AN ABSTRACT OF THE THESIS OF

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Title: Evaluation of Hop (*Humulus lupulus* L.) Protoplast Inoculation with Prunus Necrotic Ringspot Virus

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Hop tissue (*Humulus lupulus* L.) was grown *in vitro* in callus, suspension, and protoplast cultures. Medium components were evaluated for optimal callus growth. Solid Murashige and Skoog (MS) medium with picloram at 1.5 uM, 6-benzylaminopurine (BAP) at 10 uM, 1 g/l casein hydrolysate, and 100 mg/l citric acid induced white, relatively friable callus from petioles of the cultivar Cascade. Suspension cultures were initiated and sustained using liquid Gamborg's B5 medium with 1 g/l casein hydrolysate, 100 mg/l citric acid, picloram at 1.5 uM, BAP at 10 uM, 250 mg/l L-glutamine, and 250 mg/l NH_4NO_3.

Protoplasts were isolated from suspension cultures to test procedures and conditions for inoculation with Prunus necrotic ringspot virus (NRSV). Successful infection was not verified; however, 0.0 to over 1,000 ng of virus were adsorbed per 50,000 protoplasts, the amount adsorbed

depending on the inoculation treatment. Poly-L-ornithine and protamine sulfate, included with inoculum as infection facilitators, both adversely affected protoplast viability, particularly with protamine sulfate concentrations above 10 ug/ml. This effect was moderated by adding 147 mg/l $CaCl_2$ to inoculum buffer.

Enzyme linked immunosorbent assay (ELISA), an immunofluorescent slide assay, and nucleic acid hybridization were used to detect NRSV in or on protoplasts. ELISA was the most reliable and sensitive method. Results from hybridization using a cDNA probe, reverse transcribed from NRSV RNA, were difficult to interpret because of non-specific hybridization with host RNA. Extraction with 2M LiCl was found to be an efficient way to extract total RNA from small plant samples. Possible reasons for negative results from protoplast inoculation are discussed.

Evaluation of Hop (*Humulus lupulus* L.) Protoplast Inoculation with Prunus Necrotic Ringspot Virus

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EVALUATION OF HOP (*HUMULUS LUPULUS* L.) PROTOPLAST INOCULATION WITH PRUNUS NECROTIC RINGSPOT VIRUS

INTRODUCTION

Hop Plants and Prunus Necrotic Ringspot Virus

Hop plants (*Humulus lupulus* L.) in the Pacific Northwest are frequently infected by two ilarviruses, Prunus necrotic ringspot (NRSV) and apple mosaic (ApMV) and three carlaviruses, hop mosaic (HMV), hop latent (HLV), and American hop latent (AHLV). Of these viruses, NRSV causes the most significant economic loss by decreasing quality, or alpha-acid content, of the hop cone and cone yield of the plant (A. Haunold, unpublished results; Thomas, 1980, Neve, 1973).

NRSV is possibly spread by several means in hops: a) vegetative propagation, b) seed transmission, c) root grafting, d) pollen transmission, e) flower thrips, and f) mechanical abrasion. A major source of NRSV spread is through vegetative propagation and distribution of infected rhizomes or cuttings. This mechanism of spread is important because of the importance of vegetative propagation for hop culture. NRSV is seed transmissible in many hosts, including *Prunus* species such as cherry, prune, and peach (Mink and Aichele, 1984; Megahed and Moore, 1967) and hops (Hampton, unpublished results; Thresh and Manwell, 1979). Although seeds are used in the hop industry only for breeding, transmission of NRSV

through the seed is a common means by which the virus is introduced into new hop clones. The potential for distributing NRSV between countries in seed is obvious. Spread within the hop yard is normally confined to local spread between neighbouring plants (Thresh, 1983; Thresh and Edwards, 1982) but occasionally, especially in certain areas, spread is believed to be rapid (Dr. A. Haunold and Dr. C. Skotland, personal communication; Thresh, 1972). Eppler (1983) proposed that NRSV is spread in the field by a) bees and other insects mechanically abrading flowers or leaves and leaving infected/infested pollen in the abrasions or b) by plant contact or mechanical abrasion during farming practices.

NRSV-infected plants can be rendered virus-free through meristem culture and/or heat treatment (Adams, 1975; Vine and Jones, 1969). Re-infection, however, may occur after therapy, as outlined above. A more permanent solution would be to incorporate genetic resistance into promising cultivars. Sources of resistance need to be found and studied for possible use.

Resistance to seed transmission of NRSV has been found. The source of this apparent resistance was hop accession 21130M. In 1977 and 1981, Cascade female plants were pollinated in Corvallis, Oregon with pollen from four male accessions, 21130M, 60023M, 19173M, and 19005M. Progeny were tested for the presence of NRSV. Results

showed that rates of virus seed transmission differed depending on the male accession used for pollination (Dr. R. O. Hampton, unpublished results). In 1977 crosses, progeny of accession 21130M X Cascade contained no NRSV whereas all progeny of accession 60023M X Cascade contained NRSV. In 1981 crosses, 12% of 60023M progeny contained NRSV but all 21130M progeny were again NRSV-free. Because Cascade was permissive to seed transmission, suppression was due to male accession 21130M.

Infection of isolated protoplasts provided a model system to investigate the nature of suppression of NRSV seed transmission by male accession 21130M. Protoplast infection is also a possible means of studying other mechanisms of resistance and for screening new hop genotypes for resistance to viruses.

Plant Protoplasts

Plant protoplasts were first isolated mechanically in 1892, but were not isolated enzymatically until 1960 (Hanke, 1980). Since the first isolation in 1960, enzymatic digestion of plant cell walls has helped make protoplast isolation routine. Protoplasts can now be used to facilitate studies on plant biochemistry and development by allowing a large population of cells to be subjected to treatments simultaneously, ready uptake of chemicals from solution, and precise monitoring of events

in the cell. Protoplasts are also uniquely suited for development of novel genotypes by protoplast fusion (Shepard et al., 1983) and for studying events of viral infection after *in vitro* inoculation (Motoyoshi, 1985; Sarkar, 1977).

Protoplast Infection with Virus

Protoplast inoculation has been used to examine multiplication of viruses in protoplasts of non-hosts (Furusawa and Okuno, 1978; Huber et al., 1977; Maekawa et al., 1985), multiplication of defective or variant virus strains (Mayo and Barker, 1983; Motoyoshi et al., 1974), and mechanisms of resistance and interaction between resistant plants and viruses (Hyber et al., 1977; Beier et al., 1977 and 1979; Kiefer et al., 1984; and Motoyoshi and Oshima, 1979). These examples help demonstrate how protoplasts can facilitate plant virus studies, especially mechanisms of resistance to viruses.

In fact, infection of protoplasts from resistant varieties or non-hosts has illustrated different mechanisms of resistance to plant viruses. Resistance can be expressed within each cell, and thus in isolated protoplasts, or only in the intact plant, requiring cell-cell interaction and thus not in protoplasts. Therefore, depending on the mechanism of resistance, the host range of some viruses can be artificially extended using protoplasts. Protoplasts may support viral infection when

the whole plant may be classified as immune or resistant (see Beier et al., 1977 and Motoyoshi and Oshima, 1979).

Knowledge of mechanisms of resistance in hops to NRSV could help generate interest to incorporate the resistance into breeding lines. Most breeding programs for disease resistance in hops have been been conducted without knowledge of the mechanisms involved or their genetic control (Royle, 1976). Protoplast infection would provide the opportunity to characterize the genetics and biochemistry of resistance of hop accessions to NRSV, including mechanisms of resistance to seed transmission, without having to spend years on conventional crosses. If a common mechanism for resistance of hops to NRSV operates within the cell rather than through cell-cell interaction, accessions could be screened for resistance by infecting protoplasts. Before protoplast culture can be applied for these research objectives, however, methods to infect a high percentage of hop protoplasts with NRSV need to be defined.

The purpose of this study was to develop methods precursory to the use of protoplasts for investigation of resistance in hops to NRSV. Major goals included: a) defining conditions for _in vitro_ culture of hop callus, suspension culture, and protoplasts, b) investigation of the usefulness of protoplast inoculation strategies available in the literature to the hop protoplast-NRSV

combination, and c) evaluation of methods to detect NRSV in protoplasts.

CHAPTER 1

GREENHOUSE AND *IN VITRO* CULTURE OF HOPS

INTRODUCTION

Optimizing *in vitro* culture conditions was a research objective for obtaining high yields of healthy hop callus tissue and suspension culture cells as a source of experimental protoplasts and subsequent culture of protoplasts. In previous studies, medium mineral salts, vitamins, and hormones were evaluated for efficacy of hop meristem growth (Adams, 1975; Vine and Jones, 1969; C. Goldstein and A. Sadler, unpublished results). Murashigi and Skoog (MS) mineral salts appeared to give the best results. These media were suitable for meristem culture but not for callus or suspension cell culture. Connell and Heale (1985) used Gamborg's B5 medium supplemented with 3 g/l casein hydrolysate and 1 mg/ml 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) for hop callus, suspension, and protoplast culture.

I found that 2,4-D was not suitable for critical *in vitro* studies, however, because it caused an undesirable texture and browning of callus. Therefore, two auxins, naphthaleneacetic acid (NAA) and picloram were compared with 2,4-D, in various ratios with BAP, to optimize hop callus growth in MS medium containing citric acid and casein hydrolysate. Results are given along with

conditions for *in vitro* culture of cell suspensions and protoplasts. The main research objective was to define culture conditions to obtain white, metabolically active cells and friable or soft, loose callus tissue.

MATERIALS AND METHODS

Hop Plant Culture

Clones of cv. Cascade, and USDA accessions 60023M, 21130M, 19005M, and 19173M were initiated by potting pieces of rhizomes (at least two dormant buds per piece) taken in early spring from plants grown in the hop germplasm repository at Corvallis. Plants were also initiated with softwood cuttings using the following method. Portions of stem (5 to 10 cm) including one pair of leaves with axillary buds were excised and the basal portion (5-7 cm) was dipped for three seconds in rooting solution[1]. The stem was then placed in wet 80 grit silica sand. The cuttings were kept in a mist chamber (12 second mists at 2.5 minute intervals) for 2-3 weeks until roots 1.2-2.5 cm long had formed. The rooted cuttings were potted in soil, left under mist for another two to four days, then placed in the greenhouse. Greenhouse temperatures ranged from 70-90°C during the day and 60-70°C at night.

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[1] Rooting Solution: 1,000 ppm of indolebutyric acid (IBA) and boric acid

a. prepare boric acid solution at 2 g/l distilled water
b. prepare IBA solution at 2 g/l 95% ethanol; stir
c. slowly combine the two solutions while stirring, keeping solution from heating up too much

Seed of Cascade X 21130M progeny (8118) were germinated using a method outlined by Dr. A. Haunold (personal communication). Seeds stored at 4°C were soaked for 5 minutes, with agitation, in a solution of 10:4:2 water:commercial bleach:95% ethanol. The seeds were then rinsed in cold running tap water for 15 minutes with agitation and placed onto qualitative-grade filter paper. The filter paper with seeds was layered over blotting paper and 1/4" foam rubber in a petri dish. Seeds were moistened with 15 ml water plus 5 ml of a solution of 30-35 ppm Captan and incubated for 6-8 weeks at 4°C. When most of the seeds began to germinate, the petri dishes were placed into an incubator with a day-night cycle of 16 hours of light at 25°C and 8 hours of dark at 10-15°C (alternating temperature not critical). The light quality approximated that of daylight. When roots and shoots were approximately 1 cm long, seedlings were planted in pots, covered with coarse silica sand (80 grit) and subirrigated until sturdy (approximately one week).

Supplemental lighting in the glasshouse was provided using a mixture of Gro-Lux (Sylvania) and Cool White (General Electric) fluorescent tubes to extend day length to 12-14 hours. Plants were fertilized with Ra-pid-Gro (23-19-17 N-P-K, RA-PID-GRO Corporation) every 7-14 days during the growing season and periodically with Osmocote (14-14-14 N-P-K, Sierra Chemical Co.).

Hop Callus Culture

Hop callus cultures were established from petioles of cultivars Cascade, Nugget, Galena, and Hallertauer progeny, USDA male accessions 21130M, 60023M, 19005M, 19173M, and 8118 (the progeny of 21130M X Cascade). Vigorously growing plants, some from the field but mostly from the greenhouse, were a source of petiole tissue. Callus was easier to obtain from vigorous rather than poorly growing explant tissue (see also Wetter and Constabel, 1982). In preparation for starting callus cultures, petioles were surface sterilized by washing 30 seconds in 70% ethanol, then 5 minutes in 1.05% sodium hypochlorite (20% commercial bleach) with 10 drops Tween 20 added per 100 ml solution. They were then washed in sterile distilled water. Middle sections of the petiole (5-10 mm) were placed on Murashige and Skoog (MS) medium (Musashige and Skoog, 1962; Wetter and Constabel, 1982). Citric acid (100 mg/l) and casein acid hydrolysate (1.0 g/l) were added, respectively to reduce browning and stimulate growth. Glucose (20 g/l) was used as a carbon source. Medium was solidified using 2.5 g/l Gelrite (Kelco Inc.). Auxin was supplied in the form of picloram (1.5 uM final concentration) and cytokinin as 6-benzyl-aminopurine (BAP) (10 uM final concentration). Both hormones were added in 25 ul DMSO (not more than 0.1% DMSO added to media) after autoclaving. Other auxins tried

were 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA) at various levels and ratios with BAP (see Tables 2 and 3, Results). Three replicate 125 ml flasks, each with three petiole sections/flask, provided callus-performance estimates for each hormone-ratio treatment. Fresh weight of callus was measured 2 months after petioles were planted on media.

Suspension Culture

Suspension cultures were initiated by shaking clumps of vigorously growing callus in 50 ml of supplemented Gamborg's B5 medium at 100 cycles/min on a gyrotory shaker. Ambient temperature averaged 22-24°C. To enhance rapid division and healthy metabolism of disaggregated cells, Gamborg's B5 medium (Gamborg et al., 1968; Wetter and Constabel, 1982) was supplemented with ribose (0.25), L-glutamine (0.58), NH_4NO_3 (0.25), casamino acids (1.0), and citric acid (0.1 g/l). Hormone supplements were the same as for callus cultures. After about 2 weeks, suspensions were subcultured by diluting 1:1 with new medium. Once suspended cells began rapidly dividing, they were subcultured weekly at ratios of 1:2 (21130M) or 1:4 (8118, 60023M, and Cascade), old to new medium.

Protoplast Isolation

Protoplasts were isolated from suspension cultures which were up to a week old or occasionally from vigorously growing callus which was not more than 2 months

old. Suspension culture cells collected by centrifugation (120 X g) were added to a filter sterilized enzyme mixture and incubated overnight (12-14 hrs) at 22-24°C. The enzyme mixture was similar to that used by Xu et al. (1982). It consisted of CPW13M solution[2], 3.0% Meicelase (Meiji Seika Pharma Int. Ltd, Tokyo, Japan), 1.5% Rhozyme (Genencor, South San Francisco), and 0.05% bovine serum albumin (BSA). Protoplasts were purified by sucrose floatation by mixing 37% sucrose in CPW salts 1:1 with the enzyme/protoplast solution and centrifuging at 120 X g for 10 minutes. Protoplasts were then washed once or twice by centrifugation (first time at 120 X g then at 50 X g) and resuspended in Kao's (Wetter and Constabel, 1982) or Gamborg's supplemented B5 medium (with total osmolarity made to 0.7 M with mannitol) or inoculum (described in Chapter 2). Viability was assessed by light microscopy of protoplast samples treated with either Trypan Blue (Wetter and Constabel, 1982) or 0.01% fluorescein diacetate (Windholm, 1972).

Protoplast Culture

Unless otherwise noted, protoplasts were cultured in the dark at 25°C at a concentration of 2 X 10^5 protoplasts/ml in plastic petri dishes (Falcon 1029, 100 X

[2]CPW salts consists of 101 KNO_3, 27.2 KH_2PO_4, 240 $MgSO_4$, and 1480 mg/l $CaCl_2$ buffered by 5 mM MES, pH 5.8. 13M indicates that the solution was 13% mannitol for high osmotic potential to maintain protoplast integrity.

15 mm dishes) in 0.1 ml drops or in 6- (Falcon 3069) or 24- (Corning 25820-24) well tissue culture plates. Plates were kept in humid chambers. Picloram (1.5 uM) and BAP (10 uM) were added to media to induce cell division.

The concentration of protoplasts for optimal cell viability was evaluated by incubating protoplasts at 9.24, 4.62, 2.31, 0.924, or 0.115 X 10^5 protoplasts/ml in Kao's media. Viability of protoplasts was also tested for 48 hours in six experimental media. The media tested were: 1) CPW13M, 2) supplemented B5 (used for suspension culture) with osmolarity adjusted to 0.7 M with mannitol (a) with and (b) without hormones, 3) supplemented B5 medium with 0.7 M mannitol and with phosphate level decreased to 0.2 mM, and 4) Kao's (a) with and (b) without hormones.

RESULTS

Growth of Callus

Callus weights from MS medium containing different levels of BAP and picloram, 2,4-D, or NAA are presented in Tables 1, 2, and 3 respectively. Of all hormone ratios used, picloram:BAP ratios of 0.1:20, 1.0:10, 1.0:20, and 10:20 uM induced the greatest amount of friable, white callus. Although it may appear otherwise from information in Tables 1, 2, and 3, at any level of 2,4-D or NAA, callus was less healthy (light colored, friable, rapidly growing) as with the above levels of picloram. Callus grown on medium containing NAA developed many roots.

Other medium components also influenced callus growth and health but were not evaluated quantitatively. At any level of auxin/BAP, cultures tended to brown unless citric acid was included at 100 mg/l. Activated charcoal at 0.1 to 0.3% was also tried as an anti-oxidant. No callus growth occurred on medium containing charcoal. Slight differences in growth were found when glucose and sucrose were compared as carbon sources. Flasks with glucose yielded slightly larger masses of callus. Also, callus grown on medium containing sucrose browned more readily than on medium containing glucose. There was no noticable difference in callus growth on MS or Gamborg's B5 medium. Casamino acids at 1 g/l appeared to slightly enhance the

Table 1A. Hop cultivar Cascade callus weight (g) in response to different ratios of picloram and 6-benzylaminopurine (BAP) on Murashige and Skoog (MS) medium[1].

