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thesis entitled

REGENERATION OF CAPSICUM ANNUUM AND SCREENING OF THE R1 GENERATION FOR RESISTANCE TO CUCUMBER MOSAIC VIRUS AND A SURVEY OF PEPPER VIRUSES IN MICHIGAN

presented by

Janette Lynn Jacobs

has been accepted towards fulfillment
of the requirements for

M.S. degree in Plant Pathology

Christine Stephens
Major professor

Date October 28, 1991

REGENERATION OF *CAPSICUM ANNUUM* AND SCREENING OF THE R1 GENERATION FOR RESISTANCE TO CUCUMBER MOSAIC VIRUS AND A SURVEY OF PEPPER VIRUSES IN MICHIGAN

By

Janette Lynn Jacobs

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1991

ABSTRACT

REGENERATION OF CAPSICUM ANNUUM AND SCREENING OF THE R1 GENERATION FOR RESISTANCE TO CUCUMBER MOSAIC VIRUS AND A SURVEY OF PEPPER VIRUSES IN MICHIGAN

By

Janette Lynn Jacobs

Differences in morphogenetic response of 58 pepper (Capsicum annuum) genotypes was examined on Murashige and Skoog (Physiol. Plant. 53:319-326) medium (MS) supplemented with indole-3-acetic acid (3 mg/L), 6-benzylaminopurine (4 mg/L), and silver nitrate (10 mg/L). Explants were incubated at 28 C for one month, transferred to MS medium supplemented with napthaleneacetic acid (0.1 mg/L), with whole plants regenerated from 19 of 58 genotypes. A total of 95 R1 generation lines from the sexual offspring of plants regenerated from C. annuum PI178849 were screened for resistance to cucumber mosaic virus (CMV). Extensive variability in infection of plants was observed between inoculation and screening methods. Resistance to CMV was not observed among the 95 R1 lines. A survey of randomly-selected pepper from commercial fields in Michigan in 1989 and 1990 demonstrated that CMV is an important virus endemic in Michigan and tomato spotted wilt virus is not presently endemic, but imported to the state in pepper transplants.

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Christine T. Stephens, my major professor for her support and guidance throughout my studies at Michigan State University. I would like to thank Dr. Donald Ramsdell and Dr. Joseph Saunders for their time and guidance while serving on my graduate committee.

My sincere gratitude is extended to Dr. Harry Murakishi and Dr. Richard Allison for their friendship and for kindly providing the use of their laboratory.

I would like to express sincere thanks to my husband, family, and friends for their constant encouragement and support.

In addition, I am grateful for the technical assistance from Sheila Linderman, Gongzhi Li, and John Kusnier with my research project.

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LITERATURE REVIEW

In terms of economic importance, pepper (Capsicum annuum) is a significant crop placing fifth in the world-wide production of vegetables. The pepper crop in the United States has a value of 169 million dollars annually. There are 110,000 acres in pepper production and 686,750 tons of pepper fruit are harvested each year (Marshall, 1977). Michigan has a relatively small acreage (3850) of pepper with a value of 3.2 million dollars annually (Michigan Agricultural Statistics, 1989). The majority of peppers grown in Michigan are of the bell fruit type and are produced for fresh market. Peppers are mostly grown in the southwest, central and southeast regions of the Lower Peninsula.

Internationally, severe losses in the pepper industry occur due to the presence of virus diseases. Virus diseases damaging to pepper have been reported in California (Abdalla, 1985), Florida (Anderson and Corbett, 1957; Zitter, 1973), Georgia (Benner et al, 1985), Texas (Villalon, 1975, 1981) and in several countries including the Netherlands (Rast, 1982), Italy (Conti, 1977), Canada (Lana and Peterson, 1980), and India (Deol and Rataul, 1978). Due to virus infection, pepper crops are occasionally abandoned without harvesting in some of the major production areas in the southwestern United States (Villalon, 1981). Pepper production has been eliminated from some growing regions in Massachusetts (Moorman and Woodbridge, 1983) and Texas (Villalon, 1975) as a direct

result of virus disease pressure.

Thirty six viruses are known to infect pepper (Villalon, 1975), but only alfalfa mosaic (AMV), cucumber mosaic (CMV), pepper mottle (PeMV), potato virus X (PVX), potato virus Y (PVY), tobacco etch (TEV), and tobacco mosaic (TMV) viruses have been economically important (Marco and Cohen, 1979; Horvath and Nienhaus, 1982; Agrios et al., 1985; and Villalon, 1981).

Although not serologically confirmed, a preliminary field study demonstrated that CMV was involved in natural infection of pepper in Michigan (C.T. Stephens and T. Stebbins, personal communication). CMV is a highly destructive plant pathogen known to have a number of strains. CMV attacks a wide range of crops (191 species in 40 families), and is world-wide in distribution. CMV is transmitted in a nonpersistent manner by more than 60 species of aphids (Francki et al., 1979). The virus belongs to the cucumovirus group and has a tripartite genome encapsidated in three types of isometric particles of about 28 nm in diameter. All three components are necessary for infection (Francki et al., 1979).

Pepper is one of the major economic crops affected by CMV. In virus surveys, CMV has been detected in pepper fields in Texas (Villalon, 1975), Florida (Simons, 1957), Louisiana (Barrios et al., 1971), New Jersey (Doolittle andnd Zaumeyer, 1953), Georgia (Benner et al., 1985), and Quebec (Lana and Peterson, 1980). Symptoms of CMV on pepper

include mottling, chlorosis, oak-leaf or circular necrotic markings on the leaves, and tan rings or spots on the fruit. These rings become brown and necrotic and result in fruit spotting. Leaf symptoms develop when peppers are infected at a young stage of growth (Simons, 1957). Fruit symptoms are not always present, particularly when plants become infected at an early or late stage of development (Pasko et al., 1984).

The major strategy for virus disease control is the use of vigorous aphid control programs. Various cultural practices have been developed for reducing the incidence of aphid transmitted viruses in pepper: oil sprays which decrease the ability of aphids to acquire and transmit stylet-borne viruses (Vanderveken, 1977); soil mulching with reflective surfaces which repel the aphids (Smith and Webb, 1969); and attraction of the aphids to yellow sticky polyethylene traps, thus keeping the aphids from the plants (Cohen and Marco, 1973). Each of the above mentioned practices, however, has certain limitations. Additionally, aphid control programs for virus disease management are usually unsuccessful because virus transmission only requires one short feeding.

Genetic resistance remains the principal solution for controlling virus diseases in pepper. Disease-resistant crop species have long been produced by identifying resistant genotypes and then crossing individuals exhibiting resistance with cultivars that possess horticulturally

acceptable traits. Traditional breeding has been successful and in many cases, resistance to PeMV, PVY, TEV, and TMV has been found and introduced into different pepper cultivars (Villalon, 1986; and Holmes, 1937). Capsicum frutescens cultivar LP-1 showed a high degree of tolerance to CMV, and the tolerance was determined to be a recessive trait (Barrios et al., 1971).

Limitations do exist, however, to conventional plant breeding methods. For example, the desired resistance is not always available, it may only exist in a species that is sexually incompatible, the genes for resistance may be tightly linked to undesirable traits, or may be multigenic and difficult to transfer (Knott and Dvorak, 1976; Wenzel, 1985). Also, virus-resistance is subject to breakdown due to the appearance of other viral strains. Significantly, in spite of active pepper breeding programs in the United States, few lines are commercially available that contain multiple virus resistance.

Genetic variability is essential to plant breeding; continual input of new genes is vital for the improvement of commercial crop species. A relatively recent method of developing new genetic material is through the use of plant cell culture-generated genetic variability, which is termed somaclonal variation (Larkin and Scowcroft, 1981). Plant cell culture generates genetic variation as well as epigenetic variation in regenerated plants. Genetic variation is the result of pre-existing genetic differences

in somatic cells in the explant tissue, or evolves during cell proliferation on the explant. Genetic variant characters that are expressed in plants regenerated from plant cell culture are transmissable to progeny in sexually propagated crops (Evans and Sharp, 1983). Examples of somaclonal variation include cytoplasmic gene changes, chromosome rearrangements, mitotic crossing over, and changes in gene expression (Evans, 1989). These genetic changes are either unique to somaclonal variation or occur at a much higher frequency than in spontaneous or induced mutagenesis. Epigenetic changes are induced by specific components of the cell culture medium, and although almost unique to somaclonal variation, they are not useful for crop improvement, as they are not expressed in the R1 progeny (Evans, 1989). Examples of epigenetic variation include, dwarfing, alteration in leaf shape, or other changes in growth habit.

As a result of genetic changes, somaclones with altered genotypes may express levels of disease resistance which are greater than the parent genotype. The first report recognizing the potential of tissue culture-derived variants as a source of variability for crop improvement occurred with sugarcane (Heinz and Mee, 1969) and later with potatoes (Shepard et al., 1980). In these studies researchers foundthat plants regenerated from callus and protoplast cultures varied in a number of traits, including morphological characteristics, maturity date, yield and

response to pathogens. Although this early work was done with asexually propagated crops, other investigators have since shown that somaclonal variation occurs in many plant species, including tobacco, rice, carrot (Larkin and Scowcroft, 1981), alfalfa (Latunde-Dada and Lucas, 1983), celery (Wright and Lacy, 1985), and tomato (Evans and Sharp, 1983).

The causes of somaclonal variation are still being investigated, but a number of facts are known. Somaclonal variation is usually genetic and heritable, and variants occur at a high frequency. In addition, changes occur both in monogenic and polygenic traits, and in both nuclear and organelle genomes (Daub, 1986). In somaclonal studies, the frequency of variation has been estimated to be as high as 30-40% for the number of plants showing some type of variation, and from 0.2 to almost 3% for variation in a particular trait (Daub, 1986). Somaclonal variation frequencies are high enough that desirable variants can be identified and selected if an efficient screening system exists at the whole-plant level.

Somaclonal variation may be used to introduce the best available varieties into plant cell culture and to select among regenerated plants (RO) or their self-fertilized progeny (R1) for incremental improvements in resistance overexisting commercial varieties. This procedure has been utilized in selecting tomato somaclones resistant to TMV (Barden, 1986), and sugarcane subclones with a greater

degree of resistance to Fiji virus (Krishnamurthi, 1974).

The phenomenon of somaclonal variation offers a means to select variability without exposure to a mutation agent. The use of protoplasts offers a means of directly exposing single plant cells to a selection agent. Utilization of plant protoplasts in an *in vitro* selection system may offer a means of creating or isolating plant genotypes with increased resistance to disease. Screening for resistance to plant pathogens at the single cell level offers unique advantages. Millions of cells can be screened in a short time, the technique holds promise for selection of resistance from horticulturally susceptible cultivars (Shepard, 1981), and may allow for the recovery of increased levels of disease resistance (Murakishi and Carlson, 1982).

To date, viral pathogens offer the most promise as direct selection agents in the use of *in vitro* screening systems because protoplasts can be synchronously infected with viruses (Hibi et al., 1986; Maule et al., 1980; Motoyoshi and Oshima, 1975; Nishiguchi et al., 1987). Infection of protoplasts has been demonstrated in a variety of plant species such as barley, *Brassica*, cowpea, cucumber, tobacco, and tomato (Okuno and Furusawa, 1978; Maule, 1983; Koike et al., 1977; Maule et al., 1980; Hibi et al., 1986; Nishiguchi et al., 1987; Motoyoshi and Oshima, 1975). Also, studies using protoplasts isolated from resistant host plants of tomato and cucumber infected with TMV and CMV respectively, revealed that resistance to the pathogen

functions at the single cell level (Maule et al., 1980; Motoyoshi and Oshima, 1975). In addition, Murakishi and Carlson (1982), working with protoplasts isolated from systemically infected tobacco plants, demonstrated that viral pathogens can be used as selection agents at the single cell level to isolate variant cells, if conditions are manipulated to allow for uniform infection and for the preferential growth and selection of virus-resistant cells.

