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PROJECT No. US-156-79

Identification and Serology of Strawberry Mild Yellow-Edge Virus

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3. Abstract

Progress was made in the following areas of BARD project US 159-79:

1. Purification and determination of physical properties, serology, and aphid transmission properties of strawberry mild yellow-edge virus (SMYEV);
2. Elimination of SMYEV by heat therapy and tissue culturing;
3. Occurrence and aphid relationships of SMYEV in Israel; and
4. Identification of nucleic acids specific to SMYEV.

SMYEV is an isometric (23 nm) virus with an in vivo thermal inactivation point 45-50 C. Partial purification of SMYEV from infected strawberry tissue was by comminution in liquid nitrogen followed by differential and successive rate-zonal sucrose density gradient centrifugations. Polyclonal rabbit antisera prepared against SMYEV identified it in crude preparations by indirect (F(ab')₂) ELISA. SMYEV was thus identified in elite clones of several major US strawberry cultivars testing negative by conventional bioassays. SMYEV was distantly serologically related to barley yellow dwarf virus but not to 5 other known luteoviruses. Aphid transmission of SMYEV was efficient if the virus isolate and Chaetosiphon fragaefolii colony found together in nature were used together in the greenhouse. SMYEV transmission by micro-injecting aphids was successful using viruliferous aphid hemolymph, but purified SMYEV could not be transmitted by aphid injection or membrane feeding. Several heat-treated, tissue-cultured clones of leading western US strawberry cultivars that had repeatedly indexed negative for SMYEV on the standard strawberry virus indicator tested positive for SMYEV by ELISA and exhibited density gradient peaks and viruslike particles as seen by electron microscopy typical for the standard isolate of SMYEV used in this study. This evidence that a virus resembling SMYEV remained undetected by standard bioassay procedures in heat-treated meristemmed clones of several leading strawberry cultivars was probably one of the more important results to come out of this project.

SMYEV was found to infect most of the Israeli stock of its leading cultivar, Aliso. Three separate negative indexings of a suspect plant are needed to state with 95% confidence that it is free of SMYEV. With that as a criterion, two linear relationships were found: a) between the length of time an SMYEV-infected plant is held at 38 C and the % of infected, regenerated 4 mm explants; b) between explant length and % of SMYEV infected, regenerated explants from plants grown at greenhouse temperatures.

Five aphids found on Israeli strawberries, Acyrtosiphon solani, A. porosum, Aphis gossypii, Macrosiphum euphorbiae, and Myzus persicae, failed to transmit SMYEV experimentally, but the worldwide known vector of SMYEV, Chaetosiphon fragaefolii, was collected on strawberry in Israel during the closing months of the BARD project.

A method of preparing double stranded RNA from SMYEV was developed to the point where distinct bands unique to infected plants were detected in polyacrylamide gels.

4. Body of Report

- A. Purification, some properties, and serology, of strawberry mild yellow-edge virus.

ABSTRACT

Strawberry mild yellow-edge virus (SMYEV), a persistent, aphid-borne virus transmitted by several Chaetosiphon spp., is common and serious in cultivated strawberries in western USA. Oregon SMYEV isolate MY-18, readily transmitted by a colony of C. fragaefolii collected with it in the field, was increased in Fragaria in the greenhouse for partial purification and study. After comminution in liquid nitrogen, extraction in 0.1 M phosphate, 0.01 M DIECA, 1% thioglycollic acid (pH 7.0) and differential and rate-zonal density gradient (dg) centrifugation, an ultraviolet-absorbing dg band ($A_{254 \text{ nm}}$), not seen in control preparations, was detected in extracts from MY-18-infected plants. Isometric, 23 nm-diameter, virus-like particles were uniquely associated with this band. We were unsuccessful in numerous attempts to infect Fragaria with partially purified MY-18 preparations by means of membrane-fed or injected C. fragaefolii. MY-18 has an in vivo thermal inactivation point between 45 and 50 C as determined by feeding C. fragaefolii on detached leaves that had been immersed in water for 10 minutes at various temperatures. In ELISA tests rabbit antisera against MY-18 differentiated between extracts from healthy and MY-18-infected Fragaria. Our data support the generally held hypothesis that SMYEV is a luteovirus. However, comparative ISEM and ELISA tests failed to reveal serological relationship between MY-18 and potato leafroll, beet western yellows, legume yellows, pea leafroll, and tobacco necrotic dwarf viruses.

Strawberry mild yellow-edge virus (SMYEV) is one of the most common viruses of cultivated strawberries (13). The virus is transmitted in a persistent manner by the strawberry aphids Chaetosiphon fragaefolii (Cockerell), C. thomasi H.R.L., C. jacobi H.R.L. (12) and C. minor (Forbes) (7).

In Fragaria vesca L. indicator clones initial symptoms appear 20-28 days after inoculation depending on the strain of the virus. The first symptoms are epinasty followed by chlorosis and sometimes necrosis of the net veins of one or two leaflets of the youngest leaf. On successive leaves the necrosis is more general and affected leaves soon die. Two months after inoculation the symptom pattern of the whole plant is quite distinct with the youngest leaves showing net vein chlorosis and necrosis and epinasty, three or four symptomless mature leaves, and a few dead mature leaves. Based on symptomatology and vector properties, SMYEV is considered a possible luteovirus (11).