PICLORAM (uM)	BAP (uM) 0.1	1.0	10.0	20.0
0.01	0.089 ± 0.027	0.064 ± 0.008	0.113 ± 0.067	0.359 ± 0.302
0.10	0.381 ± 0.365	0.508 ± 0.239	1.648 ± 0.793	2.037 ± 0.619
1.00	0.408 ± 0.208	1.851 ± 0.682	2.093 ± 0.764	1.999 ± 0.633
10.0	0.276 ± 0.052	0.821 ± 0.176	1.566 ± 0.317	2.067 ± 0.463

Table 1B. Appearance of callus grown on MS medium supplemented with picloram and BAP.

PICLORAM (uM)	BAP (uM) 0.1	1.0	10.0	20.0
0.01	necrotic hard (dry)	mostly necrotic some tan; hard	necrotic hard	necrotic hard
0.10	some tan mostly hard	some tan mostly hard	tan & white[1] soft/friable	tan & white some friable
1.00	some tan some brown	tan to brown soft/friable	white & tan friable	white & tan soft/friable
10.0	necrotic ± friable[2]	necrotic ± friable	tan to brown soft/± friable	tan friable

Table 1A (cont.). Hop cultivar Cascade callus weight (g) in response to different ratios of picloram and 6-benzylaminopurine (BAP) on Murashige and Skoog (MS) medium.

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[1]Standard deviations are based on callus weights from 3 or 4 replicate flasks; each flask contained 3 calluses.

Table 1B (cont.). Appearance of Cascade callus on MS medium supplemented with 2,4-D and BAP.

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[1]White callus generally indicated more healthy callus and was thus preferred to tan callus. The color listed first indicates the color that was most common in a particular treatment.

[2]Friable or soft callus was preferred to hard callus. The ± symbol means "somewhat".

Table 2A. Hop cultivar Cascade callus weight (g) in response to different ratios of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) on Murashige and Skoog (MS) medium (see footnote explanations for Table 1).[1]

		BAP (uM)			
		0.1	1.0	10.0	20.0
2,4-D (uM)	0.01	0.051 ± 0.011	0.056 ± 0.016	0.085 ± 0.019	0.249 ± 0.188
	0.10	0.043 ± 0.013	0.095 ± 0.031	0.090 ± 0.022	0.149 ± 0.168
	1.00	0.103 ± 0.030	0.178 ± 0.051	0.275 ± 0.111	0.319 ± 0.117
	10.0	0.724 ± 0.096	0.919 ± 0.445	1.238 ± 0.256	1.367 ± 0.361

Table 2B. Appearance of Cascade callus grown on MS medium supplemented with 2,4-D and BAP (see footnote explanations for Table 1).

		BAP (uM)			
		0.1	1.0	10.0	20.0
2,4-D (uM)	0.01	necrotic hard (dry)	mostly necrotic some tan; hard	necrotic hard	necrotic hard
	0.10	some tan mostly hard	some tan mostly hard	tan & white[1] soft/friable	tan & white friable & hard
	1.00	some tan some dark	tan to brown soft/friable	white & tan hard but moist	white & tan soft/friable
	10.0	necrotic ± friable[2]	necrotic ± friable	tan to brown soft/± friable	tan soft/friable

Table 3A. Hop cultivar Cascade callus weight (g) in response to different ratios of naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) in Murishige and Skoog (MS) medium (see footnote explanations for Table 1).[1]

		BAP (uM)			
		0.1	1.0	10.0	20.0
NAA (uM)	1.0	0.227 ± 0.107	0.513 ± 0.183	0.937 ± 0.256	1.103 ± 0.227
	10.0	0.358 ± 0.239	1.152 ± 0.526	1.285 ± 0.455	1.909 ± 0.458
	20.0	0.337 ± 0.344	1.576 ± 0.795	2.229 ± 0.745	2.103 ± 0.487
	30.0	0.240 ± 0.283	1.004 ± 0.362	2.145 ± 0.739	2.187 ± 0.838

Table 3B. Appearance of Cascade callus on MS medium supplemented with NAA and BAP.

		BAP (uM)			
		0.1	1.0	10.0	20.0
NAA (uM)	1.0	necrotic; dry hard; roots	dark brown; dry hard; roots	brown; dry; roots hard-friable	brown; dry hard
	10.0	brown; dry; roots friable-hard;	brown; dry friable; roots	brown; dry friable; roots	tan-brown[1] dry; hard
	20.0	brown/tan; roots dry but soft	brown/tan; dry friable; roots	brown/tan; roots dry; friable/hard	tan; roots dry; friable
	30.0	tan/brown; roots dry; friable	tan/brown; dry friable; roots	tan; roots; dry friable-hard	tan; roots dry; friable

amount of callus growth over medium without the supplement.

Suspension Culture

Gamborg's B5 medium with its lower concentrations of macronutrients, favored initiation and growth of cell suspensions more than the MS medium used for callus induction. Generally, after several cell suspension transfers done every other week to fresh medium at a 1:1 ratio, suspension cultures of 1-10 cell aggregates could be obtained. Once established, suspensions required subculturing at 6-8 day intervals. Cells of genotypes 8118, 60023M, and Cascade all were most easily maintained by using a 1:4 ratio of culture:new medium. Growth rates for 8118 and Cascade cultures are shown in Figure 1. 21130M, Galena, Nugget, 19005M, 19173M, and Hallertauer progeny cultures grew poorly as cell suspensions under my experimental conditions and required a 1:2 ratio for renewal. To keep cultures alive, these six genotypes often needed transfer of callus clumps along with disaggregated cells.

Protoplast Culture

Sustained protoplast viability depended on the medium type (Table 5) and concentration of protoplasts in culture medium (Table 4). Based on general appearance of cultures and percent protoplast viability, 2.0×10^5 protoplasts/ml appeared to be the optimal concentration with 0.924×10^5

Figure 1. Growth rate of hop cultivar Cascade and accession 8118 cell suspensions. Growth rate is expressed as daily change in packed cell volume (cell suspensions centrifuged at 121 X g).

Figure 1.

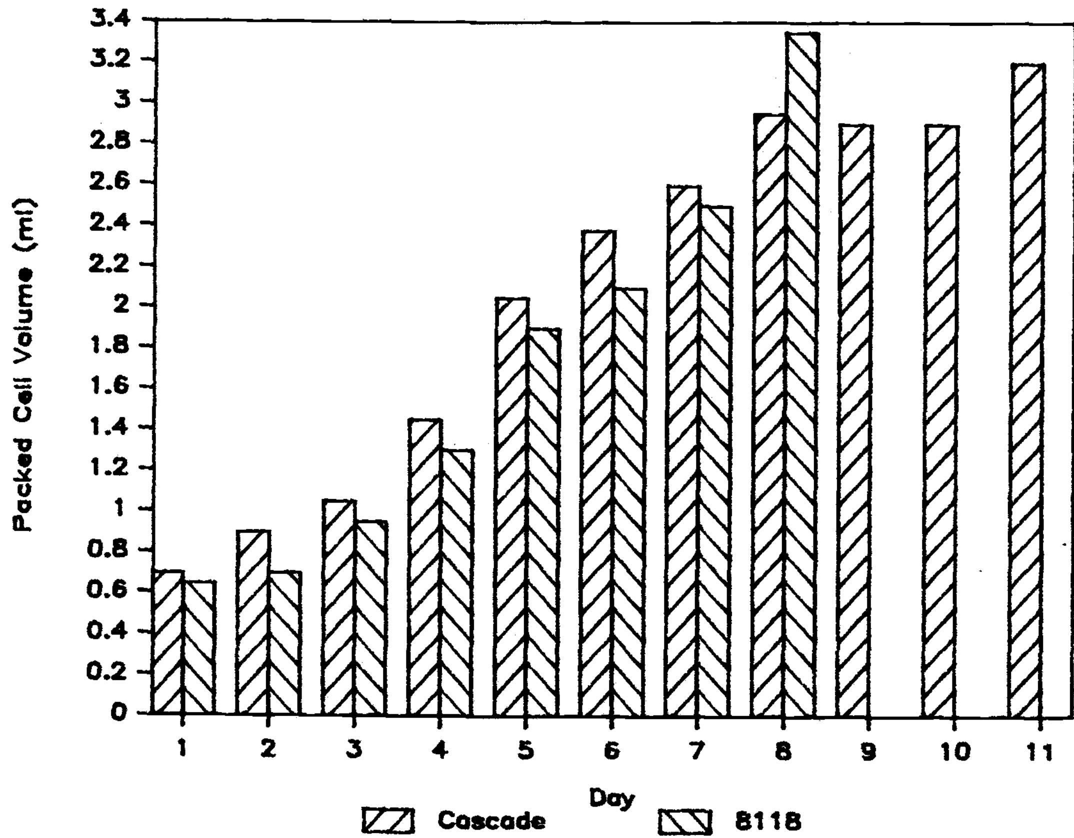

Table 4. Viability of cultured protoplasts as influenced by protoplast concentration.

Media	Protoplasts/ml ($X\ 10^{-5}$)	% Viability[1] 1 week	2 weeks
CPW13M	4.62	2 ± 0.4	<1
Kao's	9.24	7 ± 3.0	<1
"	4.62	20 ± 3.8	5 ±3.0
"	2.31	41 ±12.2	8 ±4.7
"	0.924	30 ± 6.2	23 ±8.5[2]

[1]Standard deviations based on 3-5 samples. Viability was determined by light microscopy after staining with fluorescein diacetate (Windholm, 1972).

[2]Although % viability was higher than with $2.31\ X\ 10^5$ protoplasts/ml, general appearance of cells and cultures did not appear to be better.

Table 5. Viability of cultured protoplasts as influenced by cultural medium.

Medium[1]	% Viability[2] 6 hours	48 hours
CPW13M	>70	34 ± 13
B5, 0.2 mM PO_4^-	40-50	18 ± 8
B5 w/ hormones[3]	40-50	30 ± 8
B5 w/o hormones	40-50	23 ± 14
Kao's w/ hormones	39	26 ± 4
Kao's w/o hormones	50	22 ± 5

[1]Respective medium contained 2 X 10^5 protoplasts/ml.

[2]Standard deviations based on 3-5 samples. Viability was determined by light microscopy after staining with fluorescein diacetate (Windholm, 1972).

[3]Picloram at 1.5 uM and 6-benzylaminopurine at 10 uM.

protoplasts/ml second. At a concentration of 0.115 X 10^5 protoplasts/ml, protoplasts did not maintain their viability over 1-2 weeks.

Protoplast viability was highest after 48 hours in CPW13M (Table 5). This viability, however, did not last for 1 or 2 weeks (Table 4). B5 and Kao's media, with or without hormones, were about equal for maintaining 20-30% of protoplasts viable for 48 hours. Protoplasts cultured for 48 hours in CPW13M fluoresced more brightly after treatment with fluorescein diacetate and in general appeared more healthy than protoplasts cultured in B5 or Kao's media.

DISCUSSION

Callus and Suspension Cell Growth

Less browning with more abundant, relatively friable callus was observed with cv. Cascade hop callus on medium with picloram as compared to medium with 2,4-D or NAA. The large amount of roots which formed on callus grown on medium containing NAA made that callus unsuitable for suspension culture initiation or protoplast isolation. This response to picloram occurs with other plant species. Mok and Mok (1977) found that picloram was better than 2,4-D, NAA, or IAA for inducing rapidly growing, friable callus with *Phaseolus* cultivars.

Even though hop tissue responded well to picloram, several transfers of selected callus onto fresh medium were required to obtain uniform, rapidly growing callus . This effect was more apparent with 21130M, Hallertauer progeny, Nugget, and Galena than with Cascade, 8118, or 60023M. Whether this was a conditioning response or just selection of callus from vigorous regions on the petiole was not determined, but taking into account the number of transfers needed with some genotypes, a conditioning response seems likely.

Browning of hop petiole callus tended to occur when sucrose rather than glucose was used as a carbon source. This was also observed for *Sequoia sempervirens* callus by

Ball (1953). Ball conjectured that hydrolysis of sucrose to glucose and levulose contributed to callus browning. Adams (1975) also found that meristems of cv. Bullion grew better with glucose than with sucrose and even that 2% sucrose, despite the presence of glucose, was inhibitory to meristem growth.

Citric acid at 100 mg/l in medium reduced callus browning. Callus failed to grow when medium contained activated charcoal, presumably because charcoal absorbed vitamins and hormones necessary for cell division.

As with callus, tissue selection and numerous transfers were required to obtain rapidly growing 'single' cell suspension cultures. Again, the effect was most apparent with 21130M, Hallertauer progeny, Nugget, and Galena. All of these genotypes required a 1:2 ratio of old to new media, often with callus transfer needed, rather than the 1:4 ratios used with Cascade, 8118, and 60023M. The fact that the former genotypes required more care in transfers than the latter genotypes indicates that either they didn't grow well in a disaggregated form or that hormone ratios or concentrations used were not optimal for them.

Protoplast Culture

Protoplast viability differed greatly depending on the concentration of protoplasts in culture medium. When the concentration was 9.24 or 4.62 X 10^5/ml, cultures

browned in less than one week. At these high concentrations, waste products or secondary metabolites may have been the limiting factor to healthy cell growth. When cultures were diluted to as low as 1.15 X 10^4 protoplasts/ml however, cells lost viability by two weeks. Gleba and Sytnik (1984) emphasized that one of the most important criteria for protoplast culture is maintaining the proper cell density. They stated that 10^4 to 10^5 cells/ml is optimum in most cases. Hanke (1980) cited references, however, which stated that protoplasts survived best when grown at concentrations greater than 10^6/ml.

In addition to protoplast concentration, cell viability depends on medium constituents and the cultural procedure. The protoplast plasmalemma tends to leak cell metabolites for a short time after isolation (Kao and Michayluk, 1975; Hanke, 1980) because of plasmolysis and/or cellulase activity (Taylor and Hall, 1976). Complex media (Kao and Michayluk, 1975) compensate for or retard the loss of these metabolites from freshly isolated protoplasts. Also, protoplasts of some genotypes regenerate into cell colonies when incubated in ul amounts of medium (Gleba and Sytnik, 1984), which also may compensate for metabolite loss. Caboche (1980) found that haploid tobacco protoplasts turned brown and died if cultured at an initial concentration less than 5 X 10^3

protoplasts/ml. Cells remained healthy, however, if the protoplasts were grown for 3-4 days at high densities with a high concentration of auxin. I found that at a concentration of 2 X 10^5 protoplasts/ml protoplasts survived equally well over the time interval tested in either supplemented B5 or Kao's medium. L-glutamine, thought to be the most important component of casein acid-hydrolysate for plant cell culture, is included at fairly high concentration in supplemented B5. This may contribute to the lack of observed difference between the two media. Also, because the protoplasts were not routinely cultured at concentrations less than 10^5 protoplasts/ml, a complex medium such as Kao's was not essential. I routinely cultured protoplasts in Kao's medium when maintaining cells for over one week, however, because of the outside chance that it might have enhanced culture survival and growth.

CHAPTER 2

EVALUATION OF CONDITIONS FOR INOCULATION OF HOP CUTTINGS AND PROTOPLASTS WITH PRUNUS NECROTIC RINGSPOT VIRUS

INTRODUCTION

Protoplasts were first successfully inoculated with a plant virus in the late 1960's (Takebe et al., 1969). Since this time, numerous investigators have inoculated protoplasts to study stages of virus multiplication, ultrastructure of infected cells, resistance to plant viruses, and interaction between viruses within one host (see reviews by Takebe, 1984, and Motoyoshi, 1985).

Each virus-host combination requires somewhat unique conditions for successful protoplast inoculation. Variations in inoculation conditions include polycation types and concentration, polycations vs. polyethylene glycol, buffer salts at various concentrations, concentration of non-buffering salts, buffer pH, and concentration of virus. The purpose of this investigation was to explore conditions requisite to successful NRSV inoculation of hop protoplasts. Because protoplast inoculation treatments may affect success of subsequent protoplast culture, viability was tested after some inoculation treatments. Mechanisms of protoplast infection are also discussed.

Susceptibility of protoplasts is often different from that of intact plants, depending on the mechanism of

resistance in the intact plant (Beier et al., 1977; Motoyoshi and Oshima, 1979; Maekawa, et al., 1985). Therefore, attempts were made to mechanically inoculate hop cuttings with NRSV to assess susceptibility of five hop accessions as a control for potential differences between susceptibility of protoplasts and intact plants.

MATERIALS AND METHODS

Hop Cutting Inoculations

Potted plants grown from soft-wood cuttings were mechanically inoculated with NRSV to determine susceptibility of Cascade, 21130M, 60023M, and 8118 to NRSV. When plants were showing 2-10 new leaf pairs (1-2 pairs fully expanded), they were inoculated by dusting with carborendum and rubbing inoculum on leaves with cotton swabs. Inoculum consisted of 1 g infected cucumber cotylendons ground in 10 ml 0.02M sodium phosphate buffer, pH 7.0 containing 0.1% thioglycollic acid. Other phosphate buffer additions were 2% nicotine (from Skotland, personal communication) and 0.01 M DIECA (Fulton, 1970). Leaves of different ages, from youngest to oldest on the plant, were inoculated and each inoculated leaf was marked. After 4-6 weeks under greenhouse conditions, uninoculated leaves near those that had been inoculated were assayed for NRSV by ELISA (Clark and Adams, 1977). Some plants were reassayed the next year in mid-spring to test the possibility that initial titers precluded detection by ELISA.