Although studies examining the manipulation of pepper in vitro have been moderately successful, plant regeneration has often restricted to only a few genotypes. To date, the regeneration of whole plants from somatic cells is not sufficiently routine for efficient application of technology such as somaclonal variation and genetic transformation.

Manipulative variables in pepper regeneration systems include factors such as growth medium components, light duration and intensity, and temperature. Experiments in the literature involving pepper plant regeneration utilize the same basal medium but disagree on the type and concentrations of hormones in the regeneration process. Shoot bud formation of hypocotyl and cotyledon sections was reported on Murashige and Skoog (MS) medium supplemented with 1 mg/L indole-3-acetic acid (IAA) and 2 mg/L 6-benzylaminopurine (BAP) (Gunay and Rao, 1976), but, quantitative response data were not included in this report, and 58 - 62% of cultures produced a mass of callus overgrowth which suppressed shoot primorida elongation in

another study using the same medium (Fari and Czako, 1981). A continuous light and constant temperature treatment at 28.5 C was reported as stimulatory for extended shoot and root organogenesis in pepper tissues incubated in MS medium supplemented with 0.5 mg/L IAA and BAP (Phillips and Hubstenberger, 1985). Glucose as a carbon source has also been reported as stimulatory for shoot regeneration (Phillips and Hubstenberger, 1985). A concentration of 3 - 7 mg/L of BAP in MS medium induced the largest number of shoots per explant in another study, with auxin (IAA or naphthaleneacetic acid, NAA) inhibiting shoot formation when used in combination (Sripichitt et al., 1987). Finally, 5 mg/L BAP in MS medium resulted in the best shoot differentiation, however, the number of shoots increased but did not elongate (Agrawal, et al., 1989). Also, in this study, 0.1 mg per liter of IAA or NAA was critical to obtain complete plants (Agrawal et al., 1989).

One factor common to all of these reports is the low frequencies of the number of explants producing plantlets as well as the low number of plantlets obtained per explant. Also, regarding all of the above reports, regeneration of pepper genotypes was not reproducible using these methods.

A thorough understanding of how explant sources, medium components, and environmental conditions affect shoot initiation and elongation is necessary to provide a rapid regeneration technique which will be applicable to a wide range of pepper genotypes.

A factor in these studies which had not been explored, was the role of ethylene in pepper regeneration. Ethylene is a gaseous plant hormone produced from essentially all parts of higher plants, including leaves, stems, roots, fruits, tubers, and seedlings (Abeles, 1973). Ethylene production plays an important role in regulating many developmental processes, ranging from germination to senescence.

Few studies have addressed the role of endogenously produced ethylene on organogenesis in higher plants (Horner et al., 1977). However, with respect to the role of exogenously applied ethylene, it was shown that shoot forming callus tissues contained significantly lower concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (Grady and Bassham, 1982) and produce less ethylene (Huxter, et al., 1981) compared to non-shoot-forming callus tissues. Exogenously applied ethylene precursor in high concentrations inhibited shoot primordium formation in tobacco callus cultures (Huxter et al., 1981).

Also, auxins can promote the endogenous production of ethylene (Yang and Hoffman, 1984). The promotion of ethylene production by auxin was initially discovered by Zimmerman and Wilcoxon (1935). Since auxin and ethylene cause a number of similar responses, and auxin is capable of promoting ethylene production, many responses previously attributed to auxin can be traced to ethylene produced in

response to auxin treatment. In vegetative tissues, the rate of ethylene production is thought to be regulated by the internal level of free auxin (Abeles, 1973). It is known that ethylene production rate in IAA-treated tissue declines when IAA is withdrawn from the incubation medium (Yoshii and Imaseki, 1982). Auxins, which are essential for callus induction, can play a negative role in plant regeneration. The results of previous studies suggest that in non-regenerating plant tissue cultures auxin induced ethylene production may be responsible for the suppression of shoot regeneration.

Silver ions are known to inhibit ethylene action in plants (Beyer, 1976). Silver ions applied foliarly as silver nitrate, effectively blocked the action of exogenously applied ethylene in such classical ethylene responses as abscission, senescence, and growth retardation in pea, orchid, and cotton (Beyer, 1979). The basis for this protection by silver ions is unknown. It has been suggested that silver ions interfere with the incorporation of ethylene at its receptor site, but not its oxidation to CO_2, in etiolated pea seedlings (Beyer, 1979). An effect of silver nitrate on liverwort organogenesis was interpreted as a desuppression of potential development of local populations of cells suppressed by ethylene (Basile and Basile, 1983). Silver nitrate effectively promoted shoot regeneration in callus cultures of wheat and tobacco genotypes with poor regeneration ability (Purnhauser, et

al., 1987).

Another factor of importance in plant regeneration is the variation observed among genotypes of a particular species. This genotypic variation in the organogenic response is widespread among plant species. Callus cultures of monocots have poor regeneration ability, and great differences in organogenic response exist between genotypes (Sears and Deckard, 1982; Duncan, et al., 1985). Genotypic specificity for regeneration capacity in legumes is well-documented in alfalfa (Chen and Marowitch, 1987), white clover (Bhojwani et al., 1984), and soybeans (Parrott et al., 1989). Genotypic variation is even observed in Nicotiana, which has been used as a model in tissue culture systems (Purnhauser et al., 1987).

Researchers have attempted to find indicators of a genotype's ability of regenerative capacity. A study using soybean genotypes showed no relationship between maturity group, seed coat color, flower color, and disease susceptibility or resistance to a genotype's ability to produce high numbers of somatic embryos (Parrott et al., 1989). Genotype-specific capacity for regeneration has been exploited to breed alfalfa with a high regeneration capacity (Bingham et al., 1975).

The objectives of this study were to: 1) determine which pathogenic viruses are present in pepper fields in Michigan, 2) to develop a reproducible whole plant regeneration system for pepper, 3) to assess the use of

somaclonal variation as a means of selecting virus-resistant pepper germplasm at the whole plant level, and 4) to investigate those variables which influence the uptake of virus particles into protoplasts of pepper by electroporation.

LIST OF REFERENCES

Abdalla, O.A., Desjardins, P.R., and Dodds, J.A. 1985. Survey of pepper viruses in California by the ELISA technique. Phytopathology 75:1311.

Abeles, F.B. 1973. Ethylene in Plant Biology. Academic Press, New York: pp 1-302.

Agrawal, S., Chandra, N., and Kothari, S.L. 1989. Plant regeneration in tissue cultures of pepper (Capsicum annuum L. cv. mathania). Plant Cell Tiss. Org. Cult. 16:47-55.

Agrawal, S., Chandra, N., and Kothari, S.L. 1988. Shoot tip culture of pepper for micropropagation. Current Sci 57:1347-1349.

Agrios, G.N., Walker, M.E., and Ferro, D.N. 1985. Effect of cucumber mosaic virus inoculation at successive weekly intervals on growth and yield of pepper (Capsicum annuum) plants. Plant Dis. 69:52-55.

Anderson, C.W., and Corbett, M.K. 1957. Virus diseases of peppers in central Florida. Survey results 1955. Plant Dis. Reptr. 41:143-147.

Barden, K.A., Smith, S.S., and Murakishi, H.H. 1986. Regeneration and screening of tomato somaclones for resistance to tobacco mosaic virus. Plant Sci. 45:209-213.

Barrios, E.P., Mosokar, H.I., and Black, L.L. 1971. The inheritance of resistance to tobacco etch and cucumber mosaic viruses in Capsicum frutescens. Phytopathology 61:1318.

Basile, D.V., and Basile, M.R. 1983. Desuppression of leaf primordia of Plagiochila artica (Hepaticae) by ethylene antagonists. Science 220:1051-1053.

Benner, C.P., Kuhn, C.W., Demski, J.W., Dobson, J.W., Colditz, P., and Nutter, F.W. 1985. Identification and incidence of pepper viruses in northeastern Georgia. Plant Dis. 69:999-1001.

Beyer, E.M. Jr. 1976. A potent inhibitor of ethylene action in plants. Plant Physiol. 58:268-271.

Beyer, E.M. Jr. 1979. Effect of silver ion, carbon dioxide, and oxygen on ethylene action and metabolism. Plant Physiol. 63:169-173.

Bhojwani S.S., Mullins, K., and Cohen, D. 1984. Intra-varietal variation for in vitro plant regeneration in the genus Trifolium. Euphytica 33:915-921.

Bingham, E.T., Hurley, L.V., Kaatz, D.M., and Saunders, J.W. 1975. Breeding alfalfa which regenerates from callus tissue in culture. Crop Sci. 15:719-721.

Chen T.H.H., and Marowitch, J. 1987. Screening of Medicago falcata germplasm for in vitro regeneration. J. Plant Physiol. 128:271-277.

Cohen, S. and Marco, S. 1973. Reducing the spread of aphid-transmitted viruses in pepper by trapping the aphids on sticky yellow polyethylene sheets. Phytopathology 63:1207-1209.

Conti, M. 1977. Identification and prevalence of pepper viruses in northwest Italy. Phytopathol. Z. 90:212-222.

Daub, M.E. 1986. Tissue culture and the selection of resistance to pathogens. Ann. Rev. Phytopathol. 24:159-186.

Deol, G.S., and Rataul, H.S. 1978. Role of various barrier crops in reducing the incidence of cucumber mosaic virus in chilli, Capsicum annuum Linn. Indian J. Ent. 40:261-264.

Doolittle, S.P., and Zaumeyer, W.J. 1953. A pepper ring spot caused by strains of cucumber mosaic virus from pepper and alfalfa. Phytopathology 43:333-337.

Duncan D.R., Williams, M.E., Zehr, B.E., and Widholm J.M. 1985. Planta 165:322-332.

Evans, D.A. 1989. Somaclonal variation-genetic basis and breeding applications. TIG 5(2):46-50.

Evans, D.A., and Sharp, W.R. 1983. Single gene mutations in tomato plants regenerated from tissue culture. Science 221:949-951.

Fari, M., and Czako, M. 1981. Relationship between position and morphogenetic response of pepper hypocotyl explants cultured in vitro. Sci. Hortic. 15:207-213.

Francki, R.I.B., Mossop, D.W., and Hatta, T. 1979. Cucumber mosaic virus. Descriptions of Plant Viruses, No. 213. Commonwealth Mycological Institute, Association of Applied Biologists, Kew, Surrey, England.

Grady K.L., and Bassham, J.A. 1982. 1-Aminocyclopropane-1-carboxylic acid concentrations in shoot-forming and non-shoot-forming tobacco callus cultures. Plant Physiol. 70:919-921.

Gunay, A.L., and Rao, P.S. 1978. In vitro Plant Regeneration from hypocotyl and cotyledon explants of red pepper (Capsicum). Plant Sci. Lett. 11:365-372.

Heinz, D.J., and Mee, G.W.P. 1969. Plant differentiation from callus tissue of Saccharum species. Crop Sci. 9:346-348.

Hibi, T., Kano, H., Sugiura, M., Kazami, T. and Kimura, S. 1986. High efficiency electro-transfection of tobacco mesophyll protoplasts with tobacco mosaic virus RNA. J. Gen Virol. 67:2037-2042.