This paper describes a procedure for partially purifying SMYEV from strawberry tissue and presents additional evidence for including SMYEV in the luteovirus group. A preliminary report of this work has appeared (10).

MATERIALS AND METHODS.

Virus isolates. The SMYEV isolate MY-18 used in purification and characterization studies was obtained from field plots at the North Willamette Experiment Station of Oregon State University near Aurora, Oregon, by a single aphid transfer from naturally-infected strawberries. This isolate was maintained in F. vesca clone UC-6 by transferring viruliferous aphids to successive plants. Other SMYEV isolates used were two California isolates and two Oregon isolates. All isolates were grafted onto F. vesca clone UC-4 and clone UC-6 to insure that the SMYEV isolates were free of other known aphid-borne strawberry viruses that occur in the area: strawberry mottle virus (SMV), strawberry veinbanding virus (SVBV) and

strawberry crinkle virus (SCV). SMYEV produces symptoms on UC-4 and is usually symptomless on UC-6, whereas SMV, SVBV and SCV produce symptoms on UC-6 (6).

Aphid transmission. Nonviruliferous strawberry aphid (*C. fragaefolii*) colonies were derived from the single field-collected aphid which originally transmitted the MY-18 isolate to *F. vesca* L. var *semperflorens* (Duchesne) Seringe cv. 'Alpine'. After the single field-collected aphid was purged of the possible presence of the semipersistent viruses SMV and SVBV by successive 2 h feeding access times on successive Alpine seedlings, it was allowed to feed on a fourth Alpine seedling 24 h then transferred to yet another Alpine seedling where it reproduced parthenogenically to form the aphid colony we used to transmit MY-18 in the greenhouse. Newly-born nymphs were transferred to young Alpine seedlings to establish a colony free of known plant viruses.

Aphids were allowed an acquisition access period of at least 24 h on MY-18-infected Alpine seedlings and then were transferred (5 aphids/plant) to test plants. After an inoculation access period of 3 days the aphids were killed by fumigation using dichlorovos in a small airtight chamber. Membrane feeding and aphid injection were carried out as previously described (4, 16). After fumigation, plants were maintained in a glasshouse at 20-26 C.

Thermal inactivation. SMYEV is not mechanically transmitted, and our extensive aphid injections and membrane feeding attempts with partially purified preparations were unsuccessful. In vivo thermal inactivation therefore was determined by aphid and graft transmissions from detached leaves that had been immersed for 10 min in water at 20, 40, 45, 50, 55 and 60 C. Aphids were allowed a 48 h acquisition access period then transferred (10 aphids/plant) to healthy Alpine seedlings for a 3-day inoculation access period. For graft transmission tests leaves were grafted to healthy Alpine seedlings after the thermal treatment. Plants were observed for symptoms three times per week for 6 weeks after inoculation.

Virus purification. Whole infected Alpine seedlings or plants of UC-4 were harvested at the early stages of symptom development 12-18 days after inoculation. Roots and leaves were kept separate for virus purification. Virus was also purified from leaves of chronically infected strawberry cv. Hood. Tissue was ground to a fine powder in liquid N₂ then homogenized in 4 volumes of 0.1 M phosphate buffer with 0.01 M diethyldithiocarbamate (DIECA) and 1% thioglycollic acid (pH 7.0) in a Virtis mixer. The homogenate was clarified by centrifugation at 10,000 g for 3 h and a second high-speed centrifugation at 140,000 g for 3.5 h through a 6 ml cushion of 25% sucrose in a Beckman 50.2 Ti rotor. The pellets from high-speed centrifugation were suspended in 0.1 M phosphate with 0.001 M DIECA pH 7.0. The samples were then layered on 10-40% sucrose gradients and centrifuged at 4 C at 39,000 rpm for 90 min in a Beckman SW 40 rotor. Virus-containing fractions were collected with an ISCO model 640 density gradient fractionator and Model UA-5 UV analyzer.

Antisera. Antiserum was prepared against SMYEV by weekly intramuscular injections for 4 wk of 100 ug of sucrose density gradient purified virus emulsified in Freund's incomplete adjuvant, followed by a booster injection of 500 ug of virus in incomplete adjuvant 2 wk after the last regular injection. Thereafter booster injections were administered monthly. The rabbit was bled 10 days after the last regular injection and 10 days after each booster injection. Quantities of SMYEV were estimated from their UV absorption spectra assuming an extinction coefficient at 260 nm of 7.0, the value reported for carrot red leaf virus (19).

Antisera to the following six viruses were kindly supplied by the workers named: beet western yellows (BWYV) and legume yellows virus (LYV) from J. E. Duffus, pea leafroll virus (PeLRV) from H. Huttinga, potato leafroll virus (PLRV) from R. G. Clarke, soybean dwarf virus (SDV) from M. Kojima, and tobacco necrotic

dwarf virus (TNDV) from S. Kubo. ISEM tests were performed as previously described (2) using the antisera listed above as trapping or decorating antibody.