Protoplast Inoculation

Poly-L-Ornithine and Protamine Sulfate Protoplasts were isolated as described on page 11, Chapter 1. After counting with a hemacytometer, protoplasts were

centrifuged (50-120 X g) and resuspended, usually to a concentration of 4 X 10^5 protoplasts/ml, in 11-13% mannitol with or without 147 mg/l $CaCl_2$. They were mixed with an equal volume of double strength inoculum. Inoculum consisted of 12.5 ug/ml NRSV in the following buffers: (a) 0.02, 0.04, 0.05, or 0.1 M phosphate buffer, pH 5.5, 6.0, 6.5, or 7.0 with 1.0, 1.5, or 2.0 ug/ml poly-L-ornithine (PLO, MW ~200,000; Sigma) or 5.0, 10.0, 25.0, or 50.0 ug/ml protamine sulfate (PS, MW ~5,000; Grade X, Sigma) with and without 147 mg/l $CaCl_2$ or (b) 0.02, 0.05, or 0.1 M citrate buffer at pH 5.5 or 6.0 with 1.5 ug/ml PLO or 25.0 or 50.0 ug/ml PS. Virus concentrations of 0.5, 2.5, 5.0, and 25.0 ug/ml were also tested in 0.02 M phosphate buffer, pH 6.0 with 1.5 ug/ml PLO and in 0.05 M phosphate buffer, pH 6.0, with 50.0 ug/ml PS. Lyophylized NRSV was resuspended just before use with mannitol solution, usually to a concentration of 1 mg/ml. NRSV was then filter sterilized and added to inoculum buffer 10-20 minutes before protoplast solutions were added. After protoplasts were incubated with inoculum 10-20 minutes, they were centrifuged (50 X g) and washed once or twice with CPW13M. They were resuspended at a concentration of 2 X 10^5 protoplasts/ml in Kao's medium or CPW13M for incubation. In one experiment, protoplasts were resuspended directly in inoculum.

Polyethylene glycol Viral inoculation in the presence of polyethylene glycol (PEG) was essentially as described by Maule et al. (1980) with a few modifications. Protoplasts were centrifuged and virus (3.04 mg/ml in 11% mannitol) was added to the pellet to achieve a final concentration of 38 ug/ml after inoculum was diluted with mannitol as described below. Pelleted protoplasts + virus were gently shaken and transferred to PEG (0.1 ml of 40% PEG (MW 6,000-8,000), pH 5.8, with 441 mg/l $CaCl_2$, per 1 to 6 X 10^5 protoplasts). Protoplasts were shaken for 10 seconds and 2-3 drops of 11% mannitol solution was added. After another few seconds, more 11% mannitol was added dropwise over 5 minutes with intermittent mixing until 1.0 ml (for 0.1 ml PEG solution) was added. For one treatment, mannitol diluent included 0.05 M potassium phosphate, pH 6.5. The suspension was incubated for 15, 30, or 60 minutes, then the protoplasts were centrifuged (50 X g), washed once with 11% mannitol and resuspended at 2 X 10^5 protoplasts/ml in CPW11M for 24 and 72 hour incubations.

Detection of NRSV in Protoplasts

To evaluate susceptibility of hop genotypes or effectiveness of inoculation, two time samples were taken

(0 and 48 or 72 hours)[1] and assayed by enzyme linked immunosorbent assay (ELISA). The ELISA technique was performed as outlined by Clark and Adams (1977). Samples, consisting of protoplasts in culture medium, were frozen in liquid nitrogen, lyophylized, and then resuspended in ELISA sample buffer at 5.0×10^4 cells/0.2 ml buffer (for 5.0×10^4 cells in one ELISA plate well). After all of the sample was suspended by vortexing, cells were homogenized with a 25 gauge needle with plastic syringe.

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[1]Usually, three replicates of 1×10^5 cells were taken for each sampling time. Each of these replicates were split into two samples and each sample put in one ELISA plate well (0.5×10^5 cells/well). Promising treatments were repeated for two or three separate inoculations.

RESULTS

Cutting Inoculations

Of 130 hop cuttings (49 Cascade, 31 21130M, 27 8118, 11 19005M, and 12 19173M) mechanically inoculated with NRSV, none were infected 6 weeks after inoculation. The following spring, after plant dormancy, 15 cuttings of Cascade, 11 of 21130M, and 10 of 8118 were again tested for the presence of NRSV. None contained detectable NRSV. All 60023M cuttings, propagated from NRSV-infected plants, contained detectable NRSV. ELISA tests showed that 19005M and 19173M field plants were NRSV-free when rhizomes were taken for greenhouse plants. Testing in subsequent years showed that both plants had become infected in the field.

Protoplast Inoculation

No virus synthesis resulting from infection was observed when Cascade protoplasts and NRSV were incubated with PLO, PS, or PEG. Various amounts of virus were adsorbed to protoplasts, however, depending on the amount of PLO or PS (Table 6 and Table 7 respectively), concentration and type of buffer (Tables 8, 9, and 10), and concentration of NRSV as inoculum (Table 6).

Poly-L-Ornithine With no PLO present, the amount of NRSV adsorbed was equal to or greater than the amount adsorbed with 1.0 or 1.5 ug/ml PLO (Table 6). Differences between samples incubated with 1.0 vs. 1.5 ug/ml PLO appeared to be negligable. The amount of virus retained

Table 6. Effect of poly-L-ornithine (PLO) and Prunus necrotic ringspot virus (NRSV) concentrations in inoculum buffer on adsorption of NRSV to Cascade protoplasts (buffer was 0.02 M phosphate, pH 6.0 with 147 mg/l $CaCl_2$).

[PLO] (ug/ml)	[Inoculum NRSV] (ug/ml)	ELISA A_{405}, T_0 [1]	Estimated[2] Adsorbed NRSV (ng)	ELISA A_{405}, T_{48} [1]	Estimated[2] Retained NRSV, T_{48} (ng)
0.0	0.0	0.000	--	0.000	--
0.0	5.0	0.102 ± 0.006	1.65	0.033 ± 0.003	1.48
0.0	12.5	0.323 ± 0.039	2.34	0.090 ± 0.012	1.62
0.0	50.0	0.877 ± 0.039	5.65	0.233 ± 0.089	2.03
1.0	12.5	0.189 ± 0.021	1.89	0.059 ± 0.013	1.54
1.0	50.0	0.616 ± 0.106	3.73	0.260 ± 0.010	2.12
1.5	2.5	0.136 ± 0.009	1.75	0.021 ± 0.005	1.45
1.5	5.0	0.157 ± 0.019	1.81	0.057 ± 0.026	1.54
1.5	12.5	0.125 ± 0.027	1.71	0.028 ± 0.006	1.47
1.5	12.5	0.320 ± 0.026	2.33	0.067 ± 0.003	1.56
1.5	50.0	0.568 ± 0.041	3.46	0.189 ± 0.019	1.89

Table 6 (cont). Effect of poly-L-ornithine (PLO) and Prunus necrotic ringspot virus (NRSV) concentrations in inoculum buffer on adsorption of NRSV to Cascade protoplasts (buffer was 0.02 M phosphate, pH 6.0 with 147 mg/l $CaCl_2$).

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[1] T_0 and T_{48} refer to the number of hours protoplasts were incubated after inoculation before samples were frozen for assay by ELISA.

[2] The amount of NRSV adsorbed to protoplasts was estimated, as described in the Appendix, using standard curves for absorbance at 405 nm of samples that contained defined amounts of partially purified NRSV.

by protoplasts for 48 hours was less than the amount adsorbed but remained at 1.45 to 2.12 ng. The differences in amount retained between treatments were small and depended more on the concentration of NRSV during inoculation than on the level of PLO.

Protamine Sulfate The effect of PS at three concentrations in four inoculation buffers on retention of NRSV by protoplasts was estimated by post-inoculation assays of protoplasts by ELISA (Table 7). Estimated amounts of NRSV adsorbed ranged from 4.3 to 1,103 ng depending on the buffer and the concentration of PS in the buffer. Increasing the concentration of PS from 10 to 25 or 50 ug/ml resulted in a dramatic increase in virus adsorbed. Although this association was consistent with different buffers, the actual amounts adsorbed were not consistent. The large difference in NRSV adsorbed in experiment 2 (Table 7) between the two samples with 10 ug/ml PS is probably not due to treatment. A more likely reason is inaccurate calculation of amounts adsorbed from A_{405} values. These two samples were in different ELISA batches which had different substrate incubation times (see Appendix). The estimates, however, were done using the same standard curve, resulting in a large error. For this same reason, comparisons of the amount of NRSV adsorbed to protoplasts due to PS concentration should be

Table 7. Effect of protamine sulfate (PS) concentration in different buffers on adsorption of Prunus necrotic ringspot virus (NRSV) to Cascade protoplasts.[1,2]

Buffer A., experiment I. 0.02 M phosphate, pH 6.5, 12.5 ug/ml inoculum NRSV, conjugate 2[3].

[PS] (ug/ml)	A_{405}[4]	Estimated[5] Adsorbed NRSV (ng)
10.0	0.672 ± 0.042	6.2
25.0	1.324 ± 0.118	79.9
25.0	1.302 ± 0.040	73.3
50.0	1.596 ± 0.029	232.0

Buffer A., experiment 2. 0.02 M phosphate, pH 6.5, 12.5 ug/ml inoculum NRSV, conjugate 2[3].

[PS] (ug/ml)	A_{405}[4]	Estimated[5] Adsorbed NRSV (ng)
10.0	1.186 ± 0.099	46.5
10.0	1.666 ± 0.085	305.0
25.0	1.903 ± 0.129	772.0
25.0	1.869 ± 0.107	676.0
50.0	1.994 ± 0.198	1,103.0

Buffer B. 0.02 M phosphate, pH 6.5, 1.0 mM $CaCl_2$, 12.5 ug/ml inoculum NRSV, conjugate 3[3].

[PS] (ug/ml)	A_{405},T_0[4]	Estimated[5] Adsorbed NRSV (ng)	A_{405},T_{48}[4]	Estimated[5] Retained NRSV (ng)
0.0	0.010 ± 0.005	4.9	0.000	0.0
10.0	0.221 ± 0.023	26.7	0.076 ± 0.020	8.4
25.0	0.310 ± 0.021	54.2	0.174 ± 0.018	18.3

Table 7 (cont.). Effect of protamine sulfate (PS) concentration in different buffers on adsorption of Prunus necrotic ringspot virus (NRSV) to Cascade protoplasts.[1,2]

Buffer C., experiment 1. 0.05 M phosphate, pH 6.0, 12.5 ug/ml inoculum NRSV, conjugate 2[3].

[PS] (ug/ml)	A_{405}[4]	Estimated[5] Adsorbed NRSV (ng)
10.0	0.853 ± 0.010	12.6
25.0	1.212 ± 0.103	51.5
25.0	1.295 ± 0.160	71.3
50.0	1.119 ± 0.087	35.8
50.0	1.428 ± 0.045	120.1

Buffer D., experiment 1 0.05 M phosphate, pH 6.5, 12.5 ug/ml inoculum NRSV, conjugate 2[3].

[PS] (ug/ml)	A_{405}[4]	Estimated[5] Adsorbed NRSV (ng)
10.0	0.578 ± 0.009	4.3
25.0	0.705 ± 0.025	7.1
50.0	0.939 ± 0.022	17.7

Table 7 (cont.). Effect of protamine sulfate (PS) concentration in different buffers on adsorption of Prunus necrotic ringspot virus (NRSV) to Cascade protoplasts.[1,2]

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[1]Several buffers and experiments are given separately because data between experiments are not directly comparable. Comparisons for differences in adsorption due to PS concentration should be made only within each buffer set.

[2]Data only for NRSV adsorbed are presented in most buffer sets because protoplasts were not viable if treated with concentrations of PS greater than 10 ug/ml if Ca^{++} was not present (see text).

[3]Conjugate references refer to particular batches of anti-NRSV IgG-alkaline phosphatase conjugate. A_{405} readings differed depending on the conjugate batch. [4]Preparation of three conjugate batches was required during the course of experiments because batches were used up.

[4]Standard deviations are based on separate ELISA readings for three samples.

[5]The amount of NRSV adsorbed to protoplasts was estimated, as described in the Appendix, using standard curves for absorbance at 405 nm of samples that contained defined amounts of partially purified NRSV.

made within each buffer grouping presented in Table 7.

Less NRSV was present 72 hours after inoculation with Buffer B than originally adsorbed. The amount retained, however, was proportional to the amount initially adsorbed.

Less than one percent of protoplasts were viable if inoculated with PS concentrations above 10 ug/ml if no Ca^{++} was present. For this reason, estimates only of virus adsorbed are given for buffers without Ca^{++} in Table 7.

PS appears to hold more potential than PLO to facilitate infection of Cascade protoplasts with NRSV. This is based on the fact that more NRSV was adsorbed to protoplasts in the presence of PS than with PLO.

Buffer Concentration and Type There were no obvious differences in the amount of NRSV adsorbed by Cascade protoplasts when PLO was included with 0.02, 0.05, or 0.01 M phosphate buffer. In one experiment, (Table 8, Set I), only about 2.5 ng NRSV was adsorbed, less than expected on the basis of previous experiments, and none was retained in any treatment. In the other experiment (Table 8, Set II), more NRSV was adsorbed with 0.05 M than with 0.02 M phosphate buffer. Although less virus was adsorbed with 0.1 M phosphate, increased adsorption was not consistently associated with increasing buffer concentration. This difference may be due to sample variation. Alternatively,

Table 8. Effect of phosphate buffer concentration on the estimated amount of Prunus necrotic ringspot virus (NRSV) adsorbed and retained by Cascade protoplasts with 1.5 ug/ml poly-L-ornithine[1].

Set I. Phosphate buffer, pH 6.5, 25 ug/ml inoculum NRSV, conjugate 2[2].

[Buffer]	A_{405}, T_0 [3]	Estimated[4] Adsorbed NRSV (ng)	A_{405}, T_{48} [3]	Estimated[4] Retained NRSV (ng)
0.02 M	0.031 ± 0.008	2.56	0.000	0.00
0.05 M	0.028 ± 0.004	2.52	0.000	0.00
0.10 M	0.032 ± 0.011	2.58	0.000	0.00

Set II. Phosphate buffer, pH 6.0, 12.5 ug/ml inoculum virus, conjugate 1[2].

[Buffer]	A_{405}, T_0 [3]	Estimated[4] Adsorbed NRSV (ng)	A_{405}, T_{48} [3]	Estimated[4] Retained NRSV (ng)
0.02 M, 1 mM Ca^{++}	0.320 ± 0.026	2.33	0.067 ± 0.003	1.56
"	0.125 ± 0.027	1.71	0.028 ± 0.006	1.47
0.05 M	0.837 ± 0.201	5.29	0.025 ± 0.006	1.46
0.10 M	0.345 ± 0.049	2.43	0.020 ± 0.003	1.45

Table 8 (cont). Effect of phosphate buffer concentration on the estimated amount of Prunus necrotic ringspot virus (NRSV) adsorbed and retained by Cascade protoplasts with 1.5 ug/ml poly-L-ornithine.

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[1]Data are presented in two sets because data between sets are not directly comparable. Comparisons for differences in adsorption due to buffer concentration should be made only within each set.

[2]Conjugate references refer to particular batches of anti-NRSV IgG-alkaline phosphatase conjugate. A_{405} readings differed depending on the conjugate batch. Preparation of three conjugate batches was required during the course of experiments because batches were used up.

[3]T_0 and T_{48} refer to the number of hours protoplasts were incubated after inoculation before samples were frozen for assay by ELISA. Standard deviations are based on separate ELISA readings for three samples.

[4]The amount of NRSV adsorbed to protoplasts was estimated, as described in the Appendix, using standard curves for absorbance at 405 nm of samples that contained defined amounts of partially purified NRSV.

the presence of Ca^{++} in the 0.02 M buffer may have altered effects due to only buffer concentration and thus confused results.

More NRSV was adsorbed using 0.02 M phosphate than with 0.05 or 0.1 M phosphate when PS was included in the inoculum buffer (Table 9). This trend of reduced adsorption in higher concentrations of phosphate is consistent for both experiments which involved PS.

More NRSV was adsorbed to protoplasts using phosphate than citrate as inoculum buffer (Table 10). Also, more NRSV was adsorbed using 0.02 than 0.05 M citrate.

Inoculation Buffer pH Amount of virus adsorbed depended very little on pH of the buffer in which protoplasts were inoculated (inoculation buffer). No consistent changes in the amount adsorbed were observed with phosphate buffer pHs of 6.0, 6.5, or 7.0 and citrate buffer pHs of 5.5 and 6.0. Any differences observed appeared to be due to test-to-test variation.

Polyethylene Glycol A_{405} values from samples taken 3, 24, 48, or 72 hours after inoculation in polyethylene glycol (PEG) showed that no detectable NRSV successfully attached to, penetrated, or infected Cascade protoplasts. These results applied to all treatments including 15 or 30 minute or 1 hour incubation periods when PEG was diluted dropwise with 11% mannitol, or 1 hour incubations with rapid dilution or with 11% mannitol diluent buffered with

Table 9. Effect of phosphate buffer, pH 6.5, and protamine sulfate concentrations on the estimated amount of Prunus necrotic ringspot virus (NRSV) adsorbed by Cascade protoplasts. Protoplasts were inoculated with 12.5 ug/ml inoculum NRSV and assayed with conjugate batch 2[1,2].

Experiment I.

[Buffer]	[PS] (ug/ml)	A_{405}[3]	Estimated[4] Adsorbed NRSV (ng)
0.02 M	25	1.324 ± 0.118	79.9
0.02 M	25	1.302 ± 0.040	73.3
0.05 M	25	0.705 ± 0.025	7.07
0.10 M	25	0.815 ± 0.045	10.9
0.02 M	50	1.596 ± 0.029	232.0
0.05 M	50	0.939 ± 0.022	17.7
0.10 M	50	0.885 ± 0.049	14.3

Experiment II.

[Buffer]	[PS] (ug/ml)	A_{405}[3]	Estimated[4] Adsorbed NRSV (ng)
0.02 M	25	1.869 ± 0.107	676.0
0.02 M	25	1.903 ± 0.129	772.0
0.05 M	25	0.970 ± 0.019	20.0
0.10 M	25	0.605 ± 0.013	4.8

Table 9. Effect of phosphate buffer, pH 6.5, and protamine sulfate concentrations on the estimated amount of Prunus necrotic ringspot virus (NRSV) adsorbed by Cascade protoplasts. Protoplasts were inoculated with 12.5 ug/ml inoculum NRSV and assayed with conjugate batch 2[2].