Holmes, F.O. 1937. Inheritance of resistance to tobacco mosaic virus in the pepper. Phytopathology 27:637-642.

Horner, M., McComb, J.A., and Street, H.E. 1977. Ethylene production and plantlet formation by Nicotiana anthers cultured in the presence and absence of charcoal. J. Exp. Bot. 28:1365-1372.

Horvath, J., and Nienhaus, F. 1982. Separation of viruses from inhibitor-containing pepper leaf extracts (Capsicum annuum L.) with controlled-pore-glass exclusion chromatography. Acta Phytopathol. Acad. Sci. Hung. 17:41-45.

Huxter T.J., Thorpe, T.A., and Reid, D.M. 1981. Shoot initiation in light-and dark-grown tobacco callus: the role of ethylene. Physiol. Plant. 53:319-326.

Jourdan, P.S., and Earle, E.D. 1989. Genotypic variability in the frequency of plant regeneration from leaf protoplasts of four Brassica spp. and of Raphanus sativus. J. Amer. Soc. Hort. Sci. 114:343-349.

Koike, M., Hibi, T., and Yora, K. 1977. Infection of cowpea mesophyll protoplasts with cucumber mosaic virus. Virology 83:413-416.

Krishnamurthi, M., and Tlaskal, J. 1974. Fiji disease resistant Saccharum officianarum var. Pindar subclones from tissue cultures. Proc. Int. Soc. Sugarcane Technol. 15:130-137.

Lana, A.F., and Peterson, J.F. 1980. Identification and prevalence of pepper viruses in southern Quebec. Phyto-protection. 61:13-18.

Latunde-Dada, A.O., and Lucas, J.A. 1983. Somaclonal variation and reaction to verticillium wilt in Medicago sativa plants regenerated from protoplasts. Plant Sci. Lett. 32:205-212.

Larkin, P.J., and Scowcroft, W.R. 1981. Somaclonal variation -- a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60:197-214.

Lieberman, M. 1979. Biosynthesis and action of ethylene. Ann. Rev. Plant Physiol. 30:533-539.

Marco, S., and Cohen, S. 1979. Rapid detection and titer evaluation of viruses in pepper by enzyme-linked immunosorbent assay. Phytopathology 69:1259-1262.

Maule, A.J., Boulton, M.I., Edmunds, C. and Wood, K.R. 1980. Polyethylene glycol-mediated infection of cucumber protoplasts by cucumber mosaic virus and virus RNA. J. Gen. Virol. 47:199- 203.

Maule, A.J. 1983. Infection of protoplasts from several Brassica species with cauliflower mosaic virus following inoculation using polyethylene glycol. J. Gen. Virol. 64:2655-2660.

Mendoza, A.B., and Futsuhara, Y. 1990. Varietal differences on plant regeneration by tissue culture in mungbean Vigna radiata (L.) Wilczek. Japan. J. Breed. 40:457-467.

Michigan Agricultural Statistics. 1989. Vegetables. pp.37-41. Michigan Dept. Agric., Lansing, MI.

Moorman, G.W., and Woodbridge, W.C. 1983. Morphogenesis of cucumber mosaic virus-induced crystalline inclusions in peppers. Phytopathology 73:1106-1108

Motoyoshi, F., and Oshima, N. 1975. Infection with tobacco mosaic virus of leaf mesophyll protoplasts from susceptible and resistant lines of tomato. J. Gen. Virol. 29:81-91.

Murakishi, H. and Carlson, P. 1982. In vitro selection of Nicotiana sylvestris variants with limited resistance to tobacco mosaic virus. Plant Cell Rep. 1:94-97.

Nishiguchi, M., Sato, T. and Motoyoshi F. 1987. An improved method for electroporation in plant protoplasts: infection of tobacco protoplasts by tobacco mosaic virus particles. Plant Cell Rep. 6:90-93.

Okuno, T., and Furusawa, I. 1978. The use of osmotic shock for the inoculation of barley protoplasts with brome mosaic virus. J. Gen. Virol. 39:187-190.

Parrott, W.A., Williams, E.G., Hildebrand, D.F. and Collins, G.B. 1989. Effect of genotype on somatic embryogenesis from immature cotyledons of soybean. Plant Cell Tiss. Org. Cult. 16:15-21.

Pasko, P.J., Nicklow, C.W., and Moorman, G.W. 1984. Factors influencing cucumber mosaic virus symptom development in pepper. HortScience. 19:586-587.

Phillips, G.C., and Hubstenberger, J.F. 1985. Organogenesis in pepper tissue cultures. Plant Cell Tiss. Org. Cult. 4:261-269.

Purnhauser, L., Medgyesy, P., Czako, M., Dix, P.J., and Marton, L. 1987. Stimulation of shoot regeneration in Triticum aestivum and Nicotiana plumbaginifolia Viv. tissue cultures using the ethylene inhibitor $AgNo_3$. Plant Cell Rep. 6:1-4.

Rast, A.TH.B. 1982. Resistance of Capsicum species to tobacco, tomato and pepper strains of tobacco mosaic virus. Neth. J. Pl. Path. 88:163-169.

Sears, R.G., and Deckard, E.L. 1982. Tissue culture variablity in wheat: callus induction and plant regeneration. Crop Sci. 22:546-550.

Shepard, J.F. 1981. Protoplasts as sources of disease resistance in plants. Ann. Rev. Phytopath. 19:145-155.

Simons, J. N. 1957. Three strains of cucumber mosaic virus affecting bell pepper in the Everglades area of south Florida. Phytopathology 47:145-150.

Smith, F.F. and Webb, R.E. 1969. Repelling aphids by reflective surfaces: New approach to control of insect-transmitted viruses. in: Maramorosch, M. (Ed.) Viruses, Vectors and Vegetation. pp. 631-639. Interscience Publishers, New York, NY.

Sripichitt, P., Nawata, E., and Shigenaga, S. 1987. In vitro shoot-forming capacity of cotyledon explants in red pepper (Capsicum annuum L. cv. Yatsufusa). Japan. J. Breed. 37:133-142.

Vanderveken, J.J. 1977. Oils and other inhibitors of non-persistent virus transmission. in: Harris, K.F. and Maramorosch, K. (Eds.) Aphids as Virus Vectors. pp. 435-454. Academic Press, New York, NY.

Villalon, B. 1976. Virus diseases of bell peppers in South Texas. Plant Dis. Reptr. 59:858-862.

Villalon, B. 1981. Breeding peppers to resist virus diseases. Plant Dis. 65:557-562.

Villalon, B. 1986. 'Tambel-2' bell pepper. Hortscience 21:238.

Wright, J.C., and Lacy, M.L. 1988. Increase of disease resistance in celery cultivars by regeneration of whole plants from cell suspension cultures. Plant Dis. 72:256-259.

Yang, S.F., and Hoffman, N.E. 1984. Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant Physiol. 35:155-189.

Yoshii, H., and Imaseki, H. 1982. Regulation of auxin-induced ethylene biosynthesis. Repression of inductive formation of 1-aminocyclopropane-1-carboxylate synthase of ethylene. Plant Cell Physiol. 23:639-649.

Zimmerman, P.W., and Wilcoxon, F. 1935. Several chemical growth substances which cause initiation of roots and other responses in plants. Contrib. Boyce Thompson Inst. 7:209-229.

Zitter, T.A. 1973. Further pepper virus identification and distribution studies in Florida. Plant Dis. Reptr. 57:991-994.

CHAPTER 1

SURVEY OF CUCUMBER MOSAIC, POTATO VIRUS Y, TOBACCO ETCH, AND TOMATO SPOTTED WILT VIRUS IN PEPPER IN MICHIGAN USING ELISA

ABSTRACT

Cucumber mosaic (CMV), potato virus Y (PVY), tobacco etch (TEV), and tomato spotted wilt virus (TSWV) were detected in Michigan pepper fields in 4/6, 2/6, 1/6, and 3/6 respectively in 1989. CMV and TSWV only were detected in 1/7 and 2/7 fields in 1990. CMV was detected throughout the four major pepper growing regions in the state while the other viruses were only detected in one or two geographic regions. TSWV was detected for the first time in Michigan, early in the growing season on pepper transplants from the southeastern United States. CMV, PVY, and TEV were not detected on Michigan-grown transplants until later in the growing season. The data suggest that CMV is a virus endemic to Michigan and TSWV is not presently endemic, but has been imported to the state in pepper transplants.

INTRODUCTION

The importance of virus diseases in pepper production has been recognized for many years. A number of surveys in the United States have been conducted on virus diseases of pepper; these studies reported that cucumber mosaic virus

(CMV), pepper mottle virus (PMV), potato virus Y (PVY), tobacco etch virus (TEV), and tobacco mosaic virus (TMV) were most frequently responsible for economic losses in pepper production (Abdalla et al., 1985; Agrios et al., 1983; Anderson and Corbett, 1957; Benner et al., 1985; Makkouk and Gumpf, 1974; Steepy and Averre, 1971; Steepy et al., 1967; Villalon, 1975; and Zitter, 1973). Other naturally occurring pepper viruses identified to be present in the United States at lower frequencies included alfalfa mosaic virus (AMV), aster ringspot virus (ARSV), potato virus X (PVX), tobacco ringspot virus (TRSV), and tomato spotted wilt virus (TSWV) (Abdalla et al., 1985; Anderson and Corbett, 1957; Makkouk and Gumpf, 1974; and Villalon, 1975).

In Michigan, reports of an increase in the incidence and severity of mosaic or virus-like symptoms have been received from commercial producers of pepper in recent years. In general, efforts to control aphid introduction and movement in the field have not been successful. Accurate virus identification and information regarding which viruses are most likely to be prevalent helps producers make informed decisions on disease management. In this study, a survey was conducted to determine the presence and prevalence of the four viruses most likely to be found in Michigan pepper fields, those being CMV, PVY, TEV, and TSWV. AMV, ARSV, PMV, PVX, and TRSV have not been found in the midwestern United States, and most common pepper

cultivars are now resistant to TMV (Sherf and Macnab, 1986); therefore, these viruses were not included in the survey. The natural distribution of the viruses, and their seasonal occurrence was also investigated.

MATERIALS AND METHODS

Field Survey Procedures

Surveys were conducted during the growing season (June-September) of 1989 and 1990 in six and five fields respectively, encompassing the four pepper-producing geographic localities in Michigan. These fields represented a total of 480 acres or 12.5% of the state pepper production area. The fields were selected prior to or at the time of planting. All fields had been planted with local or southern-grown transplants of common commercial fresh market and processing pepper cultivars (Bell Tower, Cheese, Harris, Jalapea, Jupiter, Keystone Resistant Giant, Marengo and Mayata). None of these cultivars are resistant to any of the viruses in the study.

Sample Collection

Prior to planting, transplants were tested for all viruses. In 1989, the fields were sampled four times during the growing season at approximately three week intervals. One field was sampled two additional times late in the season due to high incidence of CMV infection. In 1990, fields were sampled eight times during the growing season at two week intervals. The number of samples increased in 1990 because fields were sampled both earlier and later in the

growing season. Samples were collected along intersecting field diagonals and consisted of 50 randomly chosen leaves taken from shoot apices of 50 individual plants. Also, some leaves were collected from plants showing virus-disease symptoms as a separate sample. Additional pepper fields not included in the survey in which virus infection was suspected were sampled late in the growing season during 1989. Leaf samples were combined, placed in plastic bags, and transported to the laboratory on ice for testing.