Indirect $F(ab')_2$ ELISA tests conducted with antisera to SMYEV and several other luteoviruses were performed in standard Immulon® plates (Dynatech Laboratories Inc., Alexandria, Virginia) according to the method of Barbara and Clark (1) except that Protein A alkaline phosphatase conjugate was used (Zymed Laboratories, South San Francisco, California) and the substrate was p-nitrophenyl phosphate. Microtiter plates were coated with $F(ab')_2$ at 5 ug/ml in 0.05 M sodium carbonate buffer (pH 9.6) overnight at 4 C. Either partially-purified virus or infected tissue homogenized 1:9 (w/v) in PBS-Tween 20 with 2% polyvinylpyrrolidone (mol wt 10,000) and 0.2% ovalbumin (virus buffer) served as sources of viral antigens.

Heterologous $F(ab')_2$ indirect ELISA tests were used with the luteovirus antisera listed above in attempts to detect heterologous reactions between SMYEV and several members of the luteovirus group. Each of the luteovirus antisera was used: a) as detecting antibody after trapping with SMYEV antisera, b) as trapping antibody when SMYEV antisera was the detecting antibody, and c) as trapping and detecting antibody. The detecting and trapping antibodies were used at concentrations of 0.2 to 10 ug/ml. Root tissue of SMYEV-infected UC-6 homogenized 1:9 in virus buffer served as the source of antigen.

RESULTS

Transmissions, mechanical. Attempts were made to transmit SMYEV to Cucumis sativus L., Nicotiana tabacum L. cv. 'Xanthi', Gomphrena globosa L., Catharanthus roseus (L.) G. Don, Chenopodium quinoa Willd., C. amaranticolor Coste and Reyn., Vigna unguiculata (L.) Walp. subsp. unguiculata, Cyamopsis tetragonoloba (L.) Taub. and Alpine seedlings from leaves and roots of infected Alpine seedlings at monthly intervals from February through June. Inoculation techniques included using

carborundum as an abrasive and 0.05 M phosphate and 2% polyvinylpyrrolidone (mw 10,000) which worked well when transmitting tomato ringspot virus from strawberry (9). All attempts to transmit SMYEV mechanically failed.

Aphid. The MY-18 isolate of SMYEV was transmitted readily in a persistent manner by the strawberry aphid (C. fragaefolii) from infected strawberry to healthy strawberry plants. However, four isolates of SMYEV that had been maintained by graft transmission in the glasshouse for long periods of time (5 years or more) were not transmitted by this aphid. The MY-18 isolate was no longer aphid transmissible after three serial graft transmission spaced 2 months apart when 40 aphids were transferred from test plants after a 3-day acquisition access period to each of five Alpine seedlings. MY-18 maintained by aphid transmission during the same time was readily transmitted when five aphids were transferred from test plants to each of five Alpine seedlings. Symptomatology of aphid transmissible and nontransmissible MY-18 is identical.

We were unsuccessful in over 40 attempts (using at least 20 aphids per test) to infect Alpine seedlings with partially purified or crude MY-18 preparations by means of membrane-fed or injected aphids. However, in 2 of 10 attempts we did transmit SMYEV from viruliferous aphids to nonviruliferous aphids by injecting haemolymph from viruliferous to nonviruliferous aphids.

Thermal inactivation. SMYEV was aphid- and graft-transmissible from detached, infected leaves after immersion in a water bath for 10 min at 45 C or less but not from leaves treated at 50 C or higher. Aphids fed on leaves treated at each temperature. This indicates a thermal inactivation or inactivation of transmissibility between 45 and 50 C.

Purification. Early attempts to purify SMYEV by homogenization of fresh leaf tissue directly in various buffers and clarifying by centrifugation or organic solvents all resulted in mucilaginous preparations from which virus yields were very

low. Grinding the strawberry tissue in liquid nitrogen to a fine powder using a Waring blender with a stainless steel canister greatly reduced the problem with mucilaginous material. Clarification with organic solvents reduced virus yield as measured by 260 absorbance when compared to low-speed centrifugation. Pelleting the virus through a 6 ml cushion of 25% sucrose helped to clean up the preparations considerably before sucrose density gradient centrifugation.

The use of Driselase (Kyowa Hakko Kogyo Co., Ltd., Tokyo) which has been reported to improve virus yields of several luteoviruses by degrading the phloem tissue and releasing the virus particles (17, 19), resulted in dark-colored preparations during enzymatic digestion at 25 C even when 2% thioglycollic acid and 0.01 M DIECA were used as reducing agents. All attempts to purify SMYEV with a Driselase digestion step resulted in no detectable virus peak in the sucrose gradients.

