BIOLOGIA PLANTARUM (PRAHA)

33 (5):377–385, 1991

Attempts at Multiplication, Purification, Electron Microscopy, and Characterisation of Three Isolates of the Strawberry Mottle Agent

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Abstract. Three isolates of strawberry mottle agent (SMA) from strawberry plants were regularly maintained and multiplied by mechanical inoculation on *Chenopodium quinoa* plants showing mosaic and mottle symptoms. The use of 5 mM borate buffer pH 8.6 or tap water pH 6.6–7.9 with 4 % (m/v) charcoal for homogenization resulted usually in 100 % infection. The total of 2090 plants were infected from 2264 inoculated ones under the same conditions. The infectivity of SMA isolates in crude sap of *C. quinoa* was retained from 48 h to 72 h at 20 °C. The dilution end points of SMA isolates were 10^{-3} while the inactivation temperatures were between 50 and 55 °C. The infectivity of SMA isolates in frozen leaves of *C. quinoa* was detected still after six months.

Purification procedure of SMA is based on using low molar 25 mM borate buffer pH 8.3 with cysteine hydrochloride, DIECA and Tween 20 for homogenization of infected *C. quinoa* leaves, polyethyleneglycol precipitation, clarification with octanol, low and high speed centrifugation and sucrose density-gradient centrifugation. Partially purified preparations are highly infectious, causing mosaic, mottling and tip necrosis of *C. quinoa* plants. The agent could not be completely separated from host proteins and it could not be concentrated to a high extent. Isometric virus-like particles 14–16 nm were observed in partially purified preparations.

Strawberry mottle, the most widespread strawberry disease probably of viral origin, although known already 50 years (Harris 1938), till now has not been sufficiently characterized and the infectious agent has not been purified yet. Frazier and Posnette (1958) showed the strawberry mottle agent (SMA) to be a semi-persistent aphid-borne virus. Experimentally SMA was transmitted by various species of aphids, by grafting and by dodder. It can be transmitted mechanically with difficulty to some *Chenopodiaceae* plants (Frazier 1968). Polák and Bezpalcová (1987) succeeded in transmitting SMA to *Chenopodium quinoa*. Adams and Barbara (1986) reported unreliable sap transmission of SMA from *C. quinoa* to *C. quinoa*, particularly in mid-summer and in mid-winter.

In attempts to identify the causal agent of strawberry mottle disease, Kitajima *et al.* (1971) found aggregates of isometric virus-like particles in thin sections of *Fragaria vesca* leaves infected with SMA. The electron dense portions of these particles were 17 to 22 nm in diameter. Similar results were obtained by

Received August 29, 1990; accepted February 5, 1991

TABLE 1

The effect of buffers on the infectivity of extracts from *C. quinoa* plants infected with SMA isolates.

Buffer (3 g leaf was extracted in 9 ml of buffer)	SMA isolate	No. of plants inoculated	No. of plants infected	Infected plants [%]
0.025 M phosphate pH 7.5 with 1 % (m/v) PEG 6000	SM-1134	23	12	52.2
	SM-1279	38	22	57.9
Tap water (pH 6.6 to 7.9) with 4 % (m/v) charcoal	SM-1134	40	39	97.5
	SM-1279	56	56	100.0
	SM-RG	17	17	100.0
0.005 M borate buffer, pH 8.5, with 4 % (m/v) charcoal	SM-1134	18	18	100.0
	SM-1279	17	17	100.0
	SM-RG	18	18	100.0

Leistner and Graichen (1986) and by Polák and Bezpalcová (1987), who found these particles in infected *C. quinoa* leaves, too. Adams and Barbara (1986) tried to purify SMA from infected *C. quinoa* plants, but had no success. Hepp and Converse (1987) reported in abstract form the partial purification of SMA from infected *C. quinoa* leaves. This resulted in preparations containing isometric particles 30 nm in diameter.

This paper describes procedures for mechanical transmission and multiplication of three SMA isolates in *C. quinoa,* as well as some physical properties and attempts to purify SMA and to visualize it by electron microscopy.

MATERIAL AND METHODS

The isolates SM-1134 and SM-1279 originated from strawberry sources in the Netherlands and were selected from a group of 13 SMA isolates that had been transmitted from strawberry to *C. quinoa* by strawberry aphids (*Chaetosiphon fragaefolii*). They were chosen because sap transmission with both isolates consistently resulted in high percentages of infection whereas most of other isolates could not be maintained by weekly manual inoculation from *C. quinoa* to *C. quinoa.* The Czech SMA isolate, SM-RG has been transmitted from the strawberry cultivar "Red Gauntlet", locality Zbraslav, to *C. quinoa* by mechanical inoculation (Polák and Bezpalcová 1987). All three isolates of SMA were characterized as different in some physical properties and biological characteristics on *C. quinoa* plants.