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[1]Data are presented in two experiments because data between experiments are not directly comparable. Comparisons for differences in adsorption due to buffer concentration should be made only within each experiment.

[2]Conjugate reference refers to the particular batch of anti-NRSV IgG-alkaline phosphatase conjugate. A_{405} readings differed depending on the conjugate batch. Preparation of three conjugate batches was required during the course of experiments because batches were used up.

[3]Standard deviations are based on separate ELISA readings for three samples.

[4]The amount of NRSV adsorbed to protoplasts was estimated, as described in the Appendix, using standard curves for absorbance at 405 nm of samples that contained defined amounts of partially purified NRSV.

Table 10. Effect of citrate (cit) and phosphate (phos) buffers containing poly-L-ornithine (PLO) or protamine sulfate (PS) on adsorption and retention of Prunus necrotic ringspot virus (NRSV) by Cascade protoplasts. Buffer contained 12.5 ug/ml NRSV as inoculum but no $CaCl_2$ was included[1].

Buffer	pH	Polycation (ug/ml)	A_{405}, T_0 [2]	Estimated[3] Adsorbed NRSV (ng)	A_{405}, T_{48} [2]	Estimated[3] Retained NRSV (ng)
0.02 M cit	5.5	PS @ 50	2.006 ± 0.160	33.7		
0.02 M phos	5.5	PS @ 50	2.916 ± 0.136	142.3		
0.05 M cit	5.5	PS @ 50	1.410 ± 0.066	13.1		
0.05 M phos	6.0	PS @ 50	2.851 ± 0.114	128.4		
0.05 M cit	5.5	PLO @ 1.5	0.351 ± 0.029	2.5	0.111 ± 0.024	1.7
0.05 M cit	6.0	PLO @ 1.5	0.206 ± 0.011	2.0	0.066 ± 0.009	1.6
0.05 M phos	6.0	PLO @ 1.5	0.837 ± 0.201	5.3	0.025 ± 0.006	1.5

Table 10 (cont). Effect of citrate (cit) and phosphate (phos) buffers containing poly-L-ornithine (PLO) or protamine sulfate (PS) on adsorption and retention of Prunus necrotic ringspot virus (NRSV) by Cascade protoplasts. Buffer contained 12.5 ug/ml NRSV as inoculum but no $CaCl_2$ was included[1].

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[1] Because no $CaCl_2$ was included for inoculation, protoplasts incubated with PS were not viable. Estimates of retained NRSV are given only for treatments with PLO (see text on protoplast viability after inoculation treatments).

[2] A_{405} values given for ELISA tests of protoplasts incubated 0 (T_o) or 48 (T_{48}) hours after inoculation. Standard deviations were based on separate ELISA values for three samples, 2 ELISA wells/sample.

[3] The amount of NRSV adsorbed to protoplasts was estimated, as described in the Appendix, using standard curves for absorbance at 405 nm of samples that contained defined amounts of partially purified NRSV.

0.05 M phosphate. Clumping and fusion of protoplasts was facilitated by PEG.

Hop Genotype There were only slight differences in the amounts of NRSV adsorbed in some experiments when protoplasts of different hop genotypes were inoculated but there was no consistent pattern (Table 11).

NRSV Adsorption to Protoplasts

The percentage of NRSV in the inoculum which was adsorbed to 5.0×10^4 protoplasts depended on other components in the inoculation buffer, as stated in sections above. The most realistic estimates for percentage of NRSV adsorbed include 1.87×10^{-3} % with 0.02 M phosphate buffer, pH 6.0 with 1 mM Ca^{++} and no PLO, 0.43 % with 0.02 M phosphate, pH 6.5, with 1 mM Ca^{++} and 25.0 ug/ml PS, and 3.7 % with 0.05 M citrate, pH 5.5, with 1 mM Ca^{++} and 25.0 ug/ml PS.

Protoplast Viability after Inoculation Treatments

Tables 12 A, B, and C, show data on the viability of protoplasts after inoculation treatments[1]. Protamine sulfate damaged protoplasts. This effect was moderated by including 147 mg/l $CaCl_2$ (1 mM Ca^{++}) to buffers with inoculum. In buffers containing 25 or 50 ug/ml PS without Ca^{++} less than 1% of protoplasts were viable immediately

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[1] Protoplast viability was assessed by light microscopy of protoplast suspensions after staining with fluorescein diacetate or trypan blue (see Chapter 1, Methods, p13).

Table 11. Effect of hop genotype on adsorbance of Prunus necrotic ringspot virus (NRSV) to protoplasts in phosphate buffer, 25 or 50 ug/ml protamine sulfate, and 12.5 ug/ml inoculum NRSV.

Experiment I.

[Buffer]	pH	[PS] (ug/ml)	Hop Genotype	A_{405} [1]	Estimated[2] Adsorbed NRSV (ng)
0.02 M	6.0	25	60023M	1.787 ± 0.176	490.0
0.02 M	6.0	25	Cascade	1.787 ± 0.136	490.0
0.02 M	6.5	25	60023M	1.860 ± 0.123	652.0
0.02 M	6.5	25	21130M	1.438 ± 0.027	125.0
0.02 M	6.5	25	Cascade	1.903 ± 0.129	772.0
0.02 M	6.5	25	Cascade	1.869 ± 0.107	676.0
0.05 M	6.0	50	60023M	1.425 ± 0.055	119.0
0.05 M	6.0	50	Cascade	1.045 ± 0.071	26.8
0.05 M	6.5	25	60023M	1.152 ± 0.091	40.7
0.05 M	6.5	25	Cascade	0.970 ± 0.019	20.0

Table 11 (cont). Effect of hop genotype on adsorbance of Prunus necrotic ringspot virus (NRSV) to protoplasts in phosphate buffer, 25 or 50 ug/ml protamine sulfate, and 12.5 ug/ml inoculum NRSV.

Experiment II.

[Buffer]	pH	[PS] (ug/ml)	Hop Genotype	A_{405} [1]	Estimated[2] Adsorbed NRSV (ng)
0.05 M	6.0	50	60023M	1.081 ± 0.132	30.8
0.05 M	6.0	50	21130M	1.222 ± 0.042	53.6
0.05 M	6.0	50	Cascade	1.119 ± 0.087	35.8
0.02 M	6.5	50	60023M	1.614 ± 0.088	248.9
0.02 M	6.5	50	Cascade	1.596 ± 0.029	232.0

[1] Standard deviations based on 3 samples, 2 ELISA wells/sample.

[2] The amount of NRSV adsorbed to protoplasts was estimated, as described in the Appendix, using standard curves for absorbance at 405 nm of samples that contained defined amounts of partially purified NRSV.

Table 12 A. Effect of protamine sulfate (PS), calcium ions, and phosphate buffer, pH 6.5, containing 12.5 ug/ml Prunus necrotic ringspot virus, on protoplast viability.

[Buffer]	147 mg/l $CaCl_2$	[PS] (ug/ml)	% Viability[1] at 24 hours
0.02 M	+	0.0	80
"	+	10.0	80
"	+	25.0	62
"	-	0.0	75
"	-	10.0	15
0.04 M[2]	+	25.0	75
"	-	0.0	78
"	-	10.0	53

[1]% Viability was determined by light microscopy after trypan blue was added to protoplast suspensions (Wetter and Constabel, 1982).

[2]Inoculations were performed by direct resuspension of protoplasts in inoculum instead of resuspending with mannitol solution and mixing 1:1 to double strength inoculum.

Table 12 B. Effect of buffer pH and buffers containing poly-L-ornithine (PLO) and 25 ug/ml Prunus necrotic ringspot virus (NRSV) on protoplast viability.

Buffer	pH	[PLO] (ug/ml)	% Viability at 6 hours[1]
0.02 M phosphate	6.5	1.5	59
0.05 M phosphate	6.0	2.0	54
0.05 M phosphate[2]	6.5	1.5	69
0.05 M phosphate	6.5	"	65
0.05 M phosphate[2]	6.5	2.0	64
0.05 M phosphate	6.5	"	31
"	7.0	1.5	63
0.10 M phosphate	6.5	"	42
0.02 M citrate	5.5	2.0	58
0.05 M citrate	5.5	"	43
"	6.0	"	51

[1]% Protoplast viability was determined by light microscopy after staining protoplast suspensions with fluorescein diacetate (Windholm, 1972).

[2]Buffer included 147 mg/l (1 mM) $CaCl_2$.

Table 12 C. Effect of polyethylene glycol (PEG) in protoplast inoculation mixture and length of time protoplasts were incubated in PEG + inoculum on protoplast viability.

Incubation Time in PEG + Inoculum	% Viability at 3 hours[1]	% Viability at 72 hours[1]
15 minutes	93	58
30 minutes	88	61
1 hour	82	64
1 hour[2]	91	73
1 hour[3]	75	56
Control	95	52

[1]% Protoplast viability was determined by light microscopy after staining protoplast suspensions with fluorescein diacetate (Windholm, 1972).

[2]11% mannitol solution that was added to inoculum to dilute PEG included 0.05 M phosphate, pH 6.5.

[3]Protoplast/PEG inoculation mixture was diluted rapidly rather than dropwise with 11% mannitol.

after inoculation. In 0.02 M phosphate, pH 6.5, with 10.0 ug/ml PS, 15% of protoplasts were viable 24 hours after inoculation compared to 60-80% viability if Ca^{++} was present.

Although higher concentrations were not used, 2.0 ug/ml appeared to be the highest level of poly-L-ornithine tolerated well by protoplasts. Percent viability, determined by fluorescein diacetate staining, was only slightly lower for samples incubated in 2.0 ug/ml PLO than samples incubated in 1.5 ug/ml. Immediately after inoculation, however, protoplasts incubated in 2.0 ug/ml PLO showed more puckering of the plasmalemma than protoplasts incubated in 1.5 ug/ml PLO. In general, about 50-60% of protoplasts maintained their viability in the presence of either 1.5 or 2.0 ug/ml PLO (Table 12 B).

Protoplast viability was high when inoculated in the presence of polyethylene glycol, although it decreased somewhat as incubation time in inoculum increased (Table 12 C). As determined by fluorescein diacetate staining, 82% of protoplasts were viable after a 1 hour incubation in PEG inoculum, 93% with a 15 minute incubation, and 95% with the control (non-inoculated).

DISCUSSION

Hop Cutting Inoculations

Although, theoretically, all conditions were optimized for cutting inoculation, including inoculum source, virus strain, plant health, temperature, and inoculation buffer, I could not verify that any NRSV-inoculated hop cuttings became infected. Dr. R. O. Hampton (personal communication) encountered similar difficulty when attempting to transmit NRSV to hops. His inoculations were unsuccessful despite evaluation of several buffers, inoculation by both mechanical abrasion and grafting (chip budding), and inoculating from hop plants with naturally occurring NRSV isolates.

Because of Hampton's unsuccessful inoculation with naturally occurring NRSV isolates with hops as the inoculum source, the isolate I used was procured from Dr. C. Skotland at Prosser, WA. The isolate was originally taken out of hops, maintained in cucumber, and had been successfully mechanically introduced back into hops (Skotland, personal communication). This isolate was not successfully inoculated in my research.

Various buffers were also evaluated for inoculation enhancement. Mechanical inoculation from cucumber to cucumber was readily achievable by homogenizing infected cucumber in 0.02 M phosphate, pH 7.0 with or without 0.1%

thioglycollic acid. Inoculation success on cucumber was enhanced with phosphate buffer that included 0.01 M DIECA. Skotland (personal communication) successfully infected hops with his isolate with 2% nicotine buffer. D. Smith (personal communication) could infect hops with the same isolate with phosphate buffer containing 0.1% thioglycollic acid. In my research, inoculation of hops was not successful with any of these buffers.

The limiting factor for successful inoculation of hop cuttings in my research was probably the age of the cuttings. I inoculated all cuttings when plants had one to several fully expanded leaf pairs, generally 1-2 months after the cuttings were taken from the source plant. All ages of leaves at this stage of plant growth were inoculated; from small, expanding to mature, fully expanded, leaves. Apparently, though, plants at this stage are too old for successful mechanical inoculation. Both A. Eppler (1983 and personal communication) and C. Skotland (personal communication) inoculated hops as soon as small, juvenile leaves emerged from cuttings, probably 1-2 weeks after cutting. Eppler stressed the need to inoculate leaves at this stage for successful infection. He found that buffer was not necessary although improved efficiency if plants at this stage were used.

Eppler's success (1983) in infecting very young hop cuttings with NRSV by mechanical inoculation led him to

propose that natural spread of NRSV in hop yards might occur by mechanical injury from insects and/or farming practices. I believe this proposal may be challenged on the basis of the very brief period after cuttings start to grow during which NRSV could be transferred from infected to healthy plants. Hampton's results and those of the present work, dealing with slightly older plants, in which 200 to 300 plants were mechanically inoculated with NRSV without successful infection, seem to suggest that field transmission of NRSV by mechanical means would be quite restricted.

Although my mechanical inoculations were unsuccessful, ELISA tests of field-grown plants provide indirect indications of susceptibility of relevant genotypes to NRSV. Cascade, 60023M, 19005M, and 19173M were found to be infected, at one time or another, with NRSV. Accession 60023M maintains fairly high NRSV titer throughout, whereas NRSV titer seems to vary markedly among samples of plant parts of accessions 19005M and 19173M. Some samples of these latter accessions have been positive and some negative. Conversely, no samples of 21130M ever contained NRSV detectable by ELISA.

Protoplast Inoculation

Poly-L-Ornithine It appeared that PLO did not significantly increase adsorption of NRSV to Cascade protoplasts. In fact, A_{405} readings were lower when PLO

was included at 1.0 or 1.5 ug/ml. This decrease in adsorption with PLO was possibly due to an ineffective charge of virus-PLO complexes caused by high virus:low PLO ratios. This attraction due to ionic charge interaction between protoplasts and virus or virus-PLO complexes is important for infection (Sarkar, 1977). Any decrease in an optimum interaction would greatly affect infection success. A similar result was observed by Fukunaga and Furusawa (1981). They found that infection efficiency of cowpea protoplasts and cowpea mosaic virus (CPMV) was decreased if PLO was included at high virus concentrations. PLO enhanced infection effeciency, however, at low virus concentrations. Similar observa-tions held for other como viruses with cowpea protoplasts.

If it were possible to infect hop suspension protoplasts with NRSV, the results discussed above indicate that PLO may not be required. PLO is required for infection with some but not all virus/host combinations. Most are at least enhanced by PLO, such as infection of soybean suspension cell protoplasts with bean pod mottle virus (BPMV) (Lesney and Murakishi, 1981). Examples of virus-host combinations which require PLO include cowpea mesophyll protoplasts with alfalfa mosaic virus (Alblas and Bol, 1977), tobacco with raspberry ringspot and tobacco rattle viruses (Barker and Harrison, 1977), and tobacco suspension culture protoplasts with tobacco mosaic virus

(TMV) (Kikkawa et al., 1982). Examples of virus-host combinations which do not require PLO include infection of wheat, oat, maize, barley, or Japanese radish with brome mosaic virus (BMV) (Furusawa and Okuno, 1978), or infection of tobacco with BMV (Watts and King, 1984).

The possible lack of requirement of PLO for infection of hop protoplasts with NRSV may be a characteristic of the hop protoplasts rather than NRSV. The requirement, or lack of requirement, of PLO for protoplast inoculation depends not only on the virus but also on the host. Hibi et al. (1975) found that although inoculation of cowpea protoplasts with CPMV did not require PLO, inoculation of tobacco protoplasts with the same virus had a strong requirement for PLO.

The requirement of PLO for efficient infection may involve altered ionic charge of the virus or may depend on the induction of membrane lesions on the protoplasts by PLO. Protoplasts have a net negative charge because of plasmalemma components, the strength of charge depending on the plant species (Nagata and Melchers, 1978). Most viruses which do not require PLO for infection have a high isoelectric point or at least have partial positive charges at inoculum buffer pH. BMV has a pI of 7.9 (Watts and King, 1984) so will have a positive charge at pH 5.0-6.0 normally used for protoplast inoculations. BMV V_5 was also positively charged at pH 5.2 and did not require PLO

for infection of tobacco protoplasts (Motoyoshi et al., 1974). Cowpea chlorotic mottle, another bromo virus, has a net negative charge at pH 5.2, however, and requires PLO for infection of tobacco protoplasts. These same results with the same viruses were reported by Watts and King (1984). Lesney and Murakishi (1981) determined that BPMV has a pI of 4.8-5.3 but the pI of CPMV is 3.7-4.5. BPMV did not require, but CPMV required PLO to infect soybean protoplasts. BMV, as mentioned before, has a pI of 7.9. Infection of barley protoplasts was polycation independent at low pH with low ionic strength but polycation dependent at high pH with high ionic strength (Okuno and Furusawa, 1978). Infection seemed to be controlled by positive virus charge at low pH and virus-PLO complexes at high pH. The specific advantage from PLO relating to ionic charge seems to involve distinct complexes between PLO and the virus (Otsuki et al., 1974; Motoyoshi et al., 1974).

PLO is known to induce lesions in the protoplast plasmalemma (Motoyoshi et al., 1973; Burgess et al., 1973). Electron microscopic studies have shown that virus is present within and around these lesions. Whether these lesions are important for infection by inducing pinocytotic uptake (Cocking, 1966), by just allowing virus entry into the cell passively, or by some type of membrane healing process is unknown. The fact that some host-virus combinations appear to rely primarily on charge relation-

ships for successful infection, i.e. those that do not require PLO, suggests that lesion induction is not the most important factor for protoplast infection.

