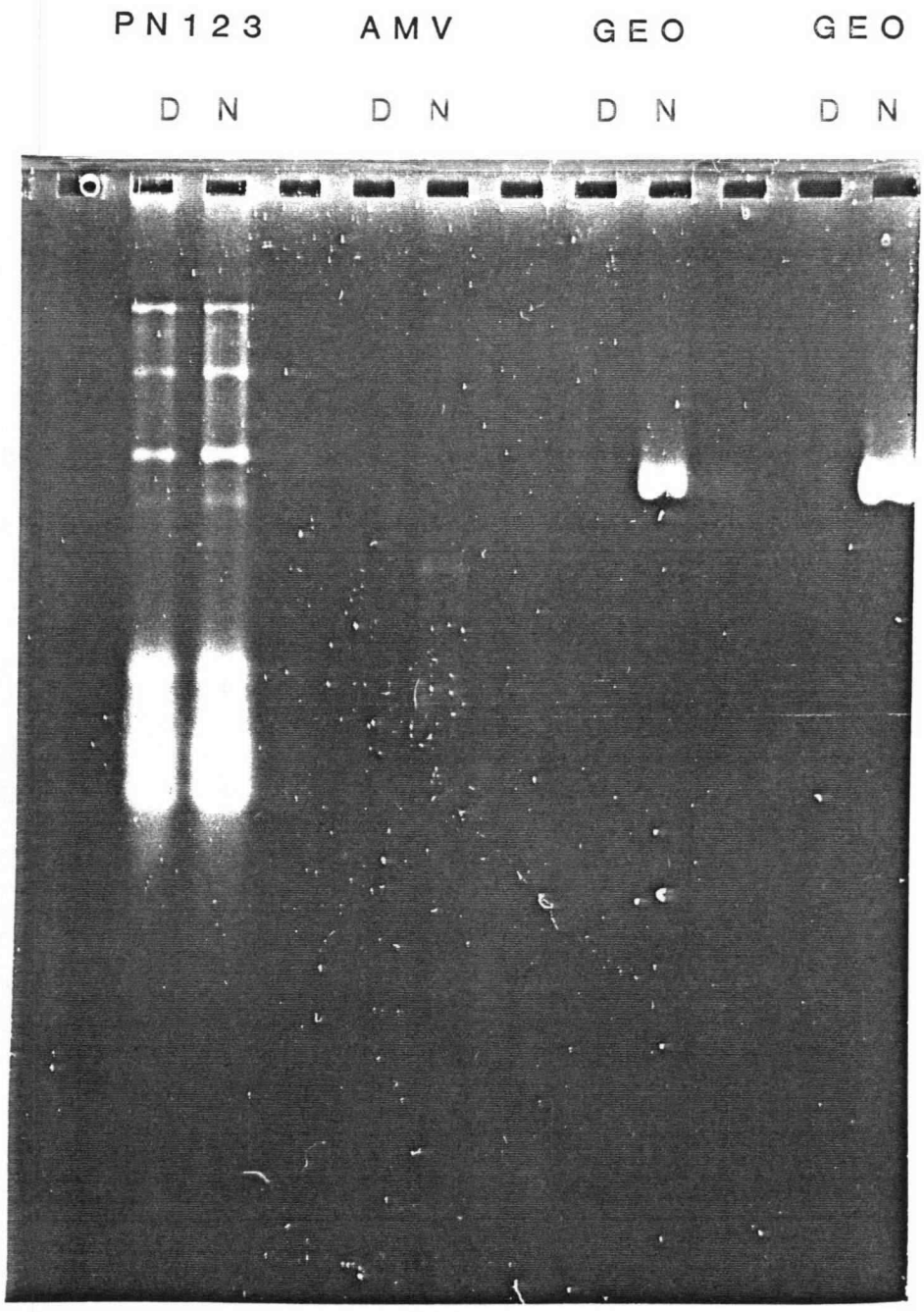

Figure 9.

Figure 10. Laser scanning densitometer readings of a photographic negative of some lanes of 1.0% agarose gel electrophoresis of native and glyoxal-denatured dsRNA. Migration is from left to right (0 to 100% migration). Samples include dsRNA from SP+ `Pinot noir' clones UCD3 (cane), UCD4 (leaves), and suspected SP+ `Pinot gris' C146 (cane), representing approximately 7 g tissue each. Also included are dsRNA molecular-weight standards:; AMV (2.1, 1.4, and 1.2 x 10^6 MW), GEO 223 (3.6 x 10^6 MW), HM9 (6.3 x 10^6 MW). Background on photograph was too intense to justify a figure in this text.