The purification procedure finally adopted for purifying SMYEV from strawberry tissue is outlined in the section on Materials and Methods. With this procedure a UV-absorbing band was present in sucrose density gradients when purifying from SMYEV-infected Hood strawberry plants but not in purifications from healthy Hood plants (Fig. 1). Virus-like particles, 28-30 nm in diameter, though somewhat swollen and distorted, were associated uniquely with this band (Fig. 2B). Assuming an extinction coefficient of 7 (19), virus yields ranged from 0.2 mg to 0.5 mg/kg of leaf tissue and up to 0.7 mg/kg of root tissue.

In an attempt to avoid the distortion and swelling observed in preparations from chronically-infected Hood tissue, attempts were made to purify SMYEV from infected indicator plants including UC-4 and Alpine seedlings. In these experiments tops and roots of plants were harvested at the early stages of symptom development, 12-14 days for UC-4 and 16-18 days for Alpine seedlings after inoculation by aphids. The purification scheme was the same as described above. The UV absorbance

of the viruslike band from F. vesca clone UC-4 leaf tissue was about 25% less than the absorbance of the viruslike band from the Hood tissues (Fig. 3).

Electron microscopy of preparations from F. vesca root and leaf tissues revealed differences in particle size and morphology. The particles from the leaf tissue looked the same as the particles from chronically-infected Hood leaf tissue (Fig. 2B), while the particles from root tissues were isometric, about 23-25 nm in diameter and showed typical angularity (Fig. 2B). The particles from the root tissues co-migrated in sucrose density gradients with LYV. All preparations were noninfectious when inoculated mechanically onto C. quinoa to check for tobacco necrosis virus (TNV) contamination (5). Also, known TNV-infected strawberry roots purified in the same manner were highly infectious on C. quinoa.

Serology. Antiserum prepared against the MY-18 isolate of SMYEV was tested against five isolates of SMYEV maintained in UC-6 and against healthy UC-6 using indirect $F(ab')_2$ ELISA. When leaf tissue homogenized 1:9 (w/v) in virus buffer was used as a source of SMYEV antigen, the A_{405} values of partially purified SMYEV preparations were about 2.5 times the threshold value of 0.131 (healthy mean plus 3 standard deviations, assuming a linear dose-response relationship in this range of absorbance).

Since root tissues gave higher yields than leaf tissues in purification studies and the particles from root preparations appeared less swollen and distorted, crude root tissues (1:9 in virus buffer) were tested as a source of antigen for the indirect $F(ab')_2$ ELISA test. With root tissues as a source of antigen the antiserum against MY-18 isolate of SMYEV reacted with the five isolates of SMYEV that were tested (Table 1). Infected crude root preparations gave A_{405} values 2-3 times higher (mean of 6 ELISA wells each) than the threshold level of 0.111 (Mean plus 3 standard deviations of A_{405} values of healthy sap in 6 ELISA wells, under the assumptions previously mentioned).

All attempts to detect SMYEV by immunospecific electron microscopy (ISEM) using homologous and heterologous antisera failed, although PLRV was detected readily by us from infected potato with homologous antisera by ISEM. However, when sap from PLRV-infected potato was mixed 1:1 with sap from healthy strawberry we could not detect PLRV in the resulting mixture by ISEM using PLRV antibody coated grids.

SMYEV was not detected in the $F(ab')_2$ indirect ELISA tests with any of the heterologous antisera used either as detecting or trapping antibody. In control tests using PLRV antisera as trapping antibody and BWYV as detecting antibody, PLRV was readily detected. but when the PLRV-infected potato leaf sap was mixed 1:1 with strawberry leaf sap PLRV was not detected in this system.

DISCUSSION

Strawberry virus purification directly from non-herbaceous hosts is rarely attempted because of tannins and in some cases dense, viscous materials in the host sap. SVBV was purified directly from strawberry by use of polyethylene glycol precipitation, sucrose-CsCl step gradients and ion exchange chromatography (14). With the procedure described here it is possible to process large amounts of strawberry tissue without polyethylene glycol precipitation, which in our experience greatly reduced SMYEV yields when used at concentrations of 6-8% with and without sodium chloride. Grinding the strawberry tissue to a fine powder in liquid nitrogen greatly reduced the problem of mucilaginous materials. Strawberry root tissue has less mucilaginous material than leaf tissue and proved to be a better source of SMYEV for purification.

Thermal inactivation points of other luteoviruses have been measured by injecting aphids or feeding them through membranes on purified virus or homogenized tissues previously subjected to various temperatures (3, 15, 18). Since we were

unable to get any transmissions from crude extracts or from purified preparations by membrane feeding or aphid injection, we used whole leaves. With both aphid and graft transmission infectivity was inactivated between 45 and 50 C. This may be explained either as damage to the host tissue that prevented aphid acquisition or graft transmission of the virus or thermal inactivation of the virus. The thermal inactivation point (TIP) between 45 and 50 C is similar to the TIP reported for soybean dwarf virus (18), but other luteoviruses such as beet western yellows virus and barley yellow dwarf virus have TIP between 60-70 C (3, 15).