To develop optimal procedures for mechanical inoculation and multiplication of SMA isolates, they were maintained in *C. quinoa* plants.

TABLE 2

Results of optimal procedure of mechanical transmission of three SMA isolates to *C. quinoa* plants.

SMA isolate	Number of trials	No. trials with all plants infected	No. of plants inoculated	No. of plants infected	Infected plants [%]	The average (variability) of incubation period [d]
SM-1134	13	7	420	373[+]	88.8	6.4 (4.6–8.2)
SM-1279	27	21	916	849	92.7	6.8 (5.0–8.5)
SM-RG	30	24	928	868	93.5	6.7 (4.9–8.5)

[+]Necrotic local lesions regularly developed after inoculation.

Systemically and locally infected leaves and infected stems of *C. quinoa* were used for homogenisation in purification attempts. Precipitation was carried out before the clarification of the SMA to reduce the volume of the preparation. All steps and variants of the purifications were checked by measuring the infectivity of the preparations on *C. quinoa* plants. Partially purified preparations were dialyzed against 2 % glutaraldehyde in borate buffer to be fixed. Gradients for density-gradient centrifuging were prepared by freezing and thawing of a 25 % sucrose solution.

Electron microscopy was performed by using a Philips CM12 or a Tesla BS-500 electron microscope. Preparations (droplets fixed by 2 % glutaraldehyde) were placed for 10 min on Formwar-coated grids. Negative staining was done by 2 % phophotungstic acid, adjusted to pH 7.0 with 1.0 M KOH. Fifty virus-like particles found in purified preparation of SMA were measured in Philips CM12 electron microscope by comparing them with a standard.

RESULTS AND DISCUSSION

Mechanical Inoculation and Multiplication of SMA

The influence of different buffers on infectivity of extracts from *C. quinoa* infected with SMA isolates is shown in Table 1. Plant extracts in phosphate buffer with 1 % (m/v) PEG 6000 resulted in a lower percentage of infection. Homogenization of leaves in tap water (with neutral or slight alkaline pH) with 4 % (m/v) charcoal usually resulted in 100 % infection of *C. quinoa* plants. Therefore, tap water with 4 % (m/v) charcoal was used in all mechanical transmissions for multiplication of SMA isolates in *C. quinoa* plants. Later on the suitability of 0.005 M borate buffer in mechanical inoculation of SMA was also tested and resulted in 100 % infection of *C. quinoa* plants. We recommend this buffer for mechanical inoculations of SMA because the pH of tap water may vary and SMA is rather sensitive to a low pH (Table 3).

TABLE 3

Effect of the homogenization buffer used in the purification procedure on infectivity of extracts from *C. quinoa* leaves infected with SM-1279.

homogenization buffer[+]	Number of inoculated plants	Number of infected plants
0.1 M citrate – 0.2 phosphate pH 4.9, with 5 mM EDTA, 10 mM DIECA, 0.05 % (m/v) Tween 20	14	0
0.01 M citrate – 0.02 M phosphate pH 4.6, with 5 mM EDTA, 10 mM DIECA, 0.05 % (m/v) Tween 20	14	2
0.1 M carbonate – bicarbonate pH 9.5, with 5 mM EDTA, 10 mM DIECA, 0.05 % (m/v) Tween 20	14	13
0.025 M borate pH 8.3 with 5 mM cysth, 10 mM DIECA, 0.05 % (m/v) Tween 20	14	14[++]
0.01 M Veronal – 0.007 M phosphate pH 6.9, with 7 mM EDTA, 10 mM cysth, 0.05 % (m/v) Tween 20	14	8

[+] = 10 g of infected leaves were homogenized in 30 ml of buffer and centrifuged at 6000 *g* for 5 min and at 12 000 *g* for 10 min. 5 ml of supernatants were taken for inoculation after 2 h of standing at 4 °C

[++] = severe systemic symptoms

EDTA = Na-ethylenediaminotetraacetate

DIECA = Na-diethyldithiocarbamate

Cysth = cystein-hydrochlotride

Darkening of plants 24 h before inoculation resulted in a higher number of infected plants when using phosphate buffer for homogenization and in a higher number of local lesions developed when using tap water with charcoal or borate buffer. Three grams of systemically infected shoots from decapitated plants are usually obtained in comparison with one gram from non-decapitated plants. *C. quinoa* plants were most suitable for inoculation in the stage of eight true leaves. Older plants showed a resistance to inoculation. Leaves with systemic symptoms, 5–12 d after inoculation, were the best source of inoculum, later on the SMA concentration decreased, especially with the isolate SM-1134, which induces necrotic local lesions.