Protamine Sulfate There was a significant difference in the amount of NRSV adsorbed to Cascade protoplasts with PS compared to PLO. PS was used in higher concentrations which may contribute to this difference. Also, a low molecular weight polycation (such as PS with MW ~5,000) may be more effective at forming complexes with NRSV than those with high molecular weights (such as PLO with MW ~200,000). Alternatively, there may be differences in the interaction between PS and NRSV due to the physical structure or charge of PS at the pHs used for inoculation. PS was tried with NRSV and hops because it had been used successfully with other virus-host combinations. Beier and Bruening (1976) began using PS because they found that PLO only from a single lot number with MW = 120,000 was effective but was no longer commercially available. PS strongly stimulated inoculation of cowpea protoplasts with CPMV virions or RNA. Takebe (1984) noted that PLO with MW < 100,000 is less efficient than PLO with larger molecular weights for facilitating infection of protoplasts with plant viruses.

The enhancement of NRSV adsorption to protoplasts with PS appears to be influenced by different buffer concentrations more than is the interaction between PLO and

NRSV. As phosphate concentration was raised, the PS-induced enhancement decreased. With a higher concentration of phosphate ions, less charge on PS molecules would be available for interaction with protoplasts and virus because of interaction with phosphate ions.

Buffer Concentration and Type Concentration of phosphate buffer appears to have had little effect on adsorption and retention of NRSV to or infection of Cascade protoplasts using PLO at 1.5 ug/ml. This observation includes buffer concentrations of 0.02, 0.05, and 0.1 M. Effect of buffer concentration was more pronounced and consistent when using PS at rates of 25 or 50 ug/ml. This may indicate a more reliable or consistent interaction between protoplasts, protamine sulfate, and NRSV. As buffer concentration was raised, the amount of NRSV adsorbed in the presence of PS decreased. Similar results were observed by others including Alblas and Bol (1977) with alfalfa mosaic virus (AMV) infection of cowpea mesophyll protoplasts, Lesney and Murakishi (1981) with BPMV infection of soybean suspension protoplasts, and by Motoyoshi and Oshima (1975) with TMV and tobacco leaf mesophyll protoplasts. Fukunaga and Furusawa (1981) also found a large effect from ionic strength of inoculum.

The possible effect of cations present in inoculation buffer was not critically examined, however K^+ or Na^+ contributed by the buffer probably did not drastically

decrease infection efficiency. Jarvis and Murakishi (1980) found that K^+, Na^+, or Li^+ did not appreciably affect infection success of soybean suspension protoplasts with CPMV and southern bean mosaic virus (SBMV). Cations of other non-buffering salts, however, can influence infection efficiency. The effect depends totally on the particular virus-host combination in question. The effect of Ca^{++} on infection of hop protoplasts with NRSV was not critically examined, but the ion does not appear to dramatically increase infection efficiency. The amount of virus adsorbed was slightly different in buffers with compared to buffers without Ca^{++}. Both Ca^{++} and Mg^{++} decreased infection of wheat, oat, maize, barley, and Japanese radish by BMV (Furusawa and Okuno, 1978). Kikkawa et al. (1982) found Ca^{++} had no effect on infection of tobacco suspension protoplasts with TMV. On the other hand, both Mg^{++} and Ca^{++} increased infection of soybean suspension protoplasts with CPMV and SBMV (Jarvis and Murakishi, 1980) and BPMV (Lesney and Murakishi, 1981).

Species of buffer ions also influence infection results. Phosphate was slightly more effective than citrate at enhancing NRSV adsorption to 'Cascade' protoplasts. Phosphate was better than citrate for infection cowpea protoplasts with AMV (Alblas and Bol, 1977) or CPMV (Beier and Bruening, 1976), and soybean suspension proto-

plasts with CPMV (Jarvis and Murakishi, 1980). Citrate was better than phosphate or Tris-Cl for infection of barley with BMV (Okuno and Furusawa, 1977) or than succinate, MES, or MOPSO for TMV infection of tobacco suspension protoplasts (Kikkawa et al., 1982). On the other hand, both citrate and phosphate were completely useless, but Tris-Cl or other organic buffers were best for infection of soybean suspension protoplasts with SBMV (Jarvis and Murakishi, 1980).

Inoculation Buffer pH The pH of the buffer in which protoplasts were inoculated had little to no effect on the amount of NRSV adsorbed to 'Cascade' protoplasts. With other virus-host combinations, pH has a dramatic effect on inoculation success. In almost all instances, infection was high at low pH and decreased with increasing pH. Examples include infection of tobacco suspension protoplasts with TMV at pH 5.2 (Kikkawa et al., 1982), barley with BMV at pH 5.5 or below (Okuno and Furusawa, 1977) and tobacco with tobacco necrotic dwarf virus at pH 4.8-5.6 (Kubo and Takanami, 1979).

Polyethylene Glycol PEG (MW 8,000) was used to enhance protoplast aggregation and hopefully cause virus precipitation onto protoplast surfaces to facilitate infection. PEG has been used successfully with several virus-host combinations including cucumber protoplasts with tobacco ringspot virus RNA and cucumber mosaic virus

nucleoprotein and RNA (Maule et al. 1980), tobacco protoplasts with TMV (Cassells and Cocker, 1980), and protoplasts from several *Brassica* species with cauliflower mosaic virus (Maule, 1983). With hop protoplasts, clumping was evident and fusions were more common in treatments with than without PEG. In any case, however, no infection occurred. Essentially no virus was adsorbed to protoplasts which may indicate that conditions in the inoculum buffer were not optimal for NRSV precipitation. Virus precipitation on the protoplast surface followed by protoplast clumping is necessary for successful infection with PEG and other protoplast fusion agents (Cassells and Cocker, 1980; Maule, 1983).

The other essential feature for successful inoculation using PEG is rapid dilution after exposure of protoplasts to PEG (Maule et al., 1980). Both methods of dilution used with hop protoplasts appeared to be equally unsuccessful.

Hop Genotype Although some differences were noted, taking into account all treatments in both experiments presented, hop genotype appears to have no effect on NRSV adsorption to protoplasts. Kiefer et al. (1984) found that the extent of binding of CPMV to protoplasts from resistant and susceptible cultivars of cowpea was similar in the concentration range 4 to 100 ug/ml. Infection rates were similar for barley, wheat, oat, maize, and

Japanese radish (a non-host plant) with BMV (Furusawa and Okuno, 1978). Presumably amount of BMV adsorbed was similar for the different hosts.

Virus Adsorption to Protoplasts

The percentage of NRSV in the inoculum which was adsorbed to 5.0×10^4 protoplasts varied tremendously, depending on other components in the inoculation buffer, but range from 1.87×10^{-3} to 3.7 %. The higher percentages compare very well to those reported in the literature. Kiefer et al. (1984) estimated that between 0.14 to 1.1 % of cowpea mosaic virus virions were adsorbed to cowpea protoplasts. This amount was sufficient to initiate infection. Motoyoshi et al. (1973) found that approximately 1 % of cowpea chlorotic mottle virus inocula became attached to tobacco protoplasts, corresponding to about 760 virus particles/protoplast. Hibi et al. (1975) estimated that about 0.1 % of cowpea mosaic virus added (approximately 10^3 virus particles) was adsorbed per protoplast. By comparison with these reports, the amount of NRSV adsorbed to hop protoplasts was in the range which should initiate infection.

Protoplast Viability After Inoculation Treatments

Protoplast viability was influenced by inoculation buffers, large decreases occuring with buffers containing protamine sulfate. Much of this loss in protoplast viability with protamine sulfate could be avoided by

including $CaCl_2$ at 147 mg/l in inoculum buffer and mannitol diluent. Ca^{++} at this concentration apparently provided enough protection to the protoplast membrane to avoid degeneration. Ca^{++} is essential for proper integrity and function of the plasma membrane, such as selectivity of ion transport, minimizing diffusive permeation, and counteracting the toxic effect of other ions (Epstein, 1972). Beier and Bruening (1976) found, however, that cowpea protoplasts suffered damage only when protamine sulfate concentrations were equal to or greater than 75 ug/ml. Loesch-Fries and Hall (1980) successfully used 25 ug/ml PS to inoculate barley protoplasts with BMV RNA. No Ca^{++} was required in the inoculum for protoplasts to maintain their viability in these studies. Hop protoplasts are apparently much more sensitive to protamine sulfate than cowpea protoplasts.

Because of widespread reference to damage of protoplasts with PLO above 2.0 ug/ml, higher concentrations were not used for hop protoplast inoculations. Some slight membrane roughness and puckering, indicating damage, was apparent visually on hop protoplasts when incubated in buffer with 2 ug/ml PLO. With other plant species, protoplasts are damaged by 1 (Alblas and Bol, 1977; Hibi et al., 1975) to 4 ug/ml PLO (Beier and Bruening, 1976). The amount of damage encountered with these levels of PLO depends on other inoculation con-

ditions. Furusara and Okuno (1978) noticed that protoplasts were more sensitive to PLO with inoculation buffers of low pH and low ionic strength.

Hop protoplast viability did not appear to be appreciably influenced by buffer concentration, pH, or buffer type under my experimental conditions. Kubo and Takanami (1979) found, however, that protoplast viability was significantly decreased by pH with incubation in inoculum buffer with pH at or below 5.2.

When hop protoplasts were cultured in CPW13M, viability remained high for 48-72 hours, but dropped by one week. Takebe (1984) recommended the use of a medium very similar to CPW13M for culture of cells inoculated with TMV. He found that richer media with carbon sources and vitamins do not improve TMV multiplication, but enhance growth of contaminating microorganisms. Because I found that hop protoplast viability in CPW13M was at least equal to or greater than viability in Kao's over 48 hours, protoplasts were cultured in either CPW13M or Kao's after inoculation with NRSV.

CHAPTER 3

PURIFICATION OF PRUNUS NECROTIC RINGSPOT VIRUS (NSRV) AND RNA AND METHODS OF DETECTING VIRUS IN SMALL AMOUNTS OF PLANT TISSUE

INTRODUCTION

Several different methods are used to detect progeny virus in inoculated protoplasts. The most commonly used method is immunofluorescence (Takebe, 1984; Takebe, 1969). For many virus-host combinations, this method is apparently reliable and easy to use, however background is often confusing. The second most commonly employed assay is with local lesion host assay. Indeed, a sensitive, reliable local lesion host provides unequivocal evidence of virus synthesis. Other methods less commonly used include detection by enzyme linked immunosorbent assay (ELISA) and by hybridization with a cDNA probe.

The method chosen for detection may depend on the virus-host combination in question and the purpose of protoplast inoculation. The only readily available local-lesion assay host for NRSV is cucumber, which is insensitive to NRSV concentration. Also, the multi-hit nature of NRSV on local lesion hosts (Loesch and Fulton, 1975) would complicate interpretation of results. ELISA is both sensitive and reliable (Zhengkaixu et al., 1984; Clark, 1981). Detection of NRSV in fusion hybrids to assess effect of combining genomes of two different plants on viral multiplication needs to be sensitive enough to

detect virus in less than 100 cells. Crossway and Houck (1986) devised a method to detect specific nucleic acid in as few as 1-10 plant cells.

Three methods were evaluated for detection of NRSV in small amounts of plant tissue: ELISA, immunofluorescence, and cDNA probe hybridization. Results are discussed in reference to specificity, sensitivity, and reliability.

MATERIALS AND METHODS

NRSV Purification

A mechanically transmissible hop strain of Prunus necrotic ringspot virus was obtained from Dr. C. Skotland, Prosser, WA. It was maintained by mechanical transfer every other week onto cotyledons of *Cucumis sativus* cv. National Pickling. Infected cucumbers were harvested 7-10 days after inoculation and virus was purified by a modification of Fulton's method (Smith and Skotland, 1986), outlined in Table 13.

Detection of NRSV in Protoplasts

Several different methods were employed for detection of progeny virus in protoplasts.

ELISA The ELISA technique was performed as outlined by Clark and Adams (1977). Details applied to my study are given in Materials and Methods, Chapter 2, pp. 34-35.

The usefulness of ELISA for detecting NRSV in protoplasts was evaluated by adding homogenate from 0.1, 0.5, and 1.0 X 10^5 inoculated 'Cascade' protoplasts in one ELISA-plate well. Also, known amounts of purified virus, from 250 pg to 50 ug/ELISA plate well, were included with homogenate from 5.0 X 10^4 non-inoculated 'Cascade' protoplasts to quantify sample A_{405} readings and to determine NRSV detection endpoint with ELISA.

Table 13. Protocol for Prunus necrotic ringspot virus (NRSV) purification modified from Fulton's method (Smith and Skotland, 1986).[1]

1. Grind tissue 2 minutes in blender with 0.01 M diethyldithiocarbamic acid, 0.02M thioglycolic acid, and 0.03M sodium phosphate buffer, pH 8.0, 4°C (1g tissue/3ml buffer).

2. Express homogenate through four layers of cheese-cloth and mix with hydrated calcium phosphate[2] (0.8 g/g tissue). Stir at 4°C 10-20 minutes.

3. Clarify by centrifugation (7,970 X g for 30-45 minutes in Sorvall GSA rotor).

4. Mix supernatant with a solution of 40% (w/v) PEG (MW 6,000-8,000) and 1M NaCl at the rate of 25ml/100ml supernatant. Stir at 4°C for 1-2 hours.

5. Pellet precipitate (7,970 X g for 50 minutes) and resuspend pellets in 0.02M sodium phosphate buffer and 0.03M EDTA, pH 8.0 to 1/15 of their original volume.

6. Clarify suspension (22,000 X g for 20 minutes in Sorvall SS-34) and pellet virus by centrifugation (100,000 X g for 2-2.5 hrs). Resuspend pellets in phosphate/EDTA buffer, pH 8.0.

7. Clarify supernatant (22,000 X g, 20 min) and layer on 10-40% (w/v) sucrose gradients. Centrifuged for 3 hrs at 24K in Beckman SW 28 rotor.

8. Collect virus and pellet (100,000 X g for 2.5 hrs). Resuspend in phosphate/EDTA buffer.

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[1] All resuspensions and as many operations as possible are performed at 4°C.

[2] Hydrated calcium phosphate: Mix 9 l of 0.1 M $CaCl_2$ by dripping slowly into 9 l of 0.1 M Na_2HPO_4 with constant stirring at room temperature. After all $CaCl_2$ has been added, the mixture is stirred 30-60 minutes longer and precipitate allowed to settle at least 4 hours. Wash precipitate 10-15 times with distilled water, allowing 4 hours settling time between washes. After 10 washes, monitor supernatant with $AgNO_3$ to detect residual chloride ion. When wash water is free of excess ions, collect precipitate by centrifugation (1,500 X g for 10 minutes). Store precipitate at 4°C in a sealed jar.

Slide Assay FITC conjugated anti-NRSV IgG was used to detect NRSV in protoplasts with a slide assay similar to that introduced by Otsuki and Takebe (1969). Protoplast suspension (2 X 10^5 protoplasts/ml culture medium) was placed on a slide smeared with freshly made Mayer's albumin (egg white and glycerol at 1:1) and dried quickly with warm air from a hair dryer. Slides were fixed in 100% acetone for 30 minutes and washed with PBS for 15-20 minutes. FITC-IgG conjugate (0.96 mg/ml protein with a fluorescein:protein ratio of 3.35) was added at a 1:10 or 1:50 dilution and slides incubated for 1 hour at 37°C in a humid chamber. They were then washed for 15-20 minutes with PBS, mounted with 40% glycerol + 60% PBS, and examined for percent infected cells on a Zeiss IM microscope equipped with a 520-560 nm pass band filter (Zeiss #487710).

NRSV RNA Isolation

NRSV RNA was phenol extracted from purified virions using a method similar to that of Peden and Symons (1973). All inorganic solutions, glassware, tubes, and instruments were autoclaved before use. When NRSV was used for RNA extraction, the sucrose gradients and final resuspension of virus purification was done in 0.2 M sodium acetate buffer, pH 7.6 with 1 mM EDTA instead of phosphate/EDTA buffer. Freshly purified virus was emulsified in a 1:1:1 mixture of virus in resuspension buffer:90% phenol con-

taining 0.1% 8-hydroxyquinoline:extraction buffer (0.2 M sodium acetate, pH 7.6 with 1 mM EDTA and 1.0% SDS (w/v)). This mixture was emulsified by shaking vigorously for 60 minutes at room temperature on a vortex mixer, then centrifuged at 10,000 X g for 10 minutes. The buffer phase was back-extracted by mixing 1:1 with fresh phenol and shaken 5-10 minutes with the vortex mixer. Likewise, the phenol phase was re-extracted by mixing 1:1 with fresh extraction buffer. Buffer phases were combined, re-centrifuged to separate out residual phenol, and mixed with 2.7 volumes of high quality, re-distilled absolute ethanol at -20°C. Tubes were kept at -20°C for 48 hours or -70°C for 24 hours to precipitate RNA. RNA precipitate was pelleted at 10,000 X g for 5 minutes. Buffer-ethanol supernatant was decanted and cold absolute ethanol added to a volume equaling that of buffer + ethanol. Contents of the tube were vortexed until the RNA pellet detached from the tube, for thorough washing in ethanol. The pellet was re-centrifuged and ethanol wash repeated twice more. The RNA pellet was stored at -20°C under absolute ethanol until needed.

Just before use of RNA, ethanol was decanted and the tube was allowed to drain dry upside down at 4°C for 10-15 minutes. Residual ethanol was removed by placing tubes under vacuum for 30 minutes with perforated parafilm covering the tube opening to prevent loss of RNA. RNA was

resuspended in sterile double distilled water or TE buffer (10 mM tris, pH 8.3 with 2 mM EDTA). Yield of RNA was estimated spectrophotometrically by absorbance of the solution at 260 nm UV light (A_{260}).

Infectivity of purified NRSV RNA was tested by mechanically inoculating cotyledons of 'Lemon' cucumber with standardized suspensions of viral RNA. For this purpose, phenol-extracted, ethanol-precipitated RNA was resuspended in TE buffer.

To check purity and verify the presence of four NRSV genomic RNAs, whole RNA was subjected to electrophoresis on 3.5% (T_0), 1.5 mm polyacrylamide gels. Gels consisted of a 20:1 acrylamide:bis ratio buffered with TBE (10X TBE is 108 Tris, 55 H_3BO_3, and 9.32 g/l EDTA) and contained 7 M urea. See Table 14 for gel recipe. Gels were often left overnight under TBE buffer after polymerization was complete. Gels were pre-electrophoresed for 60-90 minutes at 12 mA before samples were added.