Virus Identification Using ELISA

Leaf samples were weighed and ground in a 10:1 volume:weight ratio of extraction buffer which contained 2% Tween 20, 2% egg albumen, 2% polyvinyl pyrrolidone, and 0.13% sodium sulfite in 0.015M phosphate buffered saline, pH 7.2. Samples were then tested for the presence of viruses using the enzyme-linked immunosorbent assay technique (ELISA) described by Clark and Adams (1977). ELISA PathoScreen™ virus test kits were purchased from Agdia, Inc. (Elkhart, IN) and used following the instructions of the manufacturer. Strains of virus used for detection were: 1) crocus and vinca strain of CMV, 2) unknown strain of PVY, 3) unknown strain of TEV, and 4) lettuce strain of TSWV. Assay controls were sap from healthy pepper plants and sap from plants with known viruses. Samples having an absorbance reading (490 nm) of at least 3X the absorbance reading for sap from healthy plants were considered to be positive.

RESULTS

Over time, all four viruses assayed were detected in random samples from at least one or more of the geographic localities of pepper production in Michigan. TSWV was the only virus detected in transplants prior to planting. CMV, PVY, TEV, and TSWV were detected in 1989 and only CMV and TSWV were detected in 1990 in the fields sampled randomly. CMV was present in 4/6 and 1/7 fields in 1989 and 1990 respectively; PVY was present in 1/6 fields in 1989; TEV was present in 1/6 fields in 1989; TSWV was present in 3/6 and 2/7 fields in 1989 and 1990 respectively (Table 1.1). During the survey period, CMV was detected in all geographic locations sampled, TSWV was detected in locations 3 and 4, PVY was detected in locations 1 and 2, and TEV was detected only in location 1 (Fig. 1.1). In 1989, TSWV was first detected on 23 June, and PVY, CMV, and TEV were first detected 5 July, 10 July, and 31 July respectively. In 1990, TSWV was first detected on 6 July; CMV was not detected until 31 August (Fig. 1.2, 1.3).

From the four additional fields with suspected virus infection sampled, CMV was present in 2/4 and TSWV was present in 3/4 fields in 1989. PVY and TEV were not detected in these fields. The fields in which CMV and TSWV were detected were located in geographic locations 3 and 4 (Fig. 1.1).

DISCUSSION

The 1989 and 1990 survey results using ELISA support

Table 1. Identification of viruses in pepper survey samples using enzyme-linked immunosorbent assay (ELISA)

County	Locality	Field	CMV[a]	PVY	TEV	TSWV
1989						
Berrien	2	1	0/4	0/4	0/4	0/4
		2	2/5[b]	0/5	1/5	0/5
Gratiot	3	3	1/4	0/4	0/4	4/4
		4	0/4	0/4	0/4	4/4
Oceana	1	5	0/4	1/4	0/4	0/4
Saginaw	3	6	1/4	0/4	0/4	3/4
1990						
Berrien	2	2	0/8	0/8	0/8	0/8
Gratiot	3	3	0/7	0/7	0/7	5/7
Oceana	4	5	2/7	0/7	0/7	0/7
	5	12	0/8	0/8	0/8	0/8
Saginaw	3	6	0/8	0/8	0/8	6/8

[a] CMV = cucumber mosaic virus, PVY = potato virus Y, TEV = tobacco etch virus, TSWV = tomato spotted wilt virus

[b] No. of postive reactions/no. of samples tested

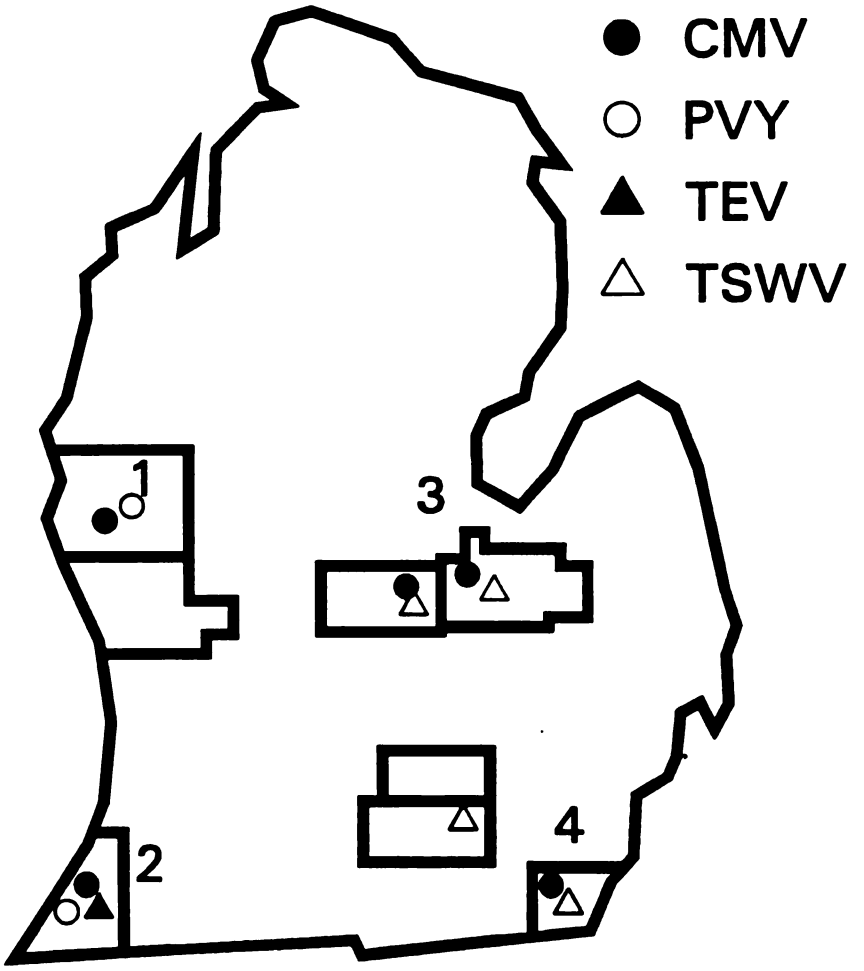


Figure 1.1 Distribution of cucumber mosaic (CMV), potato virus Y (PVY), tobacco etch (TEV), and tomato spotted wilt virus (TSWV) identified in survey and symptomatic pepper samples in 1989-1990.

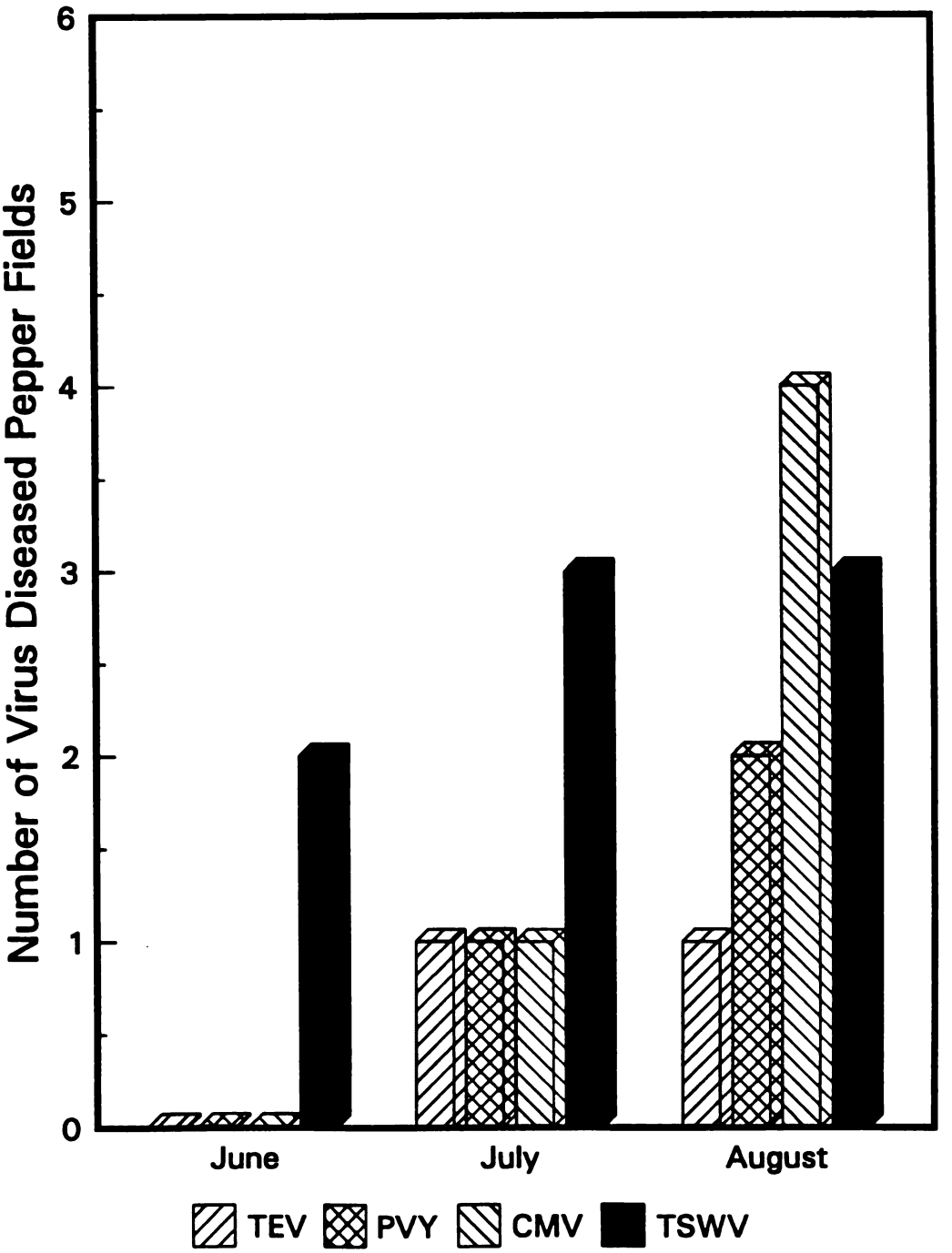


Figure 1.2 Seasonal occurrence of cucumber mosaic (CMV), potato virus Y (PVY), tobacco etch (TEV), and tomato spotted wilt virus (TSWV) in survey and symptomatic pepper samples in 1989.