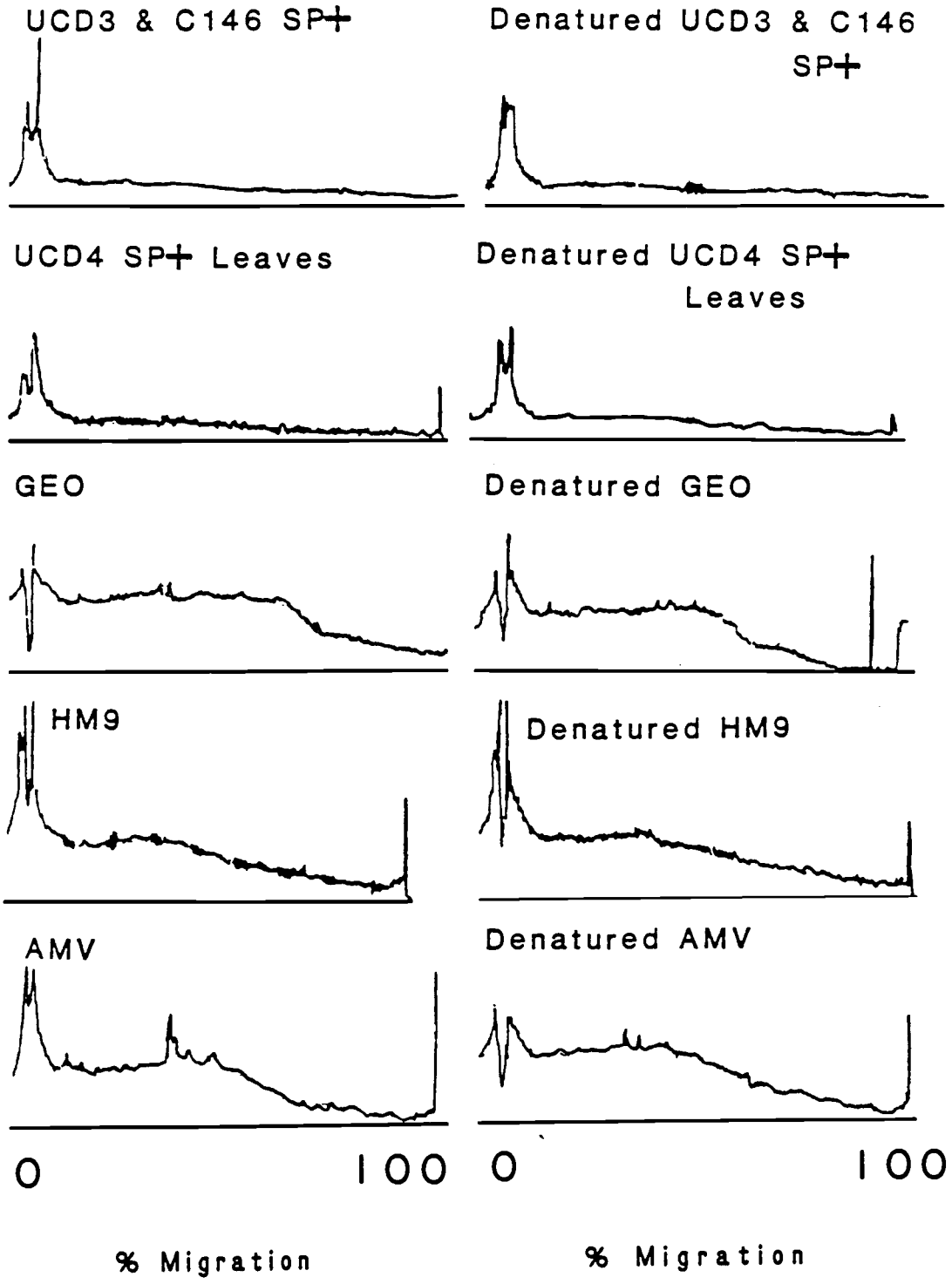
UCD3 & C146 SP+
Denatured UCD3 & C146 SP+
UCD4 SP+ Leaves
Denatured UCD4 SP+ Leaves
GEO
Denatured GEO
HM9
Denatured HM9
AMV
Denatured AMV
0
100
0
100
% Migration
% Migration

Figure 10.