Our evidence for purifying the causal agent of SMYEV is only presumptive since we were not able to reproduce the disease in strawberry using membrane-fed or injected aphids. However, the following points all suggest that the particles we have purified are SMYEV: (1) the association of 23 nm virus-like particles with the UV absorbing band that was present in preparations from infected but not from healthy strawberry; 2) the ability of the SMYEV antiserum to discriminate between SMYEV-infected and healthy UC-6 plants; 3) the aphid transmission properties and symptomatology which indicate that MY-18 is an isolate of SMYEV free of other aphid-borne strawberry viruses; and 4) mechanical transmission of MY-18 isolate of SMYEV from aphid to aphid by injection shows that SMYEV is circulative in its vectors.

The lack of aphid transmissibility of the purified preparations is not surprising since infectivity was not aphid transmissible from crude sap, or clarified preparations. This suggests that the virus is rapidly inactivated in strawberry leaf sap. The coat protein may be denatured such that it does not attach to receptor sites in the aphid salivary glands. Such a model has been proposed for loss of transmissibility of the MAV isolate of barley yellow dwarf virus after UV irradiation. Though no morphological differences were detected, serological tests indicated a rapid decrease in recognition of MAV by MAV-specific antibody with increased UV irradiation (8). This model would explain the relatively low A405

values obtained in ELISA tests and the non-discrimination between healthy and SMYEV-infected strawberry leaf tissue in ELISA tests with SMYEV antiserum. During ELISA tests the virus is in contact with inhibitory and denaturing compounds present in strawberry leaf tissue for a relatively long period of time, whereas, during purification the virus is separated from the majority of these compounds rather quickly. With root tissues, the virus may remain more serologically active in the resulting sap.

The properties of the virus purified from SMYEV-infected strawberry are consistent with its inclusion in the luteovirus group. SMYEV is transmitted in a persistent manner and is circulative in its aphid vector as shown by transmission of virus from aphid to aphid by injection. Isolation of 23 nm particles which are present in very low concentration is also characteristic of the luteovirus group. Serological affinities with other luteoviruses were not detected using indirect $F(ab')_2$ ELISA. However, if the virus is partially denatured during purification or incubation during ELISA, heterologous reactions would probably not be detected.

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Table 1. Detection of several isolates of strawberry mild yellow-edge virus from strawberry roots using $F(ab')_2$ indirect ELISA^a.

Isolate ^c	Source	$C_1/C_2 = 5/5^b$		$C_1/C_2 = 1/5^b$	
		A_{405}	infected/ healthy	A_{405}	infected/ healthy
MY-18	Oregon	.233	2.8	.199	3.2
MY-11	California (N.W. Frazier)	.249	3.0	.218	3.5
Sevy-9	California (N.W. Frazier)	.186	2.2	.136	2.2
76B	Oregon (R.H. Converse)	.193	2.3	.185	2.9
Miller's	Oregon (P.W. Miller)	.240	2.9	.156	2.5
Healthy UC-6	Frazier (6)	.084	-	.063	-

^a protein-A-alkaline phosphatase (Zymed Laboratories, South San Francisco, California, U.S.A.).

^b C_1 = coating or trapping antibody; C_2 = detecting antibody; numbers = mcg/ml.

^c All isolates were extracted from Fragaria vesca clone UC-6.

B. Evidence for an unknown latent virus in strawberry.

While attempting to purify strawberry mild yellow-edge virus (SMYEV), a clone of strawberry cv. Benton that was free of known viruses by standard indexing techniques was used as a control. Clarified extracts from these control plants, when subjected to rate-zonal density gradient centrifugation, yielded a UV-absorbing fraction that contained viruslike particles. In subsequent tests these particles (28 nm) co-sedimented with those of SMYEV. Repeated leaf graft indexing from this Benton clone to strawberry virus indicator clones Alpine, UC-4, 5, 6, 10, 11 and 12 failed to produce any symptoms, although 28 nm particles were found in grafted UC-10 and 12 after subsequent purification. This latent virus may be a mild strain of a described virus from strawberry, like SMYEV, or may be a new virus not detectable with currently used indicators. Five of six strawberry cultivars subsequently tested were found to contain similar viruslike particles, whereas the virus-indicator clones checked were free of such particles.

- C. Heat therapy and shoot apex culture to eliminate mild yellow-edge virus from Hood strawberry.

ABSTRACT

Strawberry mild yellow-edge virus (SMYEV) was eliminated from 4 mm long shoot apex explants of Hood strawberry plants held at a constant 38 C in a growth chamber. Virus detection in each regenerated plant was by means of leaf graft analysis to a maximum of three Fragaria vesca indicator plants. The percentage of virus elimination from these explants was directly related to the length of time that they had grown at 38 C. The linear equation describing this relationship ($Y = -2.08X + 80.8$) predicts elimination of SMYEV from 50% of shoot apices excised after 15 days at 38 C and 100% after 39 days. SMYEV was also eliminated from Hood shoot explants grown at greenhouse temperatures of 20-25 C. The linear equation describing this relationship ($Y = 23.35X - 2.00$) predicts elimination of SMYEV from 50% of explanted shoot apices that were 2.2 mm in length and 100% that were 90 um in length. Heat therapy and shoot apex culture contribute independently to freeing explants of SMYEV and therefore should be combined to obtained increased reliability in developing strawberry clones free of SMYEV.