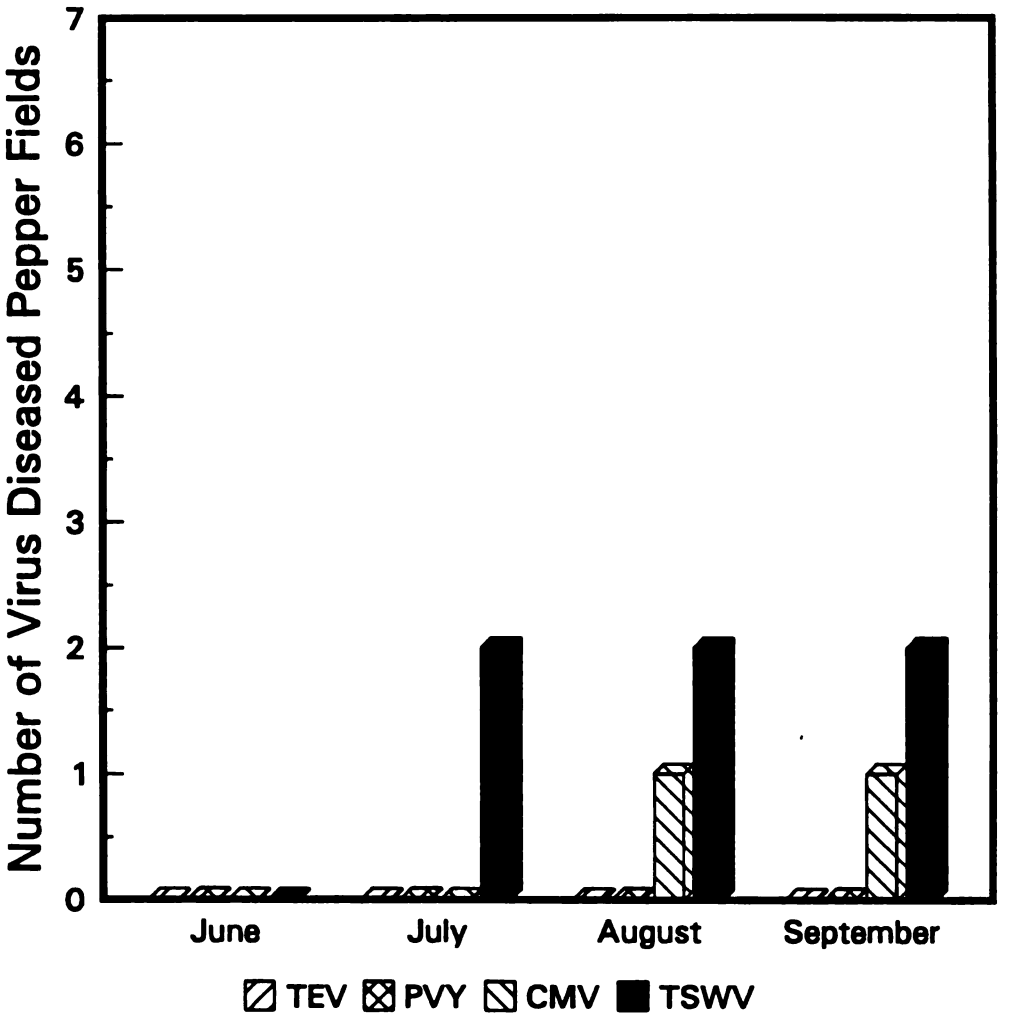


Figure 1.3 Seasonal occurrence of cucumber mosaic (CMV), potato virus Y (PVY), tobacco etch (TEV), and tomato spotted wilt virus (TSWV) in survey and symptomatic pepper samples in 1990.

the conclusion that the most widespread and principal virus disease of pepper in Michigan was caused by CMV. CMV and PVY were identified as the most predmoninant viruses of pepper in Massachussetts (Agrios et al., 1983), while PVY and TEV were identified as the most predominant viruses in pepper in California (Makkouk and Gumpf, 1974), Florida (Zitter, 1973), Georgia (Benner et al., 1985), New Jersey (Steepy et al., 1967), North Carolina (Steepy and Averre, 1971), and Texas (Villalon, 1975).

Plants with obvious disease symptoms exhibited large necrotic rings or oak-leaf markings on mature leaves and severe mosaic on the new growth. Symptoms on the fruit consisted of tan to necrotic spots, or yellowish concentric rings. Plants expressing these symptoms tested positive to either or both the crocus and vinca strain(s) of CMV.

Studies by Anderson (1959) identified virus-infected plant species bordering pepper fields as important sources of primary inoculum in initial infection of aphid-borne viruses by their vectors. They identified 16 plant species as reservoirs for CMV in Florida (Anderson, 1959). Many of the CMV-host reservoirs identified are common weed or crop plants in Michigan. CMV was predominantly detected in the latter part of the growing season with the earliest detection on 10 July in isolated plants within pepper plantings. The date of detection and increased incidence in the latter part of the growing season suggests that pepper transplants are not the source of CMV. The most likely

source of primary inoculum of CMV in Michigan is weed reservoir hosts bordering pepper fields. The increased CMV titer in reservoir plants and primary infected pepper plants, along with the movement of the aphid vectors into the fields probably accounts for the incidence of secondary spread of CMV in the latter part of the growing season. The distribution of CMV is facilitated by the number of aphid species (over 60) which are capable of vectoring the virus, and also by its naturally wide host range, (40 families which include 191 species) (Francki et al., 1979). Such factors as virus host reservoirs and the presence of efficient aphid vectors contribute to the survival and dissemination of CMV which is endemic in Michigan.

TSWV was detected in Michigan (1989) for the first time in pepper. TSWV was detected early in the growing season only in samples from plants purchased as transplants from suppliers in Georgia. In 1989, unusually high populations of the thrips Frankliniella occidentalis were known to have infested large areas of transplant production in Georgia. Pepper transplants were then shipped north with an undetermined percentage of plants infected with TSWV as well as thrips. Transplants from Georgia generally were symptomless prior to transplanting in Michigan. Transplant shock results in defoliation of transplants making it difficult to detect virus diseases until new growth is present to assay. In the 1990 survey, we were not able to detect TSWV until 6 July due to transplant shock defoliation

and unseasonally cold temperatures, which suppressed new growth on plants. In the majority of Michigan fields, the occurrence of TSWV was minor (<5% of the plant stand). In a few fields, however, TSWV infected up to 40% of the transplants. Field symptoms as observed in Michigan were severe stunting, in some cases almost complete cessation of growth, many plants did not produce any fruit; distortion and mottling of young leaves, and ringed patterns on mature leaves; necrotic markings were present on the fruit. Visual observation revealed that secondary spread of TSWV within infected fields was neglible. The lack of secondary spread may have been a result of low populations of the western flower thrips (F. occidentalis) transported with the transplants or migration of the thrips due to a lack of foliage or flowers to feed on.

TSWV is distributed worldwide largely because of its wide host range, the establishment of its main vector, the western flower thrips in new geographic areas, and movement of virus-infected plant material, especially in the floricultural and ornamental industries. Additionally, the ability of TSWV to form hybrid strains by the process of recombination has allowed its diverse strains to flourish in many environments.

Michigan has natural populations of the onion and celery thrips, which also vector TSWV, however, not as effectively as the western flower thrips. If the western flower thrips becomes established outside of greenhouses in

Michigan, TSWV could pose a serious threat to the vegetable industry. TSWV has been reported to cause severe economic losses in vegetable production in Hawaii (Milbrath and Cook, 1971).

In fields where PVY and TEV were detected, infection sites remained isolated within pepper plantings. PVY and TEV currently do not appear to seriously threaten Michigan pepper production, however, these viruses have caused major economic losses in California (Abdalla et. at, 1985, and Makkouk and Gumpf, 1974), Florida (Zitter, 1973), Georgia (Benner et. al, 1985) New Jersey (Steepy et. al, 1967), North Carolina (Steepy and Averre, 1971), Texas (Villalon, 1975), and in other countries (Conti and Masenga, 1977, and Stover, 1951). Therefore, the possibility exists that PVY and TEV may become important pathogens on pepper in Michigan in the future.

Viruses in field-infected pepper were difficult to identify based on symptom development. Plants showing classic symptoms of CMV infection, necrotic rings or oak-leaf markings on mature foliage could be diagnosed. Southern transplants infected early with TSWV could be detected based on the severe stunting and formation of concentric rings on the foliage, however, if infection took place later in the season, positive identification would be difficult. The identification of pepper viruses based on field symptom expression alone is not accurate due to symptom variability. The ELISA technique was found to be

efficient and reliable for virus disease detection in pepper field surveys.

LIST OF REFERENCES

Abdalla, O.A., Desjardins, P.R., and Dodds, J.A. 1985. Survey of pepper viruses in California by the ELISA technique. Plant Disease. 75:1311.

Agrios, G.N., Walker, M.E., Ferro, D.N., and Corredor, D. 1983. Virus incidence and spread in pepper field plots treated with reflective mulch and oil. Phytopathology 73:361.

Anderson, C.W. 1959. A study of field sources and spread of five viruses of peppers in central Florida. Phytopathology 49:97-101.

Anderson, C.W. and Corbett, M.K. 1957. Virus diseases of peppers in central Florida survey results 1955. Plant Dis. Reptr. 41:143-147.

Benner, C.P., Kuhn, C.W., Demski, J.W., Dobson, J.W., Colditz, P., and Nutter, F.W. Jr. 1985. Identification and incidence of pepper viruses in northeastern Georgia. Plant Dis. 69:999-1001.

Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Conti, M. and Masenga, V. 1977. Identification and prevalence of pepper viruses in northwest Italy. Phytopathol. Z. 90:212-222.

Francki, R.I.B., Mossop, D.W., and Hatta, T. 1979. Cucumber mosaic virus. Descriptions of Plant Viruses, NO. 213. Commonwealth Mycological Institute, Association of Applied Biologists, Kew, Surrey, England.

Makkouk, K.M. and Gumpf, D.J. 1974. Further identification of naturally occurring virus diseases of pepper in California. Plant Dis. Reptr. 58:1002-1006.

Milbrath, G.M. and Cook, A.A. 1971. Virus diseases of pepper (Capsicum sp.) in Hawaii. Plant Dis. Reptr. 55:783-785.

Sherf, A.F. and Macnab, A.A. 1986. Vegetable diseases and their control. pp 520-536. John Wiley & Sons, New York, N.Y.

Steepy, T.L. and Averre, C.W. 1971. Prevalence of pepper viruses in North Carolina. Plant Dis. Reptr. 55:751.

Steepy, T.L., Lewis, G.D., and Varney, E.H. 1967. Identification of the viruses affecting commercial varieties of pepper (Capsicum annuum) in New Jersey. Plant Dis. Reptr. 51:709-711.

Villalon, B. 1975. Virus diseases of bell peppers in south Texas. Plant Dis. Reptr. 59:858-862.

Zitter, T.A. 1973. Further pepper virus identification and distribution studies in Florida. Plant Dis. Reptr. 57:991-994.

CHAPTER 2

REGENERATION OF PEPPER (*CAPSICUM ANNUUM*) FROM PRIMARY LEAF EXPLANTS

ABSTRACT

Growth regulator combinations and silver nitrate concentrations were examined for their effect on shoot regeneration of 58 pepper genotypes. Primary leaf explants from *in vitro* seedlings were cultured on a revised Murashige and Skoog medium amended with auxin, cytokinin and 1.6% glucose. Combinations of of indole-3-acetic acid (IAA), 0-5 mg/L, and 6-benzylaminopurine (BAP), 0-5 mg/L, were tested to determine the most effective medium for shoot initiation. When cultured on MS amended with specific growth regulator combinations, the *Capsicum* genotypes examined showed varied shoot initiation and elongation responses. No specific growth regulator combination resulted in shoot regeneration of all genotypes tested. Ten mg/L silver nitrate resulted in the best shoot and leaf differentiation and also reduced hard callus formation. Differences in morphogenetic response of individual genotypes were evaluated on a single shoot initiation medium. Overall, whole plants were regenerated from 19 of 58 genotypes examined. Based on these experiments, a reproducible regeneration system for

pepper was developed with a total of over 500 plants regenerated.

INTRODUCTION

Although studies examining the manipulation of pepper *in vitro* have been moderately successful, shoot regeneration has been restricted to only a few genotypes. In some of the previous studies data presented concerned the formation of shoot buds on explants, but not shoot regeneration frequencies. To date, the regeneration of whole pepper plants from somatic cells is not sufficiently routine for the application of technologies such as somaclonal variation and genetic transformation.

Manipulative variables that have been studied in pepper regeneration systems include culture medium constituents, length of photoperiod, temperature, and explant age and source (Agrawal et al., 1989; Fari and Czako, 1981; Gunay and Rao, 1978; Phillips and Hubstenberger, 1985; Sripichitt et al., 1987). Previous studies involving pepper regeneration have utilized Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), but differed on the source of explant and in regard to the function and importance of auxin and cytokinin in the regeneration process.