Figure 11. Lanes of 1.0% agarose gel electrophoresis of SP+ UCD12 and SP+ UCD3 in H_2O or 0.3 M NaCl, with or without RNase (10ug/ml). Lanes: **A**, SP+ UCD12 dsRNA in H_2O with RNase; **B**, SP+ UCD12 dsRNA in H_2O without RNase; **C**, SP+ UCD12 dsRNA in 0.3 M NaCl with RNase; **D**, SP+ UCD12 dsRNA in 0.3 M NaCl without RNase; **E**, SP+ UCD3 dsRNA in H_2O with RNase; **F**, SP+ UCD3 dsRNA in H_2O without RNase; **G**, SP+ UCD3 dsRNA in 0.3 M NaCl with RNase; **H**, SP+ UCD3 dsRNA in 0.3 M NaCl without RNase. Electrophoresis was at 50V for 18 hrs. Gels were stained with ethidium bromide (1ug/ml), and destained with sdH_2O. DsRNA (approximately 0.32 ug) loaded into wells represented approximately 14 g tissue.

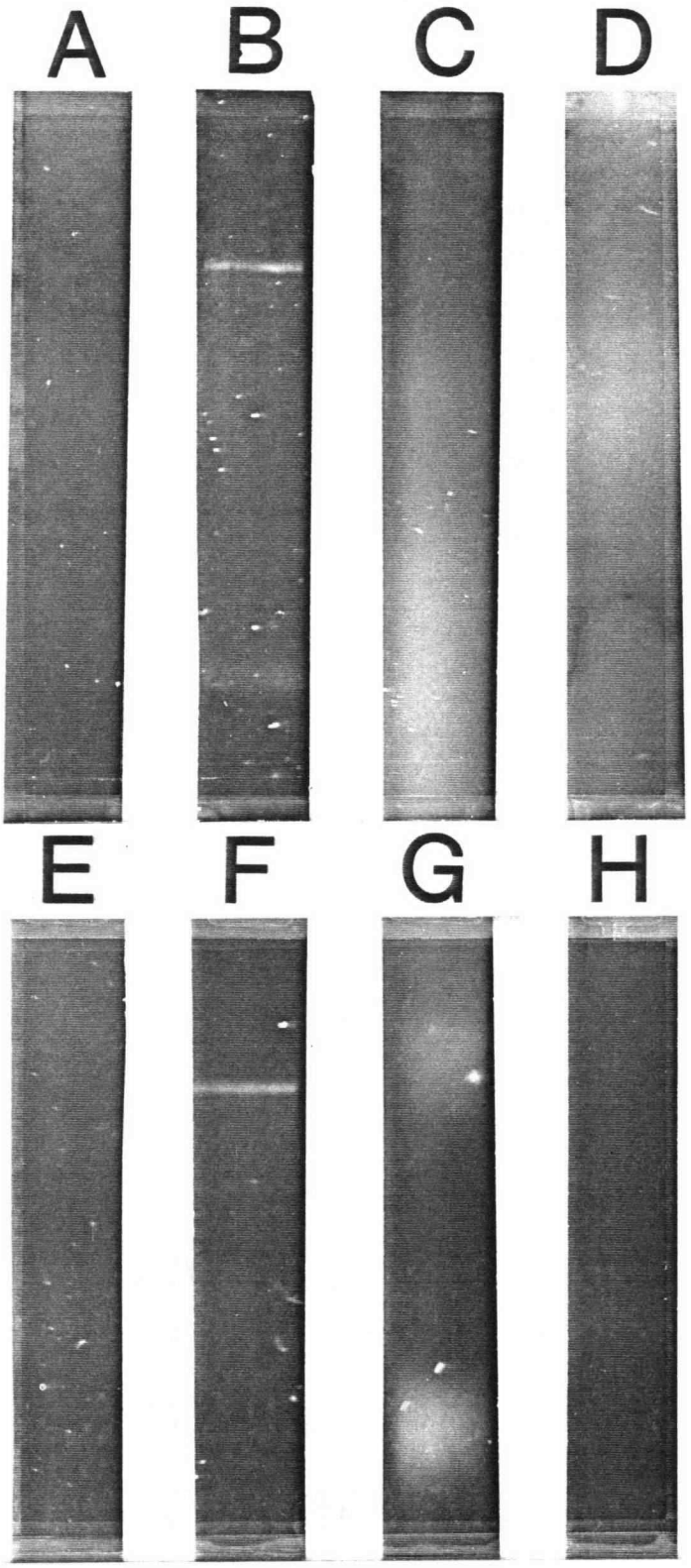

Figure 11.