Strawberry mild yellow-edge virus (SMYEV) is a persistent, aphid-borne luteovirus of cultivated strawberry in many parts of the world (6, 7, 10). Because SMYEV infection of advanced strawberry selections occurs so rapidly in breeder's field plots, it has been necessary to eliminate this virus from a number of cultivars. Clones of many cultivars have been freed of SMYEV by use of heat therapy and/or shoot apex culture but with varying degrees of success (9, 10, 11, 14, 15, 17). In the present study we determined the incidence of SMYEV after varying the length of time of heat therapy and after varying the size of shoot apices excised from plants grown at temperatures of 20-25 C.

MATERIALS AND METHODS

Isolate 76-B of SMYEV in strawberry cv. Hood from Aurora, Oregon was used in these studies. The Hood clone was found to be free from other virus or viruslike agents detectable by leaf graft analysis (3) on Fragaria vesca clones UC-4 and UC-6 (4). SMYEV-infected Hood daughter plants were propagated in a screened, insecticide-treated greenhouse. For heat therapy studies Hood plants that were well established in large pulp pots inserted into larger pulp pots filled with peat moss were brought over a 30-day period from greenhouse temperatures of 20-25 C to a constant 38 ± 1 C in a growth chamber that furnished a mixture of fluorescent and incandescent light (11,000 lux) for 16 hours per day. The plants were watered only with $\frac{1}{4}$ strength Hoagland's solution at 38 C. At intervals (Table 1) during 45 days, runner tips 4 mm long were excised and grown out to regenerate whole plants in tissue culture using previously published techniques (1, 14).

The influence of explant size on the incidence of SMYEV in runner tips taken from Hood plants grown at temperatures of 20-25 C in the greenhouse was studied by taking explants ranging in size from 0.25 to 8.0 mm in length. Whole plants were regenerated from these explants as noted above.

Regenerated plants were grown on greenhouse benches and indexed by leaf grafting to UC-4 to identify those infected with SMYEV. Plants indexing negative were re-indexed on UC-4 and on F. vesca var. semperflorens (Duch.) Ser. cv. 'Alpine' over a 9-month period. Repeated virus testing over time is desirable because there are reports (5, 9, 13, 16) that the titer of some viruses falls below detection thresholds in explants after therapy, but rises later.

RESULTS

SMYEV incidence decreased in explants 4 mm in length as the period of growth at 38 C increased (Table 1). These data fit ($r = 0.927$, $P = 0.05$ to 0.10) the linear regression equation $Y = -2.08X + 80.8$, that predicts 50% inactivation of SMYEV after 15 days at 38 C.

As the length of excised runner tips from plants grown at greenhouse temperatures of 20-25 C decreased, the incidence of SMYEV in the resulting regenerated plants also decreased (Table 2). Despite considerable variability (e.g., 2.0 mm data), the data for tips 0.25 to 4.0 mm long fit the linear regression equation $Y = 23.35X - 2.00$, that predicts 50% of the explants 2.2 mm in length would be freed of SMYEV ($r = 0.922$, $P = 0.02$ to 0.05).

In this study, the overall reliability of leaf graft indexing on Alpine and UC-4 to detect this Hood isolate of SMYEV was 73%. This figure was obtained by pooling the indexing results from known positive plants per total plants tested from all three of the graft runs (43/62, 17/23, 12/14). Total numbers decreased with each succeeding graft run because only negative plants were retested each time.

DISCUSSION

Although there are no previous time studies on the elimination of SMYEV in strawberry plants by heat therapy, Posnette and Cropley (15) obtained a daughter

plant free of SMYEV after 16 days at 37 C, while the mother plant remained infected. Vine (17) found no evidence that heat treatment helped to eliminate SMYEV from infected clones. Mellor and Fitzpatrick (9) found that mother plants remained infected after 6 months at 38 C but that small axillary buds propagated from those plants after 3 months at 38 C were often freed of SMYEV. In the present study, an inverse linear relationship was found between the length of time infected plants were held at 38 C and the resulting incidence of SMYEV in explants. The equation predicts explants taken after 39 days at 38 C should be free of graft-detectable SMYEV.

It is widely accepted for many viruses, including SMYEV, that the smaller the shoot apex excised the more likely it is to be free of the virus being studied (1, 5, 8, 14, 16). Although not relating size of the initial explant to the incidence of SMYEV in the resulting regenerated plants, several workers found that excising shoot apices 0.3 to 8.0 mm in length from SMYEV-infected plants grown at greenhouse temperatures of 20-25 C gave rise to some healthy plants (11, 12). Miller and Belkengren (11) found no evidence that brief (1-7 day) heat therapy assisted in eliminating SMYEV from explant sources; however Mullin et al. (12) found that only 25% of 0.3 - 0.8 mm explants taken from normally grown infected strawberry plants were free of SMYEV, while 82% of such explants taken from plants grown for 42 days at 36 C were free of SMYEV. The present data show that decreasing explant length from runner tips grown at greenhouse temperatures of 20-25 C causes a proportional decrease of the incidence of SMYEV in the resulting explants. The equation describing this relationship predicts that all explants less than 90 um in length are likely to be free of SMYEV.