The goal of this study was to develop a protocol for efficient regeneration of whole plants from primary leaf explants of *Capsicum* spp. The objectives of this research were: 1) to examine the role of growth hormones in pepper shoot initiation and elongation; 2) determine the effect of

silver nitrate on morphogenetic responses of pepper; 3) examine the effect of environmental conditions on *in vitro* pepper culture; and 4) screen *Capsicum* genotypes to test for regeneration capacity.

MATERIALS AND METHODS

Capsicum genotypes

The *Capsicum* genotypes surveyed encompass a wide range of vegetative morphologies and horticultural qualities including traditional open-pollinated cultivars as well as hybrids. Most of the *C. annuum* cultivars examined are commonly grown by commercial producers in the United States. The plant introductions (PI) used in the study were obtained from the Southern Regional Plant Introduction Station (Experiment, GA).

In vitro seedlings

Seeds of *C. annuum* were surface sterilized by immersion in 95% ethanol for 2 min followed by 30 min in 50% aqueous (v/v) commercial bleach (2.62% sodium hypochlorite) containing 1-2 drops/250 ml Tween 20 (Sigma Chemical Co., St. Louis, MO). The seeds were rinsed three times and imbibed for 30 min in sterile distilled water. Nine seeds per container were sown in GA-7 vessels (Magenta Corp., Chicago, IL) containing 50 ml of revised MS medium (Murashige and Skoog, 1962) modified by omitting the growth hormones, reducing the amount of NH_4NO_3 to 165 mg and Bacto agar (Difco Laboratories, Detroit, MI) to 8 g, and increasing the thiamine·HCl to 0.2 mg per liter. The pH of

the medium was adjusted to 5.8 using 1N NaOH or 1N HCl prior to autoclaving. Vessels were placed in a growth room at 25 C under cool-white fluorescent lights supplying 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on a 16 hr photoperiod to allow seed germination and seedling growth.

Source of explants

For all experiments explants (5 x 10 mm) were excised from fully expanded primary leaves of 4-6 wk-old seedlings (Fig. 2.1), resulting in 2-4 explants per leaf, depending on the genotype used.

Culture medium

For all experiments MS medium was modified by changing the carbon source to glucose (16 g), increasing the amount of nicotinic acid, pyridoxin·HCl and thiamine·HCl to 1.0 mg, and decreasing the amount of Bacto agar to 8 g per liter. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl before the agar was added, and 25 ml of medium was dispensed into 100 x 15 mm petri dishes. This modified MS medium will be designated MSc medium.

Rating system

After 30 days in culture, the explants were rated for shoot regeneration response. A rating system of 1-5 was used: 1 = no callus formation, 2 = callus formation, 3 = callus and shoot bud formation, 4 = callus, shoot bud and leaf formation, and 5 = callus and elongated shoot formation (Fig. 2.2). This rating system was employed in all

Figure 2.1 Four to six week-old seedlings of pepper (Capsicum annuum) grown in vitro.

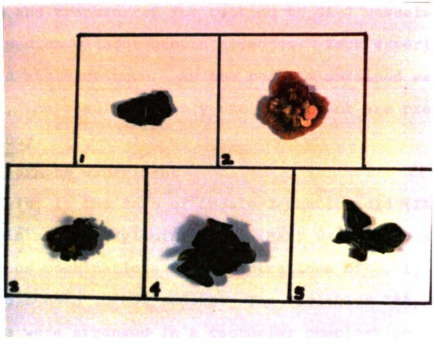


Figure 2.2 A rating system for the regeneration response of pepper (Capsicum annuum) from leaf explants after 30 days in culture. Regeneration rated on 1-5 scale; 1 = no callus formation, 2 = callus formation, 3 = callus/shoot bud formation, 4 = shoot bud/leaf formation, and 5 = elongated shoot formation.

experiments. Explants with a rating of 4 or 5 were subcultured for shoot elongation to GA-7 vessels in 50 ml of MSc medium supplemented with 2 mg/L or 4 mg/L of BAP or 0.1 mg/L NAA depending on the experiment. Elongated shoots were excised and transferred for rooting to GA-7 vessels in 50 ml of MSc medium without growth hormones. Each experiment was repeated at least once. As the results obtained were very similar, the results of only one experiment are presented in this study.

Growth hormone experiment

Auxin, in the form of indole-3-acetic acid (IAA), and cytokinin as 6-benzylaminopurine (BAP) were added alone or in various combinations at concentrations of 0, 1, 2, 3, 4, and 5 mg/L each to MSc medium. The variables IAA, BAP and genotype were arranged in a factorial combination in a completely random design. The three C. annuum genotypes used in the experiment were PI178849 and the cultivars Mayata and Paprika. Three explants were placed per petri dish on MSc medium and incubated at 26 C under cool-white fluorescent lights supplying 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on a 16 hr photoperiod (Figure 2.3). Eighteen explants were employed in each growth hormone treatment.

Silver nitrate experiment

MSc medium was amended with IAA (3 mg), BAP (4 mg) and silver nitrate at concentrations of 1, 10, 20, and 30 mg per liter. The variables silver nitrate concentration and genotype were arranged in a factorial combination in a

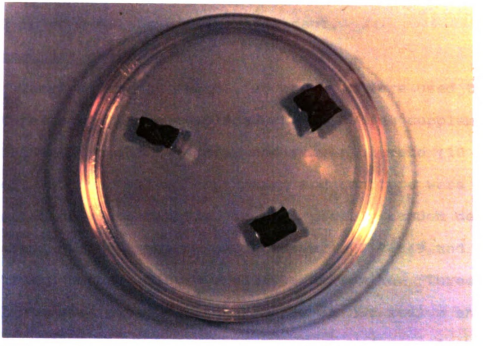


Figure 2.3 Primary leaf explants (5 x 10 mm) of pepper (Capsicum annuum) cultured on Murashige and Skoog regeneration medium.

completely random design. The four C. annuum genotypes examined were PI178849 and the cultivars Bell Star, Mayata, and Memphis. Three explants were placed per petri dish on MSc medium and cultured as above. Forty eight explants were used in each silver nitrate treatment.

Temperature experiment

Temperatures of 24, 26, 28 and 30 C were used to culture pepper explants placed on MSc medium supplemented with IAA (3 mg), BAP (4 mg), and silver nitrate (10 mg) per liter. The variables temperature and genotype were arranged in a factorial combination in a completely random design. The four C. annuum genotypes used were PI178849 and the cultivars Espana, Mission Belle, and Paprika. Three explants were placed per petri dish on MSc medium and incubated at the treatment temperature under cool-white fluorescent lights supplying 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on a 16 hr photoperiod. Forty eight explants were employed in each temperature treatment.

Shoot elongation experiment

Primary leaf explants from PI178849 were cultured on MSc medium supplemented with IAA (3mg), BAP (4 mg) and silver nitrate (10 mg) per liter. After approximately 30 days in culture explants with a rating of 4 or 5 were transferred to MSc medium supplemented with BAP (4 mg) or NAA (0.1 mg) per liter. The number of explants forming elongated shoots and the number of elongated shoots per explant were counted. Elongated shoots were excised and

rooted in MSc medium without growth hormones.

Genotype experiment

Primary leaf explants of Capsicum genotypes Bell Star, California Wonder, Crispy Hybrid, Jalapeno, Jupiter, Marengo, Mayata, Memphis, PI178849, and PIP were cultured on MSc medium supplemented with IAA (3 mg), BAP (4 mg), and silver nitrate (10 mg) per liter. Three explants were placed per petri dish on MSc medium and incubated at 28 C under cool-white fluorescent lights supplying 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on a 24 hr photoperiod. Twenty four explants per genotype were employed. Cultures were transferred onto shoot elongation medium containing 0.1 mg/L NAA.

During this study, 58 Capsicum genotypes were screened on MSc medium for their shoot regeneration capacity.

Acclimation of somaclones

Regenerated pepper somaclones were planted in moistened soilless peat mix (Baccto Professional Planting Mix, Michigan Peat Co., Houston, TX) in 2 or 3" plastic pots and placed in plastic bags. Plants were grown at room temperature (24 C) under cool white fluorescent lights supplying 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16 hr photoperiod. Plants were gradually acclimated by opening the plastic bag for increased periods of time. After plants were acclimated they were placed in the greenhouse and allowed to self pollinate and form seeds (Fig. 2.4).

RESULTS

Growth hormone experiment

An analysis of variance (Table 2.1) shows that the factors genotype, IAA, and BAP were significant at P = 0.01 level. There was also a significant interaction (P = 0.05) between factors, indicating the factors were not independent.

The following visual observations were made while rating cultures. Without the presence of auxin (IAA) and cytokinin (BAP) in the MSc medium explants of all Capsicum genotypes examined showed no morphogenetic response in culture. MSc medium containing 1-5 mg/L IAA resulted in callus and in some cases root formation on primary leaf explants. With only the amendment of cytokinin (BAP) in the MSc medium primary leaf explants of all Capsicum genotypes examined formed callus and shoot buds when BAP was included at 3-5 mg/L. When auxin (IAA) and cytokinin (BAP) were used in combination, morphogenetic responsiveness increased, however, response varied among genotypes with different IAA and BAP combinations. IAA in a range of 0-5 mg/L and BAP in a range of 3-5 mg/L resulted in better regeneration means for the genotypes tested.

Silver nitrate experiment

An analysis of variance (Table 2.2) shows that the factors genotype and silver nitrate were significant at the level P = 0.05 and 0.01, respectively. There was no significant interaction between the two factors. A Duncan's

Figure 2.4 Regenerated somaclones of pepper (Capsicum annuum PI178849) from primary leaf explants.

Table 2.1. Analysis of variance of a 3 X 6 X 6 factorial experiment concerning the effects of indole-3-acetic acid, 6-benzylaminopurine, and pepper genotype on shoot regeneration.

Source	df	mean square	F value
Genotype	2	7.35[a]	37.43*
IAA	5	0.97	4.96*
Genotype x IAA	10	0.30	1.54
BAP	5	12.34	62.82**
Genotype x BAP	10	0.38	1.91*
IAA x BAP	25	0.36	1.81*
Genotype x IAA x BAP	50	0.33	1.66*
Error	108	0.20	

[a]Regeneration rated on 1-5 scale; 1 = no callus formation, 2 = callus formation, 3 = callus/shoot bud formation, 4 = shoot bud/leaf formation, and 5 = elongated shoot formation.

*Significant difference at P = 0.05
**Significant difference at P = 0.01

multiple range test (Table 2.3) revealed that all genotypes responded the best when 10 mg/L of silver nitrate was amended to the MSc medium. Although genotype and silver nitrate treatment means were not significantly different, there were qualitative differences seen in the cultures (Fig. 2.5). Visual observations of the cultures showed a decrease in callus formation and/or the callus formed was more friable. Also, shoot buds and leaves were more differentiated with the presence of 10 or 20 mg/L silver nitrate in the medium. At 30 mg/L silver nitrate, some of the leaves looked thicker, abnormal in shape, and water-soaked.

Temperature experiment

An analysis of variance revealed that the factors genotype and temperature were not significant at the level P = 0.05 (Table 2.4) A Duncan's multiple range test showed similar regeneration means in all genotypes examined at 28 C (Table 2.5). Although treatment means were not significantly different, visual observations revealed shoot and leaf structures were more differentiated after approximately 30 days in culture at 28 C than at lower temperatures (Fig. 2.6). At 30 C some of the leaves produced on cultures began to turn yellow, showing signs of stress before being subcultured.