TABLE 4

Effect of organic solvents used for clarification in the purification procedure of SMA on the infectivity of preparations (strain SM-1279).

Organic solvent	Number of *C. quinoa* inoculated	Number of *C. quinoa* infected	Symptoms
5 % (v/v) n-octanol with 10 % (m/v) sucrose	10	10	severe mosaic, dwarfing, NLL
5 % (v/v) chloroform and 5 % (v/v) carbontetrachloride	10	10	moderate mosaic, without LL
1 % Triton X-100	10	9	moderate mosaic, without LL

NLL = numerous local lesions on inoculated leaves
without LL = without local lesions on inoculated leaves

So the optimal procedure for mechanical transmission and multiplication of SMA in *C. quinoa* plants is: Plants with eight fully developed true leaves are darkened 24 h before inoculation. Leaves of decapitated plants are dusted with Carborundum powder (600 mesh). Three grams of infected *C. quinoa* leaves are homogenized under cooling in 9 ml of tap water with 4 % (m/v) charcoal, or in 5 mM borate buffer pH 8.6 with 4 % charcoal and used for inoculation by rubbing it onto leaves.

This procedure was used for mechanical transmission and multiplication of three SMA isolates. from April till December the strains were regularly mechanically transmitted and multiplied in *C. quinoa* plants. The lower rate of infected plants occurred only in the beginning of August, in the last week of September and in the first week of October, more often in trials with the necrotic isolate SM-1134. Results of mechanical transmission of three SMA isolates to *C. quinoa* plants are given in Table 2.

Persistance of Infectivity in Crude Sap

With the isolates SM-1279 and SM-RG infectivity in crude sap of *C. quinoa* was retained after 60 h at 20 °C. Traces of infectivity were detected after 72, but not after 84 h. The isolate SM-1134 was still infective after 48, but not after 60 h at 20 °C. The dilution end points of all three isolates were 10^{-3} while the inactivation temperatures (10 min heating) were between 50 and 55 °C.

Purification of SMA

The results obtained with the different homogenization buffers used for purification are given in Table 3. A 0.025 M borate buffer pH 8.3, containing 5 mM cystein-HCl, 10 mM DIECA, and 0.05 % (v/v) Tween 20 was found to be the best one. Borate buffer with 10 mM $MgCl_2$ and 10 mM cystein-HCl was also suitable. Leaves of *C. quinoa* showing systemic symptoms were found to be the best source for the purification of SMA (Fig. 1). Leaves with local symptoms or stems of *C. quinoa* were less suitable.

The effect on the infectivity of organic solvents used for the clarification of sap are given in Table 4. The best preservation of the infectivity was reached by using n-octanol with sucrose. After further experiments the ammount of n-octanol was lowered to 4 %.

Based on the results obtained, the following procedure was used for routine purification: Leaves, buffers and organic solvents are chilled before the procedure is started. The temperature of the preparations is maintained between 3 and 5 °C during the whole procedure.

1. One hundred g of *C. quinoa* tissue is homogenized with a Pollähne press adding three volumes of 0.025 M borate buffer pH 8.3, containing 5 mM cystein-HCl, 10 mM DIECA and 0.05 % (v/v) Tween 20.
2. The homogenate is filtered through cheese-cloth and centrifuged for 5 min at 8 000 *g* and for 10 min at 23 000 *g*.
3. To the supernatant 10 % (m/v) polyethylene glycol 6000 and 25 mM NaCl is added and the solution is stirred for 30 min. The precipitate is collected by centrifuging 20 min at 10 000 *g*.
4. The sediment is resuspended in 100 ml of homogenization buffer and stirred for 30 min. Sucrose and n-octanol are added to make a 10 % (m/v) and a 4 % (v/v) solution, respectively. The solution is shaken for 3 min.
5. The green emulsion is centrifuged at 40 000 rpm for 2 h in a Beckman Ti45 rotor.
6. The yellow or yellow-brown translucent sediments are resuspended in 35 ml of 0.005 M borate buffer, pH 8.5, and stirred for 30 min. The suspension is clarified by centrifugation at 8 000 *g* for 10 min.
7. The supernatant is centrifuged at 45 000 rpm for 2 h in a Beckman Ti60 rotor.
8. The colourless or slight yellow translucent sediment and about 2 ml of the supernatant just above the sediment, are resuspended in 6 ml of 0.005 M borate buffer, pH 8.5, and stirred for 30 min. The suspension is clarified by centrifuging at 6 000 *g* for 5 min.
9. The suspension is centrifuged on top of a 10–40 % (m/v) sucrose gradient in 0.005 M borate buffer, pH 8.5 (1 ml of suspension per tube) for 2 h at 25 000 rpm in a Beckman SW28 rotor.