Both heat therapy and culturing of small shoot apices act independently to reduce SMYEV incidence in the resulting regenerated plants. It therefore appears advantageous to us to use a combination of heat therapy followed by tissue culture

of small excised tips from stolon apices or intercalary meristems in order to increase the probability of overcoming expected variations in cultivars, virus strains, and environmental influences on the elimination of SMYEV in strawberry clones.

A separate study (2) has shown that the reliability of detecting SMYEV by leaf grafting is such that three separate indexings must be negative to state with 95% confidence that the source plant is free of graft-detectable SMYEV. This present study confirms the previous report (2) on the unreliability of leaf graft indexing to detect SMYEV. Even when UC-4, a sensitive indicator for SMYEV detection, is used, the test was only 73% reliable. Therefore, three separate negative indexing tests of a given strawberry plant are needed in order to state with a confidence level between 95-99% that the plant is free from SMYEV detectable by leaf grafting. Nevertheless, in this study three unheated 4-8 mm propagants that one would have assumed were infected, each indexed negative for SMYEV in three consecutive leaf graft tests.

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Table 1. Incidence of strawberry mild yellow-edge virus in plants regenerated from excised runner tips 4 mm in length of strawberry cv. Hood grown for various periods of time at 38C.

Length of heat therapy period days	<u>Number of explants</u>		% of explants infected with SMYEV
	infected	total	
0	26	27	96
15	6	19	32
30	1	12	8
45	0	4	0

Table 2. Incidence of strawberry mild yellow-edge virus in plants regenerated from various lengths of strawberry cv. Hood runner tips grown at greenhouse temperatures of 20-25 C.

Average length of excised runner tips mm	Number of explants		% of explants infected with SMYEV
	infected	total	
0.25	1	20	5
0.5	0	12	0
1.0	6	14	43
2.0	4	15	27
4.0	26	27	96
8.0	11	12	92

D. Work in Israel by Dr. S. Spiegel.

1. The BARD project began in Israel in June 1980 with the import of the necessary strawberry indicator clones from Dr. Converse's laboratory. The indicator plants were established and propagated at the Volcani Center and serve as the basic detection system for strawberry viruses. In Israel, no data were available on the occurrence of strawberry mild yellow-edge and other viruses in commercial strawberry fields and therefore a survey was undertaken. Strawberry plants of the cultivars 'Aliso', 'Fresno', 'Tioga', and 'Nurit' were collected from commercial growing areas and were tested by leaf graft analysis on indicator plants for the presence of strawberry viruses. It was found that most plants of the cultivar 'Aliso' (the dominant cultivar at present in commercial fields) were carrying strawberry mild yellow-edge virus (SMYEV). Based on symptoms observed on indicator plants, there is a likelihood that more than one isolate of the virus is present in infected plants. Tobacco streak virus (TSV) was found in strawberry plants in one location in the northern part of the country. The virus caused typical necrotic shock symptoms in indicator plants and gave positive values in a ELISA test with an antiserum obtained from R. H. Converse. The presence of this virus in this region was not reported till now. Strawberry mottle virus (SMV) was found in strawberry plants in one location and caused typical leaf symptoms on indicator plants. Further characterization of the virus is presently being done.

2. A program for preparation of virus-tested propagation material was begun in Israel using heat-treatments and meristem culture techniques. The plants obtained are being periodically indexed for SMYEV and other virus infections.

3. Aphid collections were done throughout the growing season of fruiting fields, October-June, and in the strawberry nurseries, June-August. Aphis gossypii (Glov.) was regularly found on strawberry foliage throughout the growing season, occasionally in large colonies on the lower side of old leaves. During the spring the aphids collected mainly from young foliage were Macrosiphum euphorbiae (Thos.), Acyrtosiphon (Aulacorthum) solani (Kltb.), and Acyrtosiphon (Rhodobium) porosum (Sand.). In one instance, a few Acyrtosiphon pisum were found in a strawberry field adjoining a clover field. The main aphid vector of strawberry viruses, Chaetosiphon fragaefolii (Cock.), was not detected in Israel despite an extensive search in 1980-1982, but was detected in one location in May 1983. Transmission experiments with C. fragaefolii of the strawberry viruses present in Israel are now being conducted.

4. The possibility of isolating double stranded RNA (dsRNA) from SMYEV-infected strawberry plants as a rapid method for detecting this and other viruses was approached. Preliminary experiments indicated that the methods published for dsRNA isolation from plant tissue were unsuitable for tissues like strawberry leaves with a high level of polysaccharides and phenolic compounds which are known to interfere with isolation of RNA. Other methods were evaluated, like the method used by Dr. H.R. Cameron and his group at Oregon State University for isolation of a virus-associated RNA from cherry trees and methods for isolation of avocado sun-blotch viroid from avocado leaves. Modification of one of these methods allowed detection of several RNA bands observed in UV light following separation on polyacrylamide gels and staining with ethidium bromide. These bands were found to be single-stranded and were digested by RNAase in the presence of 0.3M NaCl. Attempts to develop a method for isolation of dsRNA from infected strawberry tissue are being continued.