Shoot elongation experiment

Both BAP (4 mg/L) and NAA (0.1 mg/L) were effective in promoting shoot elongation of shoot buds initiated on

Table 2.2. Analysis of variance of a 4 X 4 factorial experiment concerning the effect of silver nitrate and pepper genotype on shoot regeneration.

Source	df	mean square	F value
Genotype	3	4.72[a]	15.44**
Silver nitrate concentration	3	0.86	2.81*
Genotype x silver nitrate concentration	9	0.19	0.61
Error	48	0.31	

[a]Regeneration rated on 1-5 scale; 1 = no callus formation, 2 = callus formation, 3 = callus/shoot bud formation, 4 = shoot bud/leaf formation, and 5 = elongated shoot formation.

*Significant difference at P = 0.05
**Significant difference at P = 0.01

Table 2.3. Differences in the effect of silver nitrate concentration amended to Murashige and Skoog medium on shoot regeneration of four pepper genotypes[z].

Genotype	Treatment Silver Nitrate (mg/L)	Treatment mean	
PI178849	0	2.4	c[a]
PI178849	10	3.2	abc
PI178849	20	2.7	c
PI178849	30	2.8	bc
Bell Star	0	3.6	ab
Bell Star	10	3.9	a
Bell Star	20	3.9	a
Bell Star	30	3.8	a
Memphis	0	2.9	bc
Memphis	10	4.0	a
Memphis	20	3.7	ab
Memphis	30	3.8	a
Mayata	0	4.0	a
Mayata	10	4.0	a
Mayata	20	4.0	a
Mayata	30	4.0	a

[z]Means within the column followed by the same letter do not differ by Duncan's multiple range test (P = 0.05).

[a]Regeneration rated on 1-5 scale; 1 = no callus formation, 2 = callus formation, 3 = callus/shoot bud formation, 4 = shoot bud/leaf formation, and 5 = elongated shoot formation.

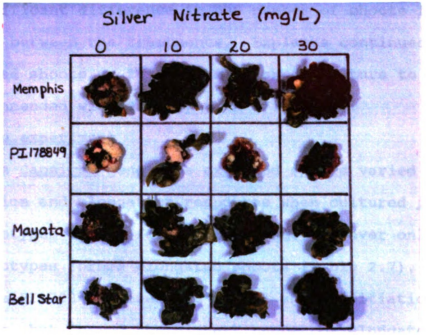


Figure 2.5 Qualitative differences in the response of four pepper genotypes cultured on Murashige and Skoog medium supplemented with 0, 10, 20, and 30 mg/L silver nitrate.

primary leaf explants of Capsicum genotypes. A larger percentage of PI178849 explants formed elongated shoots after approximately 2 wk in culture on the NAA treatment (63%) than the BAP treatment (12%) (Table 2.6). There was no significant difference in the number of shoots formed per explant between the treatments. Explants continued to form elongated shoots up to 56 days after subculture to MSc medium amended with 0.1 mg/L NAA (Fig. 2.7).

Genotype experiment

The Capsicum genotypes examined showed varied shoot initiation and elongation responses when cultured in vitro. All genotypes tested formed shoot buds, however only four of the genotypes formed elongated shoots (Table 2.7). The genotype PI178849 showed the lowest shoot initiation response, but formed the highest number of elongated shoots.

Of the 50 C. annuum genotypes cultured on MSc medium, 13 formed elongated shoots (Table 2.8). Of the 8 genotypes of other Capsicum species, 6 formed elongated shoots (Table 2.8) when cultured on MSc medium.

DISCUSSION

Successful and efficient regeneration in plant tissue culture is dependent on the thorough understanding of how the plant species, explant source, composition of the culture medium and environmental conditions affect shoot initiation and elongation. Among these factors, the composition of the culture medium and especially growth regulators play a key role in shoot initiation, elongation

Table 2.4. Analysis of variance of a 4 X 4 factorial experiment concerning the effect of temperature on shoot regeneration of four pepper genotypes cultured on Murashige and Skoog medium supplemented with 3 mg/L indole-3-acetic acid, 4 mg/L 6-benzylaminopurine, and 10 mg/L silver nitrate.

Source	df	mean square	F value
Genotype	3	0.655[a]	2.20 ns
Temperature	3	0.670	2.25 ns
Genotype x temperature	9	0.218	0.73 ns
Error	48	0.297	

[a]Regeneration rated on 1-5 scale; 1 = no callus formation, 2 = callus formation, 3 = callus/shoot bud formation, 4 = shoot bud/leaf formation, and 5 = elongated shoot formation.

ns = nonsignificant at P = 0.05 or 0.01

Table 2.5. Differences in the effect of temperature on shoot regeneration of four pepper genotypes cultured on Murashige and Skoog medium supplemented with 3 mg/L indole-3-acetic acid, 4 mg/L 6-benzylaminopurine, and 10 mg/L silver nitrate[z].

Genotype	Treatment Temperature (C)	Treatment mean
PI178849	24	2.9 bc[a]
PI178849	26	3.4 ab
PI178849	28	4.0 a
PI178849	30	3.3 ab
Mission Belle	24	3.5 ab
Mission Belle	26	3.7 a
Mission Belle	28	3.9 a
Mission Belle	30	3.8 a
Paprika	24	3.8 a
Paprika	26	4.0 a
Paprika	28	3.8 a
Paprika	30	3.8 a
Espana	24	3.6 ab
Espana	26	3.5 ab
Espana	28	3.9 a
Espana	30	4.0 a

[z]Means within the column followed by the same letter do not differ by Duncan's multiple range test (P = 0.05).

[a]Regeneration rated on 1-5 scale; 1 = no callus formation, 2 = callus formation, 3 = callus/shoot bud formation, 4 = shoot bud/leaf formation, and 5 = elongated shoot formation.

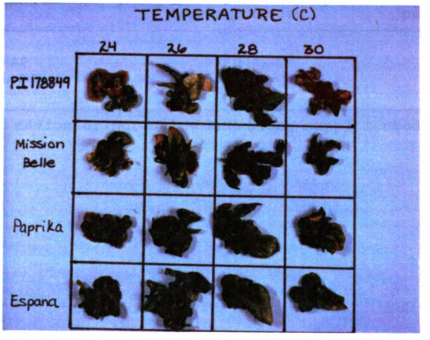


Figure 2.6 Qualitative differences in the response of four pepper genotypes cultured on Murashige and Skoog medium supplemented with 3 mg/L indole-3-acetioc acid, 4 mg/L 6-benzylaminopurine, and 10 mg/L silver nitrate at 24, 26, 28, and 30 C.

Table 2.6. Differences in the percentage of explants forming shoots and the number of shoots per explant of pepper genotype cultured on Murashige and Skoog medium supplemented with 0.1 mg/L naphthaleneacetic acid or 4 mg/L 6-benzylaminopurine.

Medium[a]	Explants forming shoots	Shoots per explant
4 mg/L BAP	4/30(12%)	3.5
0.1 mg/L NAA	19/30(63%)	3.7

[a]A basal medium of Murashige and Skoog supplemented as stated.

Figure 2.7 Shoot elongation of pepper (Capsicum annuum PI178849) on Murashige and Skoog medium amended with 0.1 mg/L naphthaleneacetic acid.

Table 2.7. Shoot initiation and elongation responses of Capsicum genotypes when cultured on Murashige and Skoog medium supplemented with 3 mg/L indole-3-acetic acid, 4 mg/L 6-benzylaminopurine, and 10 mg/L silver nitrate.

Genotype	No. explants forming shoot buds	No. explants forming elongated shoots
Bell Star	21/24	1/24
California Wonder	7/24	0/24
Crispy Hybrid	11/24	1/24
Jalapeno	14/24	2/24
Jupiter	5/24	0/24
Marengo	9/24	0/24
Mayata	12/24	0/24
Memphis	10/24	0/24
PI178849	5/24	2/24
PIP	12/24	0/24

Table 2.8. Shoot regeneration capacity of Capsicum genotypes when cultured on Murashige and Skoog medium supplemented with 3 mg/L indole-3-acetic acid, 4 mg/L 6-benzylaminopurine, and 10 mg/L silver nitrate.

Genotype	Regeneration[a]
C. annuum	
Annabelle	-
Bell Star	+
Bell Tower	-
Big Belle	-
California Wonder	-
Cadice	-
Calumet	-
Capri	+
Crispy Hybrid	+
Cuban	-
Early California Wonder	-
Espana	-
Giant Ace	-
Gloria	-
Green Boy	-
Gloria Pepper	-
Hidalgo	-
Honeybelle	-
Hybrid Hot	-
Jalapeno	+
Jupiter	+
Keystone Resistant Giants	+
Lady Bell	-
Marengo	+
Mayata	+
Memphis	+
Midal Pepper	-
Mission Belle	-
Mr. Bell	-
Orobelle	-
PI173769	-
PI174114	-
PI178849	+
PI201571	-
PI206949	-
PIP	+
Paprika	-
Pepper Mexibell	+
Pimiento Perfection	+
Purple Bell	-

Genotype	Regeneration[a]
Ranger	-
RioGrande Gold	-
Scarlet Pendant	-
Sironofi	-
Skipper	-
Summer Sweet Brand	-
Sweet Belle	-
Tambel-2	-
Yolo Wonder	-
Yolo Wonder L	-
C. baccatum	
PI238061	+
PI281407	+
PI439383	+
C. chacoense	
PI260435	+
C. chinense	
PI360724	+
C. frutescens	
PI358968	+
C. practermissum	
PI260595	-
C. pubescens	
PI355812	-

[a] + = regeneration of whole pepper plants
- = regeneration of whole pepper plants did not occur on the medium used

and rooting. In the present study, no one specific combination of IAA and BAP resulted in optimal shoot initiation in primary leaf explants of all Capsicum genotypes tested. The genotypes initiated shoots when IAA was used at 0-5 mg/L and BAP was used at 3-5 mg/L. Concentrations of 3 mg/L IAA and 4 mg/L BAP were chosen for the shoot initiation medium since all genotypes examined in the experiment formed shoot buds to some extent at these concentrations. The results of this study support previous reports concerning the importance of auxin and cytokinin in regeneration of pepper in vitro (Fari and Czako, 1981; Gunay and Rao, 1978; Phillips and Hubstenberger, 1985). These results disagree with other reports stating that IAA is inhibitory to shoot initiation in pepper (Agrawal et al., 1989; Sripichitt et al., 1987).

NAA at 0.1 mg/l induced shoot elongation with some Capsicum genotypes. Agrawal et al. (1989) reported the importance of NAA in pepper shoot elongation, however, NAA at 0.1 mg/L was not sufficient for elongation of all genotypes examined in this study. BAP at 2 or 4 mg/L also induced shoot elongation in some genotypes (data not shown).

The production of ethylene in plants is induced by wounding and external factors such as auxin. Wounding and auxin are factors which are present in most in vitro tissue culture systems. Induction of ethylene production in plants can in turn bring about important physiological consequences (Yang and Hoffman, 1984). Exogenously applied ethylene or

ethylene precursors inhibited shoot initiation in tobacco callus cultures (Huxter et al., 1981). Silver ions are potent inhibitors of ethylene action in plants (Purnhauser et al., 1987). Silver nitrate effectively promoted shoot regeneration in wheat and tobacco cultures, which previously had poor regeneration ability (Purnhauser et al., 1987). Silver nitrate did not affect shoot initiation in Capsicum leaf explants; however, shoots and leaves were more developed. Also, callus formation was reduced or the callus formed was more friable. Callus overgrowth suppressed shoot primordia in 58-62% of Capsicum cultures in a study by Fari and Czako (1981). Hard callus overgrowth was observed in Capsicum cultures on medium lacking silver nitrate. Because of positive effects on in vitro culture of Capsicum spp., 10 mg/L silver nitrate became a routime addition to the shoot initiation medium.