DISCUSSION

All `Pinot noir' and `Pinot gris' grapevines affected by stem-pitting disease (SP) contained an RNA segment, at least partly double-stranded, of molecular weight between 5.4 and 6.2 x 10^6 MW. This SP-associated dsRNA may represent the RF of a virus. The isolation of this dsRNA segment supports evidence for an 800 nm closterovirus-like particle, grapevine virus "A" (GVA), associated with SP, and containing a ssRNA of 2.55 x 10^6 MW (Boccardo and d'Aquilio, 1981). But, immunological tests did not suggest any association between SP and GVA. In ELISA tests of SP-positive samples against GVA gamma-globulins, the lack of recognition might have been because the source of antigen for antisera development (GVA-infected N. clevelandii in South Africa) was quite different than the source of SP-positive samples (grapevines from Davis, CA.). The GVA antisera may also have been prepared against an SP strain different from that affecting the UCD clones.

The acridine-orange fluorescence (green for all bands) indicated that all visible bands were made up of at least some double-stranded nucleic acid, before and after denaturation. The RNase digestion also supported

this conclusion. The denaturation was not useful in confirming the size of the dsRNA, however.

The proposed size of the SP-associated dsRNA band was an estimate based on the simple regression mentioned in the previous section. There may be a point in the migration-vs-MW curve (in agarose gels) where the function becomes non-linear. This supposed "break" point would be somewhere between the HM9 marker (at 6.3 x 10^6 MW) and the CTV marker (at 13.3 x 10^6 MW). One or two markers of 8 to 11 x 10^6 MW are still needed to make accurate estimations of MW in this area, based on fitted curves. It appears however, that the migration-vs-MW function up to about 6.3 x 10^6 MW is linear. The SP-associated dsRNA migrated faster than the HM9 marker dsRNA (at 6.3 x 10^6 MW) in all cases. Because of this, the HM9 marker was more useful as the high-MW bracketing marker than was the CTV marker (at 13.3 x 10^6 MW).

The estimated size of the SP-associated dsRNA segment suggests that it belongs to a closterovirus. Typical closteroviruses have a ratio of RNA-mass to particle-length of 2821-3437 daltons(MW)/nm (Bar-Joseph, Garnsey, and Gonsalves, 1979). The predicted size of a closterovirus particle belonging to the SP-associated dsRNA (5.4 to 6.2 x 10^6 MW) would be from 785 to 1098 nm in length (encompassing the reported size of GVA). The expected size of the dsRNA for GVA would be 5.2 x 10^6 MW.

Other closteroviruses have ssRNA genomes of size similar to GVA: apple clorotic leafspot virus (CLSV: 2.35 x 10^6 MW) (Bar-Joseph, Garnsey, and Gonsalves, 1979), heracleum latent virus (HLV: 2.3 x 10^6 MW) (Bar-Joseph, Garnsey, and Gonsalves, 1979), and potato virus T (PVT: 2.5 x 10^6 MW)(Bar-Joseph et al., 1979). The RF (dsRNA) of the above viruses would range from 4.6 to 5.1 x 10^6 MW.

The success of the dsRNA isolation suggests that this technique might work as an alternative to ELISA. For virus screening of the above-mentioned hosts, we believe it would be superior. Antisera production for ELISA requires mechanical transmission and purification of viruses. Experimental animals are expensive, and phloem-limited viruses (it has been suggested that SP and LRV are phloem-limited [Goheen, personal communication]), in woody hosts present obstacles to physical manipulation, including low viral titre and phenolic browning. Further, ELISA sensitivity to different viral strains is mainly based on differences in the viral capsid proteins: the capsid protein gene(s) is only part of the viral genome. Genomic differences (for example, in severity), not expressed in the coat protein, may be missed by ELISA (if the antigen was purified virus). It is possible that strain specificity could be better distinguished by dsRNA analysis. Double-stranded RNA patterns have recently been

shown to be strain-specific for tomato mosaic virus (Valverde, Dodds, and Heick, 1986).

As a quarantine screen, dsRNA isolation would work well for SP in V. vinifera variety `Pinot gris' and `Pinot noir' (varieties particularly important in the Northwest). The proposed SP-screen could be improved for imported grape varieties. Candidate clones of varieties not known to produce detectable SP-associated dsRNA (such as Zinfandel), could act as viral donors to `Pinot noir' indicators. The indicators could then be screened for dsRNA content.