For electrophoresis, RNA was resuspended in TE buffer (25 ul buffer/13 ug RNA) and mixed 1:1 with glycerol. Just before loading RNA preparations onto gels, they were incubated in a water bath at 80-92°C for 5-10 minutes, then quickly cooled in an ethanol ice bath. Samples were applied to wells (5-10 ug/well) and electrophoresed at 25 mA (250 volts) constant current for 3-5 hours. Bromphenol blue and xylene cyanol were used as markers; bromphenol

Table 14. Recipe for 3.5% polyacrylamide-urea gel.

	amount added
Stock Acrylamide/Bisacrylamide (10 g Ac. + 0.5 g Bis in 50 ml water)	8.4 ml
Urea	21.0 g
10 X TBE	5.0 ml
Sterile Double Distilled Water	20.0 ml
TEMED	50.0 ul
10% Ammonium Persulfate	0.5 ml

usually ran off the gel after 2.5-3 hours. Periodic adjustments were made to keep voltage at 250. Final amperage after 3-5 hours was 17 or 18 mA.

After electrophoresis, the gel was stained for 30-60 minutes in TBE with 0.5 ug/ml ethidium bromide. After staining, gels were washed once in fresh buffer, then photographed by 2-3 minute exposures, F-stop 5.6, on Polaroid Type 55 4 X 5 positive/negative land film. RNA bands were photographically enhanced by use of a yellow lens filter.

For a more precise estimation of RNA molecular weights, RNA was glyoxylated and electrophoresed on 1% agarose gels, as described by Maniatis et al. (1982). Electrophoresis-grade, standard low-melting-temperature agarose (BioRad) was mixed with 0.01 M NaH_2PO_4 + Na_2HPO_4 (pH 7.0), melted, and cooled to 50^oC. The gel was 10 cm long, 15 cm wide, and approximately 3 mm thick gel (45-50 ml gel volume).

RNA samples were prepared for electrophoresis glyoxylation, as outlined by Maniatis et al. (1982). To 3.7 ul of water containing resuspended RNA was added 2.7 ul 6 M deionized glyoxal (pH 6.0), 8.0 ul DMSO, and 1.6 ul 0.1 M NaH_2PO_4 (pH 7.0). The molecular weight standard, cucumber mosaic virus strain Pg RNA, was treated identically. Glyoxylation was allowed to proceed for 1 hour at 50^oC. Loading buffer (4 ul of 50% glycerol, 0.01 M NaH_2PO_4, pH

7.0, and 0.4% bromphenol blue) was added after RNA preparations had cooled to room temperature. At least 5 ug of RNA was added to each lane. Gels were electrophoresed in 0.01 M NaH_2PO_4, pH 7.0 at 4 V/cm (40 volts, 35-40 mA, constant voltage) with a buffer change every 30 to 60 minutes to keep pH below 8.0. Gels were stained with 0.5 ug/ml ethidium bromide in running buffer for about 1 hour then rinsed. For destaining, gel was soaked in 1 mM $MgSO_4$ for 30-60 minutes. Photographs were taken as with polyacrlyamide gels.

cDNA Probe Construction

Random cDNA to NRSV RNA was constructed by reverse transcription. AMV Reverse Transcriptase System I (Bethesda Research Laboratories [BRL]) was used with the accompanying reaction protocol[1]. NRSV RNA was resuspended to 0.2 or 1 ug/ul and heated to 65-80°C for 5-10 minutes, then immediately cooled in an ice bath. To the RNA was added 2.5 ul of deoxyribonucleotide solution (10 mM dATP, dGTP, and dTTP, and 5 mM dCTP in 10 mM Tris-HCl, pH 7.5) (sometimes added before RNA was heat denatured), 150-200 uCi a-^{32}P-dCTP (> 600 Ci/mmole), 5 ul 250 mM KCl, 5 ul 500 mM Tris-HCl (pH 8.3) with 50 mM $MgCl_2$ and 50 mM DTT, 5 ul of Oligo dT_{12-18} (100 ug/ml), 5 ul actinomycin D (250

[1]Good results were also obtained with AMV reverse transcriptase from Pharmacia at 0.5 u/ml. When Pharmacia enzyme was used, no actinomycin D was included but 0.108 ul RNA guard (800 units RNAsin/ml, Pharmacia) per ul reaction volume was added.

ug/ml), 2.5 ul 200 mM potassium phosphate, pH 7.2, and 5-10 units of AMV reverse transcriptase. The mixture was incubated for 60-90 minutes at 37°C. When the reaction was complete, contents were made 23 mM EDTA (5.5 ul 0.5 M EDTA/100 ul reaction volume) and pH raised with NaOH (10.8 ul 3 M NaOH/100 ul reaction volume). This mixture was incubated 1-4 hours at 65°C or overnight at 37°C.

Yield of cDNA was determined by comparing trichloroacetic acid precipitable counts at the end of the reaction to counts at time 0. cDNA was purified by spun column chromatography (Maniatis et al., 1982) using Sephadex G-75-40, and used directly for blot hybridization.

Extraction of Total RNA

Total RNA was extracted from inoculated protoplasts, leaf, and callus samples using guanidine-HCl, LiCl, or phenol to determine the optimal extraction method of viral RNA from small amounts of plant tissue. NRSV-infected plant sources were protoplasts, callus, and leaf tissue from hop accession 60023M and tissues from inoculated 'National Pickling' cucumber. Negative controls were non-inoculated cucumber, virus-free 'Cascade' leaf tissue, and callus from virus-free 'Cascade'.

Approximately 5.6 X 10^4 protoplasts in 1.66 ml of CPW13M medium comprised experimental protoplast samples. Each ml of protoplast suspension was treated as equivalent to 1.0 g of tissue. Each sample was divided into two rep-

licate alliquots, and applied as dots on the nitrocellulose filter. This sampling procedure was usually repeated three times. Homogenates from NRSV-infected hop leaf and callus and cucumber leaf tissues were processed similarly. NRSV-infected hop and cucumber leaf tissues were macerated with the enzyme mixture used to isolate protoplasts (see Methods, p12 of Chapter 1) at the rate of 13.5 g 60023M leaf in 90 ml enzyme. The RNA from the isolated cells was extracted in the same manner as that from protoplasts.

RNA was extracted from tissues with guanidine-HCl, as described by Cheley and Anderson (1984). Callus and leaf tissue were weighed and ground in mortar and pestle (1 ml buffer/g) after freezing in liquid nitrogen. Isolated cells were centrifuged and the pellet solubilized by adding 1 ml of 7.6 M guanidine-HCl in 0.1 M potassium phosphate buffer, pH 5.0. The suspension was aspirated with a plastic syringe with a 25 gauge needle, mixed with 0.6 volumes of 95% ethanol, cooled for 24 h at -20°C and centrifuged at 10,000 X g for 20 minutes to pellet RNA.

For phenol extraction, each gram of tissue was ground in liquid nitrogen, mixed with 1.5 ml STE buffer (0.1 M NaCl, 0.05 M Tris, pH 8.0, 1.0 mM EDTA), and placed in an eppendorf tube to which was added 33 ul of 2-mercaptoethanol, 1.0 ml of water saturated phenol (90% phenol containing 0.1% 8-hydroxyquinoline), and 1 ml chloroform. The suspension was then shaken 1 hour at room temperature.

The aqueous phase was collected after centrifuging at 10,000 X g for 20 minutes and 2.7 volumes of cold 100% ethanol was added to precipitate RNA.

A modification of the procedure by Hall et al. (1978) was used for LiCl extraction. To each gram of tissue was added 2.6 ml homogenization buffer (0.2 M Tris-HCl, pH 9.0, 0.4 M NaCl, 25 mM EDTA, and 1% SDS) at 100°C. This was ground with mortar and pestle, mixed with 1.3 mg Proteinase K (Sigma), and incubated 1 hour at 37°C. Potassium was added at the rate of 200 ul of 2 M KCl per gram of tissue. The mixture was then incubated for 30 minutes on ice and centrifuged 5 minutes at 10,000 X g. Supernatant was made 2 M with respect to LiCl using 6 M stock. After thorough mixing, RNA was precipitated at 4°C overnight and pelleted at 10,000 X g for 20 minutes at 4°C. Pellet was rinsed 2 times with 2 M LiCl then resuspended in 0.1 ml sterile double distilled water. This resuspension was made 0.3 M with NaCl (6.4 ul of 5 M stock), and RNA precipitated with 2-3 volumes of cold 100% ethanol at -20°C for 48 hours or -70°C for 24 hours. Precipitate was pelleted at 10,000 X g for 20 minutes at 4°C, then washed one or two times with cold ethanol.

Blotting

Tubes with RNA pellets (centrifuged ethanol precipitate) were drained 10-15 minutes upside-down and put in a vacuum evaporator 15-30 minutes. Pellets were resuspended

in 100 ul sterile double distilled water and 300 ul of 1:1 37% formaldehyde:20 X SSC buffer was added (20 X SSC is 175.3 g NaCl and 88.2 g sodium citrate in 1 l of water, pH 7.0). The mixture was incubated for 15-30 minutes at 65°C and blotted onto nitrocellulose using a vacuum manifold (Bio-Dot Microfiltration Apparatus, BioRad). For blotting, nitrocellulose (NEN-Gene Screen, New England Nuclear) was first wet in water then in 10 X SSC before use. After blotting, the filter was air dried, baked in a vacuum oven at 80°C for 2 hours, then hybridized. Purified NRSV RNA (homologous to that used for cDNA construction) was used as a positive control.

For slot blots, RNA was prepared as above, but blotting onto nitrocellulose was accomplished by placing paper towel absorbant and 3MM paper under the nitrocellulose to soak up buffer.

Hybridization

Prehybridization and hybridization were performed essentially according to Maniatis et al. (1982). Filters were sealed in seal-a-meal bags and prehybridized in 5 X SSC buffer, 0.1% SDS, 5 mM EDTA, 5 X Denhart's (1 X Denhart's is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll [MW 400,000]), 25 mM sodium phosphate, pH 6.5, 50% formamide and 200 ug/ml denatured salmon sperm DNA for approximately 4 hours at 42°C with shaking. After prehybridization, cDNA was added to pre-

hybridization fluid (0.03 to 0.2 ug with specific activity of 2-4 X 10^7 dpm/ug into 20-25 ml solution) and filters incubated another 12-16 hours at 42°C with shaking. Filters were then washed four times at room temperature for a total of 30 minutes; first two times in 2 X SSC buffer, 0.1% SDS, and the third and fourth washes in 1 X SSC, 0.1% SDS. Further washing was in 1 X SSC, 0.1% SDS at 37°C for 2 hours. Blots were autoradiographed for 24-72 hours at -70°C using Kodak X-Omat AR (XAR-2) film with Coronex Lightning Plus intensifying screens (Dupont). Spots on autoradiographs were quantified by densitometry.

RESULTS

NRSV Purification

The modification of Fulton's method for NRSV purification typically yielded an average of 24 mg virus per kg of fresh tissue. Yields as high as 46 and 53 mg/kg were obtained. Late spring was probably the worst time of year for good yields because cucumbers grew fast and titer seemed to be low. In general, however, good yields did not appear to depend on time of year. UV absorbance curves were typical for partially purified nucleoprotein with absorption peak at 260 nm UV light.

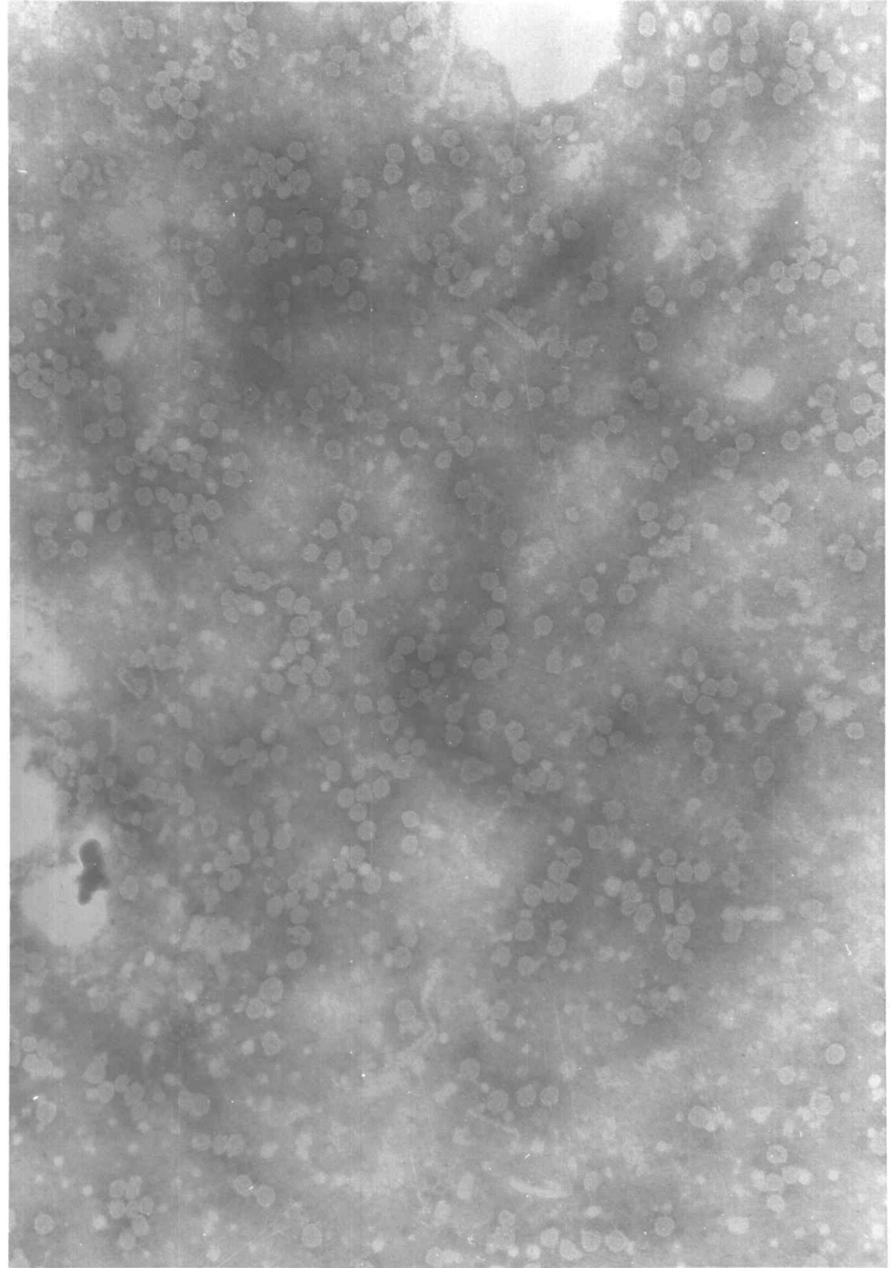
When TE or NE buffer was used for sucrose gradients and final high speed pellet resuspension, yields were between 25-30 mg/kg. Host components tended to band too closely to the upper virus band for good separation in TE gradients. This poor band separation did not occur when NE or phosphate/EDTA were used as buffers.

After lyophilization in 0.02 M phosphate buffer, pH 8.0 with 0.03 M EDTA, NRSV remained infectious at least 6 to 12 months when stored at -20°C. Likewise, NRSV also maintained normal UV absorbance characteristics during long-term storage. Titer of resuspended virus was approximately 1/500 as determined by inoculation of lemon cucumber cotyledons. An electron micrograph of purified virions stained in phosphotungstate is shown in Figure 2.

NRSV Detection with ELISA

Figure 2. Electron micrograph of Prunus necrotic ringspot virus (NRSV) purified by a modification of Fulton's method (Dennis and Skotland, 1986). X 63,000. Average particle size is 40 nm in the micrograph. The concentration of NRSV in the preparation used to make the micrograph was approximately 0.725 mg/ml, approximately 580 times the concentration used for protoplast inoculations.

Figure 2



Although in some tests less than 1.0 ng of NRSV could be detected in ELISA plate well, a more reliable or consistent detection end point was 2.5 ng/well when included with homogenate from 0.5 X 10^5 uninoculated 'Cascade' protoplasts (Table 15).

Virus was detected with 0.1 X 10^5 inoculated protoplasts/well but readings were not reliable or consistent unless homogenate from 0.5 X 10^5 inoculated protoplasts were used in each ELISA plate well. A_{405} readings were, naturally, highest when homogenate from 1.0 X 10^5 protoplasts was included in each well (Table 16).

NRSV RNA Extraction

The average percent yield of RNA from purified virions was 16.4% with a range from 9.5 to 25%. $A_{260/280}$ ratios ranged from 1.98 to 2.22 with an average of 2.04. Good RNA yields were more consistently obtained from freshly purified than from lyophylized virus. UV absorbance curves typical of RNA preparations characterized NRSV RNA preparations.

RNA precipitates from virions prepared in phosphate buffer were large and had a fluffy or chalky appearance under absolute ethanol. Purified RNA from none of the three virus purification buffers would initiate infection on 'Lemon' cucumber cotyledons.

Estimates of NRSV RNA Purity and Composition

Table 15. Sensitivity of ELISA: A_{405} readings with increasing amount of purified NRSV included with homogenate from 5 X 10^4 'Cascade' protoplasts/well[1].

ng NRSV/well	A_{405}[2]
50,000	2.044 ± 0.103
25,000	1.566 ± 0.121
2,500	1.194 ± 0.017
500	1.104 ± 0.003
250	0.927 ± 0.023
100	0.777 ± 0.069
25	0.433 ± 0.021
2.5	0.053 ± 0.005

[1] Correlation coefficient between Log_{10} ng NRSV and A_{405} is 0.981

[2] A_{405} values given ± range of values from 2 ELISA wells.

Table 16. Sensitivity of ELISA: Number of protoplasts/ELISA plate well needed for reliable detection[1].