SMYEV was purified from known infected 'Aliso' leaves collected in the field, following the procedure developed by Martin and Converse. Viruslike particles, 28-30 nm in diameter were associated with the band detected by UV scanning. Nucleic acids were prepared from the virus preparation and electrophoresed in PAGE and stained by ethidium bromide. A RNA band of high molecular weight was observed, the nature of which remains to be investigated.

E. Work in Israel by Dr. E. Tanne.

1. Three aphid species [Aphis gossypii (Glov.), Macrosiphum euphorbiae (Thos.), and Myzus persicae (Sulz.)] from local strawberry fields were tested for their ability to transmit local isolates of SMYEV. All three aphid species were given access to SMYEV with and without prior starvation to SMYEV from leaves and from purified preparations through membranes for 48 hours before being transferred to F. vesca UC-4, UC-5, and Alpine indicator plants and to a variety of herbaceous plants for inoculation feeding periods. Repeated trials along these lines through autumn and winter resulted in no positive transmissions in any case.

2. SMYEV nucleic acid extraction studies. Routine phenol extraction methods and several other methods of extraction reported in the literature were applied to healthy and SMYEV-infected strawberry leaf material. In one instance a disease-related, spreading band of presumed nucleic acid was noted.

5. Evaluation of research achievement with respect to the original research proposal

The original research plan had 9 objectives which will be restated, together with the research done to meet these objectives, in column form:

Objective	Results
A. Increase SMYEV by grafting indicators	Routinely accomplished to provide large large quantities of infected plants.
B. Investigate SMYEV membrane feeding assay	Extensive investigation failed to achieve positive results
C. Electron microscopy of SMYEV	23 nm isometric particles matched UV peaks
D. Purify SMYEV	This was done to the point of preparing several mg of purified virus
E. Evaluate purification results	This was done by electron microscopy and serology but not by membrane feeding of aphids
F. Produce SMYEV antiserum	Conventional polyclonal antisera in rabbits were produced that distinguish between SMYEV infected and healthy strawberries.
G. Evaluate serological methods for SMYEV	Indirect $F(ab')_2$ ELISA is the method of choice
H. Develop routine SMYEV detection tests	Indirect $F(ab')_2$ ELISA will distinguish between infected and healthy strawberry plants but background levels interfere with sensitivity and reliability
I. Bring to the level of practical use	SMYEV or a similar virus was found in a number of major U.S. strawberry cv. elite clones that indexed SMYEV-free by standard leaf graft bioassay.

In addition, tobacco necrosis virus was found to mimic symptoms and thus to interfere with the routine leaf graft detection of SMYEV. The reliability of leaf graft detection of SMYEV was investigated and the dynamics of its eradication by heat therapy and by tissue culture were carefully studied. Also considerable progress was made in developing dsRNA extraction as a diagnostic tool for SMYEV.

6. Description and results of cooperation

1. R. H. Converse spent the initial three months of the BARD contract in Israel instructing S. Spiegel and E. Tanne in the production of standard SMYEV indicators and leaf graft techniques currently in use to detect SMYEV and other strawberry viruses. This initial contact was the basis for the development of a standard strawberry virus indexing system by the Volcani group thereafter, and has resulted in identifying SMYEV as being very common in Aliso, the major Israeli strawberry cultivar.

2. E. Tanne spent a sabbatic year in the laboratory of R. H. Converse and initiated work that led to the identification of tobacco necrosis virus as a common greenhouse contaminant of strawberry indicators that caused symptoms that could easily be misread as virus. Subsequent use of sanitation measures and bio-detectors of TNV brought this problem under control.

3. S. Spiegel visited the laboratory of R. H. Converse twice, with support from U.S. strawberry industry sources, to instruct R. H. Converse in dsRNA extraction procedures and to develop further her skills in strawberry virus bioassay. As a result of these contacts she has identified additional viruses new to the Israeli strawberry industry. These will not be otherwise mentioned in this report because they are not related to SMYEV.

7. Thesis

In this overall project we tested the hypothesis that strawberry mild yellow-edge virus is a luteovirus. We found no data that would critically flaw this hypothesis, and a great deal of data to support it.

8. Inventions

None

9. List of published papers from this project to date

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Papers ready for publication:

- ** 1. Converse, R. H. and E. Tanne. 1983. Heat therapy and shoot apex culture to eliminate mild yellow-edge virus from Hood strawberry. (for Phytopathology.)
- ** 2. Martin, R. R. and R. H. Converse. 1983. Purification, some properties and serology of strawberry mild yellow-edge virus. 1983. (for Canadian Journal of Plant Pathology).

** See Section 4 for these manuscripts.