Mossop et al. (1985) have clearly shown that dsRNA isolation works for LRV and CB in grape. Faster, more sensitive screening for LRV, CB and SP may be possible when these dsRNA segments are used to generate radioactive probes specific to the diseases.

Stem-pitting disease could be caused by one or more viruses. It has not yet been established that the GVA virus (only isolated from N. clevelandii) is the causal agent of stem-pitting disease (Conti et al., 1980). The SP-associated dsRNA isolated in this study would suggest that there is only one particle acting as the causal agent, and that it is a ssRNA virus, of about 800 nm length. Whether or not the SP-associated dsRNA isolated in this study is part of the SP causal agent would need to be determined in one of two ways: 1) Infectivity tests

using the SP-associated dsRNA, or; 2) reinfection of the grapevine host (so far unsuccessful) with GVA, followed by recognition of the isolated ssRNA GVA nucleic acid core by a radioactive probe made from SP-associated dsRNA.

We have succeeded in isolating a high MW SP-associated dsRNA that was found consistantly in our SP-diseased hosts and may serve as the basis for a quarantine screening system.

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APPENDICES

APPENDIX A: DsRNA migration distances, from 0.7, 0.8 and 1.0% agarose gels: measured from densitometer scans, and photographic negatives. GEO-migration percentage calculated from scans are determined with respect to the total length of the scan. GEO-migration percentages calculated from photos are determined by measuring the distance from the well to the dsRNA band.

Data Source, Date	gel conc.	source dsRNA	total migration	band migration	migration as percentage of GEO migration
scan 5-20-'86	0.8%	CTV	9.4 cm	1.6 cm	48%
		HM9	9.5 cm	2.1 cm	63%
		GEO	9.5 cm	3.3 cm	100%
(=34.5%)					
		AMV 1	9.4 cm	3.9 cm	118%
		AMV 2	9.4 cm	4.2 cm	127%
		AMV 3	9.4 cm	4.5 cm	136%
		UCD12	9.1 cm	2.3 cm	73%
		UCD3	9.5 cm	2.4 cm	73%
		UCD4	9.5 cm	2.4 cm	73%
		UCD17	9.5 cm	2.4 cm	73%
		UCD10	9.1 cm	2.3 cm	73%
scan 4-24-'86	1.0%	CTV	10.0 cm	1.6 cm	51%
		GEO	10.0 cm	3.1 cm	100%
(=31%)					
		AMV 1	10.0 cm	3.9 cm	125%
		AMV 2	10.0 cm	4.1 cm	132%
		AMV 3	10.0 cm	4.5 cm	145%
		UCD4	9.3 cm	1.9 cm	66%
scan 6-2-'86	1.0%	CTV	9.2 cm	0.9 cm	42%
		HM9	9.5 cm	1.2 cm	57%
		GEO	9.5 cm	2.1 cm	100%
(=22%)					
		AMV1	9.0 cm	2.5 cm	119%
		AMV2	9.0 cm	2.7 cm	128%
		AMV3	9.0 cm	2.9 cm	138%
		UCD12	8.8 cm	1.1 cm	56%
		Colmar146	9.1 cm	1.2 cm	59%

photo 2-4-'86	0.8%	GEO	__	2.3 cm	100%
		UCD12	__	1.5 cm	65%

Appendix A cont';

Data Source, Date	gel conc.	source dsRNA	total migration	band migration	migration as percentage of GEO migration
photo 2-25'86	1.0%	GEO	__	1.2 cm	100%
		UCD3	__	0.75 cm	63%
		UCD12	__	0.75 cm	63%
photo 3-21-'86	0.7%	GEO	__	2.3 cm	100%
		UCD12	__	1.7 cm	74%
photo 3-24-'86	0.7%	GEO	__	5.4 cm	100%
		UCD12	__	3.9 cm	72%
photo 4-9-'86	0.7%	GEO	__	4.2 cm	100%
		UCD3	__	3.2 cm	76%
photo 4-13-'86	0.7%	GEO	__	4.3 cm	100%
		UCD12	__	2.7 cm	63%
photo 5-6-'86	0.7%	GEO	__	4.6 cm	100%
		UCD4	__	3.4 cm	74%

APPENDIX B: ELISA A_{405} values.