		Protoplasts/well					
		10^4		5×10^4		10^5	
[PLO] (ug/ml)	[NRSV] (ug/ml)	A_{405}[2]	ng NRSV Adsorbed[3]	A_{405}	ng NRSV Adsorbed	A_{405}	ng NRSV Adsorbed
1.0	12.5	0.035 ± 0.011	1.5	0.189 ± 0.021	1.9	0.365 ± 0.011	2.5
1.5	12.5	0.071 ± 0.013	1.6	0.320 ± 0.026	2.3	0.586 ± 0.044	3.6
1.0	50.0	0.179 ± 0.032	1.9	0.616 ± 0.106	3.7	1.100 ± 0.027	8.0
1.5	50.0	0.157 ± 0.023	1.8	0.568 ± 0.041	3.5	1.035 ± 0.045	7.2

[1] Inoculation buffer was 0.02 M phosphate, pH 6.0, with 1 mM $CaCl_2$ and poly-L-ornithine at 1.0 or 1.5 ug/ml.

[2] A_{405} values represent samples taken immediately after inoculation. Standard deviations are based on 3 samples.

[3] Conversion of A_{405} values to ng NRSV is described in Appendix.

Polyacrylamide and agarose gel electrophoresis of purified NRSV RNA preparations from phosphate, NE, or TE buffers showed bands corresponding in size to RNAs 1, 2, 3, and 4 (Figures 3 and 4). RNAs 1 and 2 were present in small amounts in comparison to RNAs 3 and 4. No contaminating RNA was visualized on the gels as stained, supporting relative purity of viral RNA suggested by $A_{260/280}$ ratios. Presence of trace contaminants was not ruled out, however, because RNA detection sensitivity in electrophoretic gels is low.

Extraction of Total Plant-RNA

Total RNA extraction efficiency differed using 1) guanidine-HCl, 2) 2M LiCl, or 3)phenol (Figure 5 and Table 19). For small samples, it was easiest to process samples with phenol, then with LiCl and then guanidine-HCl. Based on densitometer peak heights (Table 20) and spot density determined visually (Figure 5), however, LiCl extraction appeared to consistently yield more RNA in cleaner preparations than extraction with phenol or guanidine-HCl extraction. This was best observed by comparisons of peak heights for infected cucumber extractions. Two negative controls, leaves from virus-free Cascade plants and non-infected cucumbers, showed some signal on the blot but the third negative control, callus from virus-free Cascade, showed no signal (peak heights not presented in Table 20). Because extraction with LiCl gave the best preparations,

Figure 3. Polyacrylamide gel of Prunus necrotic ringspot virus (NRSV) RNA. Wheat embryo RNA and a Hind III digest of phage lambda DNA included as mobility standards. Lanes 1 and 6 contain approximately 15 and 10 ug respectively of NRSV RNA from virions purified using tris-EDTA buffer. Lane 2 contains approximately 10 ug NRSV RNA from virions purified using phosphate-EDTA buffer. Lanes 3 and 7 contain a Hind III digest of phage lambda DNA. Lanes 4 and 8 contain total RNA extracted from wheat embryo. Lane 5 contains approximately 9.5 ug NRSV RNA from virions purified in sodium acetate-EDTA buffer.

Figure 3.

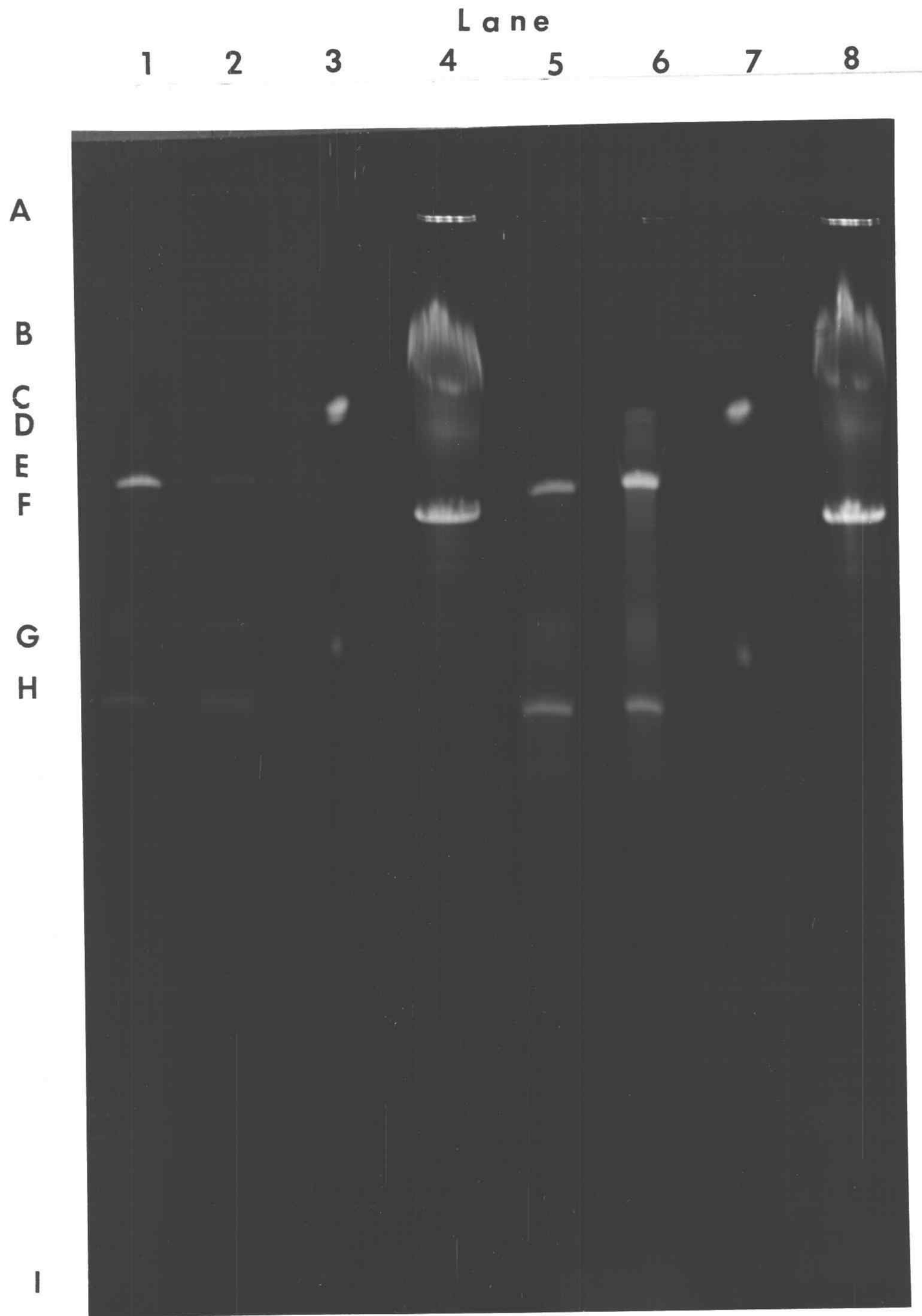


Table 17. Key to bands on polyacrylamide gel shown in Figure 3.

Band Identification Letter	Band
A	origin
B	Wheat 28S Ribosomal RNA (MW ~1.6 X 10^6)
C	Lambda phage Hind III Digest[1]
D	NRSV RNA Species 1 + 2 (MW ~1.1 to 1.3 X 10^6)
E	NRSV RNA Species 3 (MW ~0.9 X 10^6)
F	Wheat 18S Ribosomal RNA (MW ~0.65 X 10^6)
G	Lambda phage Hind III Digest[1]
H	NRSV RNA Species 4 (MW ~0.3 X 10^6)
I	Bottom of Gel

[1]Because Lambda phage Hind III digest fragments were not adequately resolved, molecular weight estimations are not given.

Figure 4. Agarose gel of glyoxylated Prunus necrotic ringspot virus (NRSV) RNA. Glyoxylated cucumber mosaic virus (CMV), strain Pg, RNA is included as a molecular weight standard. Lanes 2 and 6 contain NRSV RNA from virions purified in tris-EDTA, lanes 3 and 7 from virions purified in phosphate-EDTA, and lanes 4 and 8 from virions purified in sodium acetate-EDTA. Lanes 5 and 9 contain approximately 3.7 ug/lane of CMV RNA.

Figure 4.

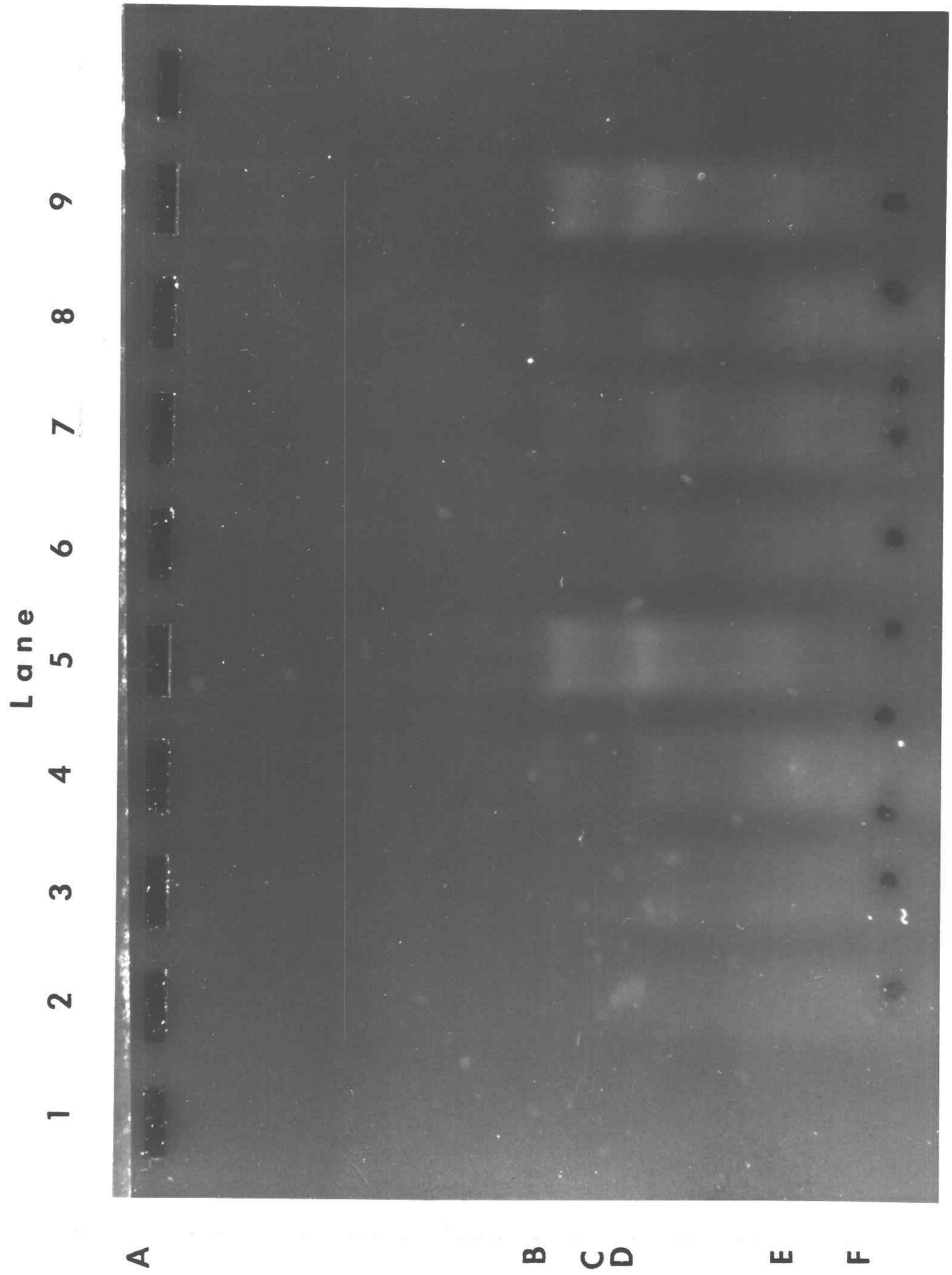
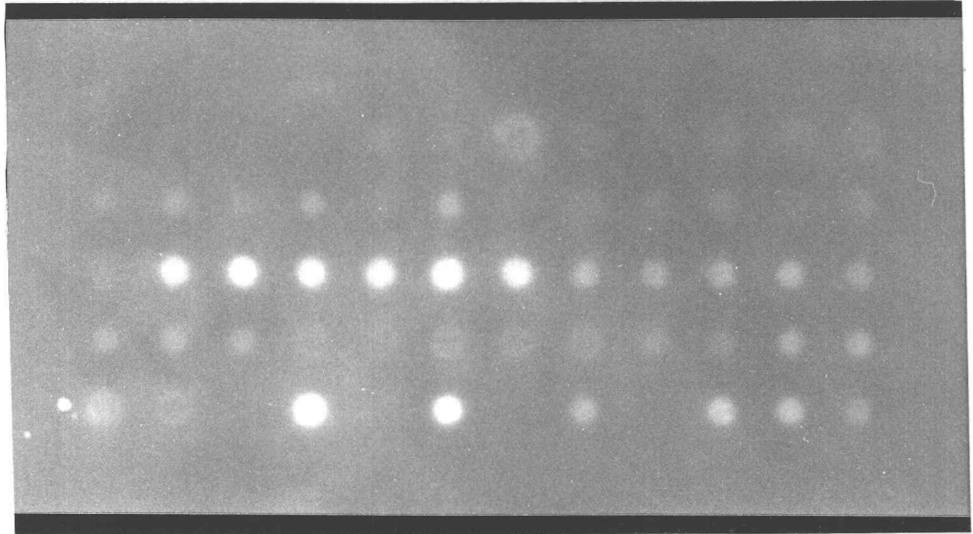


Table 18. Key to bands on agarose gel shown in Figure 4.

Band Identification Letter	Band
A	origin
B	RNAs 1 + 2 of NRSV and CMV (MW ~1.13 to 1.27 X 10^6)
C	RNA 3 of CMV (MW 0.82 X 10^6)
D	RNA 3 of NRSV (MW ~0.65 X 10^6)
E	RNA 4 of CMV and NRSV (MW ~0.35 X 10^6)
F	Migration Boundry of Xylene Cyanol

Figure 5. Dot blot with number key of total RNA extracted from various plant samples using phenol, 2M LiCl, or guanidine-HCl. Numbers in key explained in Table 19.

Figure 5.



1 2 3 4 5 6 7 8 9 10 11 12

13 14 15 16 17 18 19 20 21 22 23 24

25 26 27 28 29 30 31 32 33 34 35 36

37 38 39 40 41 42 43 44 45 46 47 48

49 50 51 52 53 54 55 56 57 58 59 60

Table 19. Key to numbers corresponding to spots in Figure 5.

A. RNA Extraction with guanidine-HCl

Dot Blot Number	Sample
1	60023M leaf 1
2	" 2
3	" 3
4	" 4
5	" 5
6	" 6
7	NRSV-Infected Cucumber 1
8	" 2
9	" 3
10	" 4
11	" 5
12	" 6
13	60023M Callus 1
14	" 2
15	" 3
16	" 4
17	60023M Protoplast 1
18	" 2
19	" 3

B. RNA Extraction with LiCl

Dot Blot Number	Sample
20	60023M leaf 1
21	" 2
22	" 3
23	" 4
24	" 5
25	" 6
26	NRSV-Infected Cucumber 1
27	" 2
28	" 3
29	" 4
30	" 5
31	" 6

Table 19 (cont). Key to numbers corresponding to spots in Figure 5.

B. RNA Extraction with LiCl

32	60023M Callus 1
33	" 2
34	" 3
35	" 4
36	60023M Protoplast 1
37	" 2

C. RNA Extraction with Phenol

38	60023M leaf 1
39	" 2
40	" 3
41	" 4
42	NRSV-Infected Cucumber 1
43	" 2
44	" 3
45	60023M Callus 1
46	" 2
47	60023M Protoplast 1
48	" 2
49	Uninoculated Cucumber
50	'Virus-Free' Hop leaf
51	'Virus-Free' Hop Callus
52	6 ug NRSV RNA from Tris-Purified Virions
53	Blank
54	0.6 ug NRSV RNA from Tris-Purified Virions
55	Blank
56	0.06 ug NRSV RNA from Tris-Purified Virions
57	Blank
58	9.5 ug NRSV RNA from Sodium Acetate-Purified Virions
59	0.95 ug "
60	0.095 ug "

Table 20. Comparison of results from three different protocols for extraction of total RNA from plant tissue. Numbers are absorbance units from densitometry of a dot blot autoradiogram.

RNA Sample	Extraction Protocol		
	Phenol	Guanidine-HCl	LiCl
60023M leaf 1	0.081	---[1]	0.049
" 2	0.047	---	0.037
" 3	0.081	---	0.036
" 4		---	0.041
" 5	0.092	0.065	0.052
" 6		0.059	0.041
Infected Cucumber 1	0.072	0.102	0.136
" 2		0.045	0.176
" 3	0.066	---	0.146
" 4		0.039	0.152
" 5	0.072	0.059	0.205
" 6		0.046	0.199
60023M Callus 1	0.061	0.066	0.075
" 2			0.047
" 3	0.041		0.070
" 4		---	0.087
60023M Protoplasts 1	0.070	0.105	0.045
" 2			0.047
" 3	0.061		

[1]Dash marks refer to samples which showed no absorbance or visible spot above autoradiogram background. Empty positions on the table indicate samples not done.

this method was chosen for RNA extraction from inoculated protoplasts.

Extraction of Total RNA from Inoculated Protoplasts

Densitometer readings from an autoradiograph of RNA from inoculated protoplasts ranged from 0.3 to 0.6 absorbance units (Table 21). The slot blot read for these absorbance units is presented in Figure 6. In Experiment 1 (Table 21), there was no difference in signal magnitude from extractions of mock-inoculated (inoculation treatment without NRSV) and inoculated protoplasts. Samples which produced no signal and those producing signal were not generally associated with time after inoculation or treatment. In Experiment 2 (Table 21), however, high absorbance readings were observed with samples inoculated with large amounts of virus. The highest absorbance, 0.552 units, was produced by samples inoculated with 100 ug/ml inoculum virus, 0.0 ug/ml PLO. Absorbance from samples of this treatment decreased to 0.346 absorbance units at 48 hours post-inoculation, indicating a decreased amount of virus retained by protoplasts. Other samples within the same experiment showed the same trend. Such a decrease observed with the cDNA probe was also observed with samples assayed by ELISA and apparently represented residual absorbed virus.