TABLE 5

Summarized results of purifications of three isolates of SMA.

	SMA isolate			Total	% of successful purifications
	SM-1134	SM-1279	SM-RG		
Number of purifications carried out	3	9	9	21	
Number of successful purifications	3	6	7	16	76.2

10. Samples are fractionated with an ISCO gradient-fractionator, using a UV detector at 254 nm. The second half of the peak (if only one is formed) or the second peak is collected, diluted 1:1 with borate buffer and centrifuged for 2 h at 45 000 rpm in a Beckman Ti60 rotor.
11. One ml of opalescent liquid from the bottom of the tube is mixed with one ml of 0.005 M borate buffer, dialyzed against 250 ml of borate buffer and is used as partially purified SMA preparation.

The purification procedure of SMA was checked after steps 2, 4 (before adding of sucrose and n-octanol), 6, 8 and 10 by biological test. The infectivity of the preparations increased during the purification procedure (Fig. 2). The procedure was used 25 times for the purification of SMA and the results are summarized in Table 5. We considered the purification to be successful when the infectivity of the preparation rose from the homogenization to the second ultracentrifugation step and was also found after the centrifugation on sucrose gradients. All three SMA isolates were purified several times. After homogenization sometimes not all inoculated plants became infected. Inoculations of *C. quinoa* plants with purified preparations resulted usually in severe infections (severe systemic mottling and mosaic, local lesions, twisting of leaves, tip necrosis, dwarfing of all inoculated plants) and the incubation period was shortened to four days. SMA was present (infective preparation) after sucrose-density-gradient centrifugation only in the second half of the peak or in the second smaller peak if it was formed (Fig. 3).

The purification procedure has been shown to be suitable for SMA, but the agent could not completely be separated from host proteins with sucrose-density-gradient centrifugations and it could not be concentrated to a high extent. When these preparations were analyzed spectrophotometrically, the obtained data indicated that they were not yet pure virus preparations.

The course of purification procedure of SMA indicated very small particles. Preparations of SMA had to be centrifuged at least for 2 h at 40 000 rpm in a Beckman Ti45 or Ti60 rotors for sedimentation of infectious agents. After

90 min of ultracentrifugation at 40 000 rpm we found some infectivity in supernatant. Virus-like particles, 14–16 nm in diameter (Fig. 4) were found in partially purified preparations. These particles were not found in preparations purified from healthy *C. quinoa* plants, using the same procedure. In spite of that fact origin of these particles can be a matter of discussion because of similarity with fraction-I protein and phytoferritin. We were not able to find any of 30 nm particles indicated by Hepp and Converse (1987) in their preliminary report.

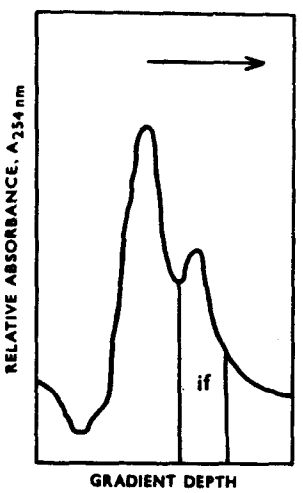

Fig. 3. UV absorbancy profile of sucrose density gradient centrifugation of SM 1279 isolate. if = infectious fraction.

We also tried to concentrate the SMA obtained after the sucrose gradient centrifugation by Cs_2SO_4 equilibrium-density-gradient centrifugation, but without success.

In attempts to further purify SMA from preparations after the second ultracentrifugation step on a column with Trisacryl GF 200, we have used 0.05 M borate (pH 7.4) as an elution buffer. None of the fractions obtained was infectious before or after concentration by ultracentrifugation. We also tried to purify SMA from infected *C. quinoa* plants using a purification procedure of Hepp and Converse which was kindly provided to us in the form of a preprint. Preparations obtained in this way caused no symptoms on *C. quinoa* plants when inoculated mechanically.

Further trials are necessary to prove identity of the strawberry mottle agent.

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Figs 1, 2 and 4 at the end of the issue