Date: 5-23-'86

dilution	gamma globulin source[a] GVA/GPV	well	cross abs. conc.[b]	antigen source[c] +/-	405 nm hv absorbtion
1000	GVA	1-2	1.0x	+	0.499
"	GVA	1-6	1.0x	+	0.607
				ave.=	**0.553**
"	GVA	1-3	0.5x	+	0.245
"	GVA	1-7	0.5x	+	0.406
				ave.=	**0.325**
"	GVA	1-4	1.0x	-	0.481
"	GVA	1-8	1.0x	-	0.613
				ave.=	**0.547**
"	GVA	1-5	0.5x	-	0.322
"	GVA	1-9	0.5x	-	0.410
				ave.=	**0.366**
500	GVA	2-2	1.0x	+	1.482
"	GVA	2-6	1.0x	+	1.655
				ave.=	**0.827**
"	GVA	2-3	0.5x	+	0.599
"	GVA	2-7	0.5x	+	0.527
				ave.=	**0.563**
"	GVA	2-4	1.0x	-	1.169
"	GVA	2-8	1.0x	-	1.621
				ave.=	**1.395**
"	GVA	2-5	0.5x	-	0.676
"	GVA	2-9	0.5x	-	0.609
				ave.=	**0.639**
1000	GPV	4-2	1.0x	+	-0.007
"	GPV	4-6	1.0x	+	0.156
				ave.=	**0.082**
"	GPV	4-3	0.5x	+	0.074
"	GPV	4-7	0.5x	+	0.102
				ave.=	**0.088**

APPENDIX B cont': ELISA A_{405} values.

Date: 5-23-'86

dilution	gamma globulin source[a] GVA/GPV	well	cross abs. conc.[b]	antigen source[c] +/-	405 nm hv absorbtion
"	GPV	4-4	1.0x	-	0.115
"	GPV	4-8	1.0x	-	0.074
				ave.=	**0.095**
"	GPV	4-5	0.5x	-	0.208
"	GPV	4-9	0.5x	-	0.035
				ave.=	**0.121**
500	GPV	5-2	1.0x	+	-0.020
"	GPV	5-6	1.0x	+	-0.023
				ave.=	**-0.022**
"	GPV	5-3	0.5x	+	0.121
"	GPV	5-7	0.5x	+	0.136
				ave.=	**0.128**
"	GPV	5-4	1.0x	-	-0.024
"	GPV	5-8	1.0x	-	-0.024
				ave.=	**-0.024**
"	GPV	5-5	0.5x	-	0.138
"	GPV	5-9	0.5x	-	0.147
				ave.=	**0.142**

Date: 5-26-'86

1000	GVA	1-2	1.0x	+	0.401
"	GVA	1-3	1.0x	+	0.405
"	GVA	1-4	1.0x	+	0.269
"	GVA	2-2	1.0x	+	0.291
"	GVA	2-3	1.0x	+	0.335
"	GVA	2-4	1.0x	+	0.327
				ave.=	**0.338**
				std.dev.=	**0.055**

dilution	gamma globulin source[a] GVA/GPV	well	cross abs. conc.[b]	antigen source[c] +/-	405 nm hv absorbtion
"	GVA	1-7	1.0x	-	0.314
"	GVA	1-8	1.0x	-	0.311
"	GVA	1-9	1.0x	-	0.309
"	GVA	2-7	1.0x	-	0.276
"	GVA	2-8	1.0x	-	0.275
"	GVA	2-9	1.0x	-	0.321
				ave.=	**0.301**
				std.dev.=	**0.020**
500	GVA	4-2	1.0x	+	1.162
"	GVA	4-3	1.0x	+	1.267
"	GVA	4-4	1.0x	+	1.141
"	GVA	5-2	1.0x	+	1.330
"	GVA	5-3	1.0x	+	1.204
"	GVA	5-4	1.0x	+	1.168
				ave.=	**1.212**
				std.dev.=	**0.073**
"	GVA	4-7	1.0x	-	0.924
"	GVA	4-8	1.0x	-	1.001
"	GVA	4-9	1.0x	-	0.989
"	GVA	5-7	1.0x	-	1.080
"	GVA	5-8	1.0x	-	1.058
"	GVA	5-9	1.0x	-	1.207
				ave.=	**1.043**
				std.dev.=	**0.097**

[a]. GVA: Antisera to grapevine virus "A" (Englebrecht, personal communication)
GPV: Antisera to grapevine potyvirus-like virus (Tanne, personal communication).

[b]. First run (5-23-'86) of cross-absorbtion was done with half-strength and full strength conjugate. Second run was done only with full stength conjugate.

[c]. Stem-pitting-positive UCD4 and UCD17 were used for positives (+). Virus-free UCD2A was used as the negative (-).