Table 21. RNA extracted with LiCl from NRSV-inoculated 'Cascade' protoplasts. Numbers are absorbance units from densitometry of a slot blot autoradiogram.

Experiment 1.

[Inoculum NRSV] (ug/ml)	Hours Post-Inoculation[1] 0	12	24	48
0.0	---[2]	0.095	0.073	---
	---	0.096	---	---
	---	0.066	---	---
	---	0.083	---	---
	---	---	---	---
	---	+[3]	0.091	0.109
	+	---	0.126	0.099
	---	+	0.113	0.142
	+	+	0.045	0.079
	---	+	0.126	
1.0	0.038	0.033	0.039	0.111
	0.036	0.118	0.033	---
	0.044	0.043	---	---
	0.027	0.053	---	---
	---	---	---	---
	0.103	+	+	0.121
	0.055	+	+	0.123
	---	+	+	0.118
	---	+	---	0.069
	---	+	---	
25	0.052	0.055	0.044	0.084
	0.054	---	0.024	0.084
	0.067	---	0.033	0.056
	0.061	---	0.036	---
	0.058	---	---	
	0.062	0.136	0.109	+
	0.062	0.153	0.069	+
	0.087	0.328	0.063	+
	---	0.204	0.080	+
	---		0.093	---

Table 21 (cont). RNA extracted with LiCl from NRSV-inoculated 'Cascade' protoplasts. Numbers are absorbance units from densitometry of a slot blot autoradiogram.

Experiment 2.

[PLO] (ug/ml)	[Inoculum NRSV] (ug/ml)	Hours Post-Inoculation 0	48
0.0	0.0	0.160	0.128
0.0	25.0		0.096
0.0	100.0	0.552	0.346
1.0	25.0	0.277	
1.5	25.0	0.238	0.108
1.5	100.0	0.282	0.152

[1]Data for five samples in each of 2 replicates are given.

[2]Dashes refer to samples which gave no signal discernable above background.

[3]Pluses refer to samples which could not be read on the densitometer but were visually identified as positive.

Figure 6. Slot blot with number key of total RNA from Prunus necrotic ringspot virus (NRSV)-inoculated and non-inoculated Cascade protoplasts. cDNA for hybridization was made by reverse transcription from purified NRSV RNA. Numbers in key are explained in Table 22.

Figure 6.

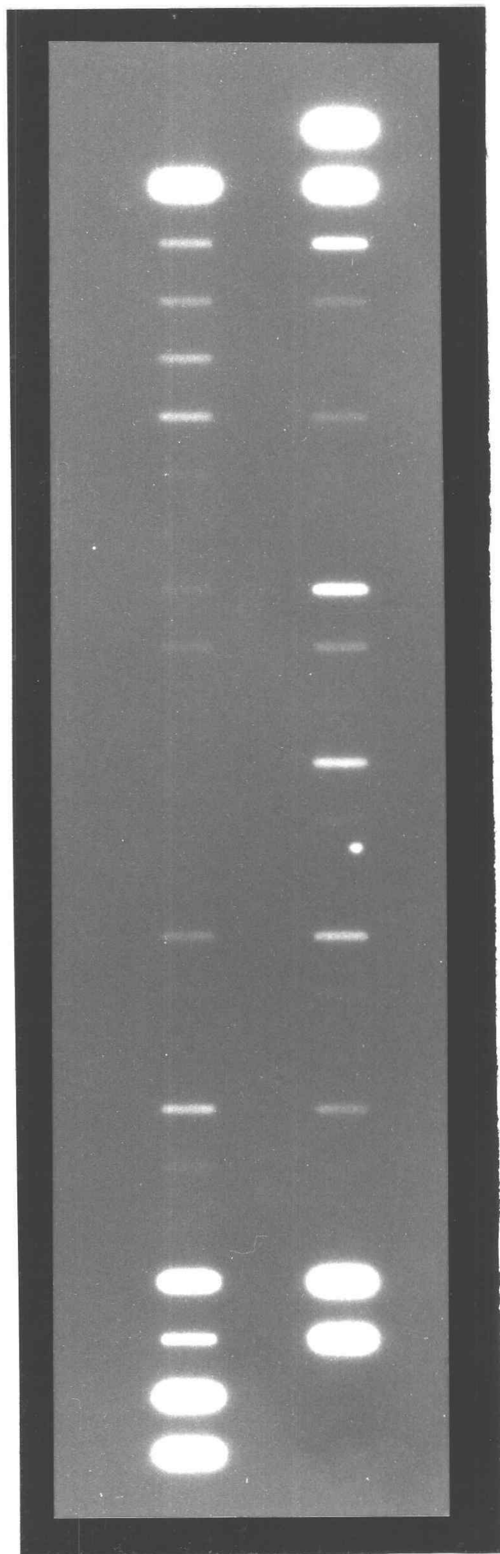


Table 22. Key to numbers corresponding to spots in Figure 6.

Spot Number	Sample
1	Unspecified amount of NRSV RNA[1]
2	250-500 ng NRSV RNA
3	2.5-5 ng NRSV RNA
4	25-50 ng NRSV RNA
5	1:100[2] of 1.0, 12.5, 0[3]
6	1:10 of 1.0, 12.5, 0
7	1.0, 12.5, 0
8	1:100 of 0.0, 12.5, 48
9	1:10 of 0.0, 12.5, 48
10	0.0, 12.5, 48
11	1:100 of uninoculated 60023M protoplasts
12	"
13	1:100 of 0.0, 0.0, 48
14	1:100 of 0.0, 0.0, 0
15	1:10 of uninoculated 60023M protoplasts
16	1:10 of "
17	1:10 of 0.0, 0.0, 48
18	1:10 of 0.0, 0.0, 0
19	uninoculated 60023M protoplasts
20	"
21	0.0, 0.0, 48
22	0.0, 0.0, 0
23	unspecified amount of NRSV RNA
24	"
25	"
26	1:100 of 1.5, 50, 48
27	1:10 of 1.5, 50, 48
28	1.5, 50, 48
29	1:100 of 1.5, 50, 0
30	1:10 of 1.5, 50, 0
31	1.5, 50, 0
32	1:100 of 0.0, 50, 48
33	1:10 of 0.0, 50, 48
34	0.0, 50, 48
35	1:100 of 0.0, 50, 0
36	1:10 of 0.0, 50, 0
37	0.0, 50, 0
38	1:100 of 1.5, 12.5, 48
39	1:10 of 1.5, 12.5, 48
40	1.5, 12.5, 48
41	1:100 of 1.5, 12.5, 0
42	1:10 of 1.5, 12.5, 0
43	1.5, 12.5, 0
44	unspecified amount of NRSV RNA
45	"

Table 22 (cont). Key to numbers corresponding to spots in Figure 6.

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[1]An unspecified amount of purified NRSV RNA was blotted as a blot marker.

[2]1:100 or 1:10 refers to dilution of the RNA extract.

[3]The first number in the series refers to ug/ml of poly-L-ornithine included for inoculation; the second number refers to ug/ml of NRSV added to inoculum buffer; the third number refers to the length of protoplast incubation, in hours, after inoculation.

DISCUSSION

NRSV Purification

Although data are not presented for different methods, I consistently got higher virus yields and cleaner preparations with the modification of Fulton's method for NRSV purification than a second method evaluated (Casper et al., 1971). Success with the method absolutely depended on properly made hydrated calcium phosphate. Problems with this purification scheme have usually been associated with this step in the purification. Casper's method depended on differential precipitation of virus and host components with PEG and NaCl (see Casper et al., 1971). Cross adsorption of host components with antibody as described in this method was, however, omitted. Lower virus yields and relatively large amounts of host material remained in final preparations from this method. Other methods, achieving host-component precipitation by reducing pH to 4.0, also gave poor yields due to partial precipitation of NRSV with host components (L. S. Loesch-Fries, personal communication).

NRSV RNA Extraction

$A_{260/280}$ ratios and A_{320} of purified NRSV RNA indicated that RNA was fairly pure; i.e. a high, narrow absorbance peak of 260 nm UV light and little light scat-

tering. Higher yields may have been possible by adding bentonite during phenol extraction.

RNA pellets purified from virions in phosphate buffer were abnormally large and had a fluffy appearance compared to RNA purified from virions in other buffers and to cucumber mosaic virus RNA. These phosphate buffer preparations may have been contaminated with some host component which was not detected by UV light absorbance. Alternatively, NRSV RNA aggregates may have formed in the presence of phosphate. Because of these possibilities, RNA from phosphate preparations was not used for reverse transcription.

NRSV RNA Purity and Composition

Unfortunately, cucumber cotyledons could not be infected with RNA pellets resuspended in TE. Specific infectivity may have been low because of preferential loss of RNAs 1 and/or 2 during virion or RNA purification. RNAs 1,2,3, and 4 are all required for infectivity of ilarviruses if no capsid protein is present (Fulton, 1983).

NRSV Detection with ELISA

As few as 10,000 protoplasts were adequate to detect adsorbed NRSV. A_{405} values from this quantity of protoplasts were usually above the detection end point of 2.5 ng/ELISA-plate well as calibrated by mixtures of purified virus and homogenates from 50,000 protoplasts (Appendix).

Homogenate from test alliquots of 50,000 protoplasts was used as experimental samples, however, because resulting A_{405} ELISA data were more consistent than with smaller numbers of protoplasts. ELISA was suitable detecting NRSV in hop protoplasts if standard curves were made for each separate group of samples assayed. Most standard curves showed a linear relationship between concentrations of virus tested and the magnitude of A_{405} values (see Appendix). Principal sources of variability were globulin-enzyme conjugates and NRSV-detection experiments. Variability among experiments was attributable substantially to different incubation times of ELISA substrate.

The application of ELISA for detection of virus in protoplasts has also been verified by others. Kiefer et al. (1984) followed cowpea mosaic virus capsid accumulation in cowpea protoplasts with ELISA. They found that a linear range of absorbancies resulting from 10-200 ng of virus was useful for quantitative comparisons. Zhengkaixu et al. (1984) used ELISA to verify protoplast infection and quantitate antigen production in tobacco protoplasts infected with liposome-encapsulated tobacco vein mottling virus (TVMV) RNA. Viral antigen was detected in protoplasts as soon as 24 hours after inoculation. They claimed detection of nanogram quantities of TVMV.

Immunofluorescent Slide Assay

Results are not presented from inoculated protoplasts analyzed by immunofluorescent assay because no fluorescence could be attributed to presence of progeny NRSV. No known-infected protoplasts were available for a positive control to verify proper activity of the anti-NRSV IgG-fluorescein conjugates. Although UV light absorption is not a definitive inticator of activity, absorption characteristics of the conjugates were normal. There were two possible reasons that no fluorescence was observed: either, 1) there was no replication of NRSV in 'Cascade' protoplasts, also indicated by ELISA, and thus, no progeny NRSV was present, or 2) progeny virus was present in low concentration and in even distribution in the protoplasts; i.e. without localization requisite to detection with immunofluorescent slide assay. Barker and Harrison (1978 a & b) found that raspberry ringspot virus antigen was dispersed within tobacco mesophyll protoplasts in such a way that fluorescence was weak and generalized and hard to differentiate from background. Interestingly, tobacco rattle virus infection of the same protoplasts enhanced the specific fluorescence of raspberry ringspot virus, apparently by inducing raspberry ringspot virus aggregation.

THESIS CONCLUSION

Some conditions were optimized for *in vitro* culture of Cascade hops. The same conditions applied to some, but not all, other hop genotypes cultured. These results should facilitate the use of tissue culture to study hop diseases and in hop breeding programs.

Although at least three basically different inoculation strategies were evaluated for infection of hop protoplasts with NRSV, no infection was observed. There are several possible reasons for these results. The first is that progeny NRSV was too difficult to detect in hop protoplasts with the three methods of detection used. NRSV may have replicated so ineffeciently in hop protoplasts that progeny virus was undetectable above backgound of adsorbed virus (and host RNA for nucleic acid hybridization). Coupled with this, virus may have been sparcely dispersed in protoplasts so that immunofluorescence was ineffective, as with raspberry ringspot virus in tobacco (Barker and Harrison, 1978).

Although many combinations of pH, buffer concentration and type, polycation, and neutral salts were explored to facilitate infection, perhaps optimal conditions were not found. With most virus-host combinations presented in the literature, slight changes in one component of inoculum will drastically affect infection success. One

notable example is the requirement for and large effect observed by both non-buffering and buffering salts on infection of soybean suspension protoplasts with cowpea mosaic or southern bean mosaic viruss (Jarvis and Murakishi, 1980). An additional complication in determining optimal conditions is the requirement for all three gemone parts of NRSV for infection. To counteract this, relatively large amounts of virus (12-50 ug/ml) were added to inoculum. No more than 1-2 ug/ml virus, however, was required for infection of turnip protoplasts with radish mosaic virus (Fukunaga and Furusawa, 1981) and of cowpea protoplasts with alfalfa mosaic virus (Alblas and Bol, 1977) in which conditions were optimized. Both of these viruses are also multicomponent.

One more possible reason why protoplast infection was not observed is because of changes in gene expression or induction of resistance mechanisms in hop tissue by in vitro culture. The conditions of culturing and protoplast isolation may induce different genetic changes (Gleba and Sytnik, 1984). Antoniw et al. (1981) were able to show that components of tissue culture media, notably IAA, 2,4-D, and BAP, induced the production of pathogenesis-related (PR) proteins in tobacco callus. Whether production of PR proteins or similar compounds occurs in other plant tissue by in vitro culture is not known. Although protoplasts from suspension cultures of other hosts have

been successfully used for virus inoculation, each virus-host combination is different.

Other potential strategies need to be explored for inoculation of hop protoplasts with NRSV. More variations in pH, buffer salts, and compounds to facilitate infection could be tried. A wider range of conditions with polyethylene glycol may prove successful. Protoplasts isolated from hop mesophyll may react differently to NRSV. Still other inoculation methods which have been used successfully in other systems and could be appied to NRSV and hops involve electroporation (Nishiguchi et al., 1986) and liposome encapsulated RNA (Watanabe et al., 1982; Zhengkaixu et al., 1984).

ELISA appears to be the most efficient method to detect NRSV in hop protoplasts. Nucleic acid hybridization may be useful if a more specific probe could be developed or if hybridization conditions were optimized to reduce non-specific hybridization. It is difficult to assess the usefulness of immunofluorescence for NRSV in hops because of lack of definitive results.

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APPENDIX

APPENDIX

Standard curves for estimation of the amount of NRSV in a protoplast sample were obtained by adding increasing amounts of purified virus to ELISA wells with or without homogenate from non-inoculated protoplasts. The A_{405} value received was taken as the x value and the Log_{10} amount of purified NRSV added was taken as the y value to construct the standard curve. A_{405} values from inoculated protoplast samples were entered into the equation for line of best fit (standard curve) as the x value. The inverse Log_{10} of the resulting y value was the estimate for amount of virus adsorbed.

A_{405} values for samples and standards varied greatly depending on the batch of alkaline phosphatase-anti-NRSV IgG conjugate used and on incubation time for p-nitrophenol substrate with conjugate-coated wells. For this reason, estimations which had a set of standards for each separate experiment were most accurate. For conjugate batch 2, only one set of standards was used for several experiments with slightly different substrate incubation times. For this reason, estimations made with this standard curve are at best approximations.

Part of the reason for inaccurate estimations with certain amounts of virus, especially very high or low amounts, is due to the principle of ELISA. ELISA increases the effective concentration of virus in a sample

by irreversibly extracting virus out of a sample. This is very effective for increasing sensitiviy of detection but makes quantitation difficult.

Sets of standards used for estimation of amounts of virus are given in Table 1 with the associated correlation coefficients.

Appendix Table 1. A_{405} values corresponding to amounts of purified NRSV included in one ELISA plate well with homogenate from 50,000 protoplasts.

Conjugate batch 1.[1] r=0.958[2] (determined with first three NRSV amounts)

Amount of NRSV Added	Log_{10} Amount NRSV Added	A_{405} (30 min)[3]
0.1275 ug	2.106	3.000
0.0159 ug	1.201	1.043
1.050 ng	0.021	0.151
0.090 ng	-1.046	0.021

Conjugate batch 2. r=0.904

Amount of NRSV Added	Log_{10} Amount NRSV Added	A_{405} (45 min)
25.00 ug	4.398	2.596
5.00 ug	3.699	1.977
0.50 ug	2.699	1.984
75.0 ng	1.875	1.842
5.0 ng	0.699	0.488

Conjugate batch 3. r=0.975

Amount of NRSV Added	Log_{10} Amount NRSV Added	A_{405} (45 min)
25.00 ug	4.398	1.172
2.50 ug	3.398	0.644
250.0 ng	2.398	0.467
25.0 ng	1.398	0.203
2.5 ng	0.398	0.021

Appendix Table 1. (cont.). A_{405} values corresponding to amounts of purified NRSV included in one ELISA plate well with homogenate from 50,000 protoplasts.

Conjugate 3. r=0.981

Amount of NRSV Added	Log_{10} Amount NRSV Added	A_{405} (35 min)
50.00 ug	4.699	2.044
25.00 ug	4.498	1.566
2.50 ug	3.398	1.194
0.50 ug	2.699	1.104
0.25 ug	2.398	0.927
0.10 ug	2.000	0.777
25.0 ng	1.398	0.433
2.5 ng	0.398	0.053

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[1]Values for conjugate batch 1 are for purified NRSV added to wells in ELISA sample buffer without homogenate from non-inoculated protoplasts.

[2]Correlation coefficients calculated with log_{10} NRSV vs. A_{405}.

[3]Time designations refer to amount of time substrate was incubated before readings were taken.