Leaf tissue of pepper has been used as an explant source in previous regeneration studies. However, explants were obtained from older plants grown in a greenhouse (Phillips and Hubstenberger, 1985), or from field grown 3-to-4 month old plants (Agrawal et al., 1989). Also, in these earlier works the data reported involves shoot bud formation on leaf explants and not data on shoot elongation or whole plant regeneration (Fari and Czako, 1981; Gunay and Rao, 1978; Phillips and Hubstenberger, 1985). The present study reports whole plant regeneration from primary leaf explants, and includes data on the number of explants

forming elongated shoots and the number of elongated shoots formed per explant.

The shoots regenerated from primary leaf explants of pepper are adventitious shoots. Microscopic observation revealed that the shoot primordia were differentiated directly on the explants around the cut edges of the leaf. This observation is in agreement with the histological evidence provided by Agrawal et al. (1989).

LIST OF REFERENCES

Agrawal, S., Chandra, N., and Kothari, S.L. 1989. Plant regeneration in tissue cultures of pepper (Capsicum annuum L. cv. mathania). Plant Cell Tiss. Org. Cult. 16:47-55.

Fari, M., and Czako, M. 1981. Relationship between position and morphogenetic response of pepper hypocotyl explants cultured in vitro. Sci. Hortic. 15:207-213.

Gunay, A.L., and Rao, P.S. 1978. In vitro plant regeneration from hypocotyl and cotyledon explants of red pepper (Capsicum). Plant Sci. Lett. 11:365-372.

Huxter, T.J., Thorpe, T.A., and Reid, D.M. 1981. Shoot initiation in light- and dark-grown tobacco callus: the role of ethylene. Physiol. Plant. 53:319-326.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473-496.

Phillips, G.C., and Hubstenberger, J.F. 1985. Organogenesis in pepper tissue cultures. Plant Cell Tiss. Org. Cult. 4:261-269.

Purnhauser, L., Medgyesy, P., Czako, M., Dix, P.J., and Marton, L. 1987. Stimulation of shoot regeneration in Triticum aestivum and Nicotiana plumbaginifolia Viv. tissue cultures using the ethylene inhibitor $AgNO_3$. Plant Cell Rep. 6:1-4.

Sripichitt, P., Nawata, E., and Shigenaga, S. 1987. In vitro shoot-forming capacity of cotyledon explants in red pepper (Capsicum annuum L. cv. Yatsufusa). Japan. J. Breed. 37:133-142.

Yang, S.F., and Hoffman, N.E. 1984. Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant Physiol. 35:155-189.

CHAPTER 3

SCREENING THE R1 GENERATION WITH CUCUMBER MOSAIC VIRUS

ABSTRACT

Four hundred plants were regenerated from primary leaf explants of pepper (Capsicum annuum PI178849). R1 generation lines of the somaclones were assessed under growth chamber and greenhouse conditions for variation in response to cucumber mosaic virus (CMV) using mechanical and natural aphid vector inoculation techniques. The screening of R1 generation lines for resistance to CMV revealed extensive variation in response among the lines and also within the parental control line. There was a poor correlation between visual ratings for symptom development and assessment using ELISA for CMV infection. R1 lines and parental controls contained a high proportion of plants which did not develop symptoms (escapes). Plants remaining uninfected were visually observed in the parent and R1 generation lines during the experiment. In view of the small sample size and the fact that parental controls also contained escapes, no significance was attached to this. A total of 2/95 lines in the study were not infected, however, repeated inoculation of these lines resulted in infection.

INTRODUCTION

Traditional methods of plant breeding in pepper (Capsicum spp.) have yielded few cultivars presently resistant to cucumber mosaic virus (CMV); none that are widely used in commercial production (Barrios et al., 1971). Traditional plant breeders utilize many sources of variability such as land races and wild species for input of new genes. Somaclonal variation holds promise as an alternative source for genetic variation in plant breeding (Larkin and Scowcroft, 1981). Somaclonal variants of plant species generally range from highly-susceptible to highly-resistant when challenged with disease organisms to which the plant was originally susceptible (Brown et al., 1986; Larkin, 1981). The phenomenon of somaclonal variation has been utilized in selecting tomato somaclones resistant to tobacco mosaic virus (Barden, 1986), lettuce somaclones with reduced susceptibility to lettuce mosaic virus (Brown et al., 1986), and sugarcane subclones with a greater degree of resistance to Fiji virus (Krishnamurthi, 1974).

The objectives of this study were 1) to determine whether variation in disease response to CMV occurred in pepper somaclones regenerated from leaf explants and 2) to compare the efficiency of aphid versus mechanical transmission of CMV to R1 pepper lines in a screening system.

MATERIALS AND METHODS

Virus

A vinca strain of CMV was provided by Dr. Robert Davis (Agdia, Inc., Elkhart, IN). This strain of CMV was used because it was virulent on pepper and was used by Agdia, Inc. to develop the CMV pathoscreen kit, which was employed in this study. The virus was increased in PI178849 pepper plants for use in inoculations. The vinca strain of CMV will be designated as CMVvi hereafter.

R1 seedlings

Seeds were collected from all mature self-pollinated fruits of each somaclonal plant and pooled. Fifty seeds from the parent and each somaclone were sown in a soilless peat mixture in 3" plastic pots covered with clear plastic wrap. The seeds were allowed to germinate at room temperature (24 C) under cool-white fluorescent lights supplying 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Thirty-six parent seedlings and twenty-four R1 seedlings of each somaclone were transplanted in soilless peat mixture in 8 oz styrofoam cups (Dart Container Corp, Mason, MI) at 12-18 days after sowing.

Inoculation of R1 seedlings

Twelve seedlings from the PI178849 parent and each R1 line were used in each treatment in studies screening for resistance to CMVvi. The two uppermost leaves of seedlings were inoculated between 24-31 days after sowing. Controls consisted of both inoculated and healthy parent plants. Seedlings of the parent and R1 lines were reinoculated if

they tested negative to CMV upon ELISA assay. These seedlings were subsequently observed visually for symptom development 14 and 28 days after mechanical and aphid inoculations respectively.

Mechanical inoculation

Leaves of seedlings were dusted with carborundum and rub-inoculated with a cotton swab dipped in a solution containing 1 g fresh weight of CMVvi infected pepper tissue which was ground in 10 ml of 0.2 M Na-phosphate buffer pH 7.0 using a mortar and pestle. The leaves were allowed to air dry and were rinsed with distilled water to remove excess carborundum and virus inoculum. After inoculation, the plants were grown in a growth chamber at 27 C under cool-white fluorescent lights at 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Aphid inoculation

Green peach aphids (Myzus persicae) were obtained from Dr. Donald Ramsdell. Aphids were reared in cages on CMVvi-infected pepper plants. Five aphids that had been reared continously on infected pepper plants were transferred to each seedling and allowed a 48 hr inoculation access period after which aphids were removed or killed. After inoculation, the plants were grown in a greenhouse under spring weather conditions.

Virus screening of R1 pepper lines using ELISA

Assays were done at approximately 14 and 28 days after mechanical and aphid inoculations, respectively. This time frame was determined necessary based on symptom development

and ELISA studies using mechanical versus aphid inoculation techniques. The uppermost leaf tissue (0.2 - 0.5 g) was placed in a plastic bag and ground at a 1:10 dilution in extract buffer. Sample aliquots of 100 μl were loaded into microtiter plates, 2 wells per sample and subjected to ELISA according to the protocol supplied by the manufacturer (Agdia, Inc., Elkhart, IN).

RESULTS

Virus infection was assessed visually for symptom expression and assessed using ELISA. Symptoms were rated on a scale of 0 - 2 where 0 = plants without symptoms, 1 = plants with indistinct symptoms, and 2 = plants with distinct, characteristic symptoms. Plants with a rating of 1 or 2 were considered CMV-positive. Plants were considered CMV-positive using ELISA if their absorbance value (490 nm) was 3X the mean of the healthy control.

Each of 95 R1 generation lines were screened with CMV using mechanical and aphid-inoculation techniques. The visual rating and ELISA absorbance values were converted into percentage of CMV-positive plants (Fig. 3.1 - 3.7). Extensive variation was observed among and within the parent and R1 lines. Also, variation was observed between the inoculation techniques and between the assessment techniques. Among the seven experiments, infection in the parental line monitored with ELISA varied from 0 to 67% and 0 to 92% using the mechanical inoculation and aphid inoculation techniques respectively. A total of 93/95 lines

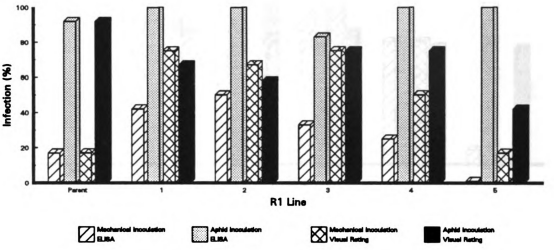


Figure 3.1 Experiment 1. A comparison of the percentage of infected plants of R1 generation lines of pepper (Capsicum annuum PI178849) obtained using mechanical or aphid-inoculation techniques and a comparison of visual rating or enzyme-linked immunosorbent assay (ELISA) analysis as detection methods for virus-infection.

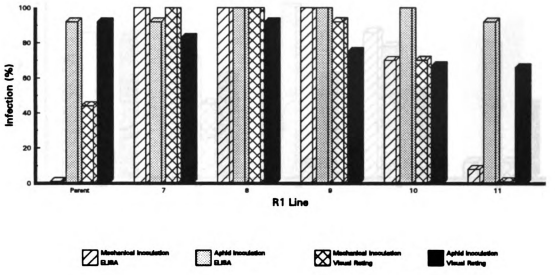


Figure 3.2 Experiment 2. A comparison of the percentage of infected plants of R1 generation lines of pepper (Capsicum annuum PI178849) obtained using mechanical or aphid-inoculation techniques and a comparison of visual rating or enzyme-linked immunosorbent assay (ELISA) analysis as detection methods for virus-infection.

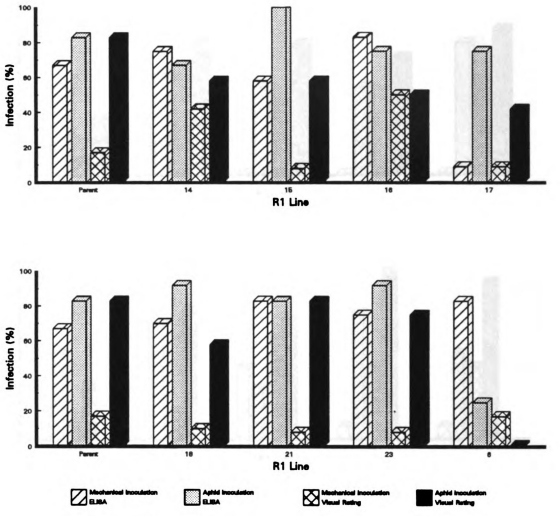


Figure 3.3 Experiment 3. A comparison of the percentage of infected plants of R1 generation lines of pepper (Capsicum annuum PI178849) obtained using mechanical or aphid-inoculation techniques and a comparison of visual rating or enzyme-linked immunosorbent assay (ELISA) analysis as detection methods for virus-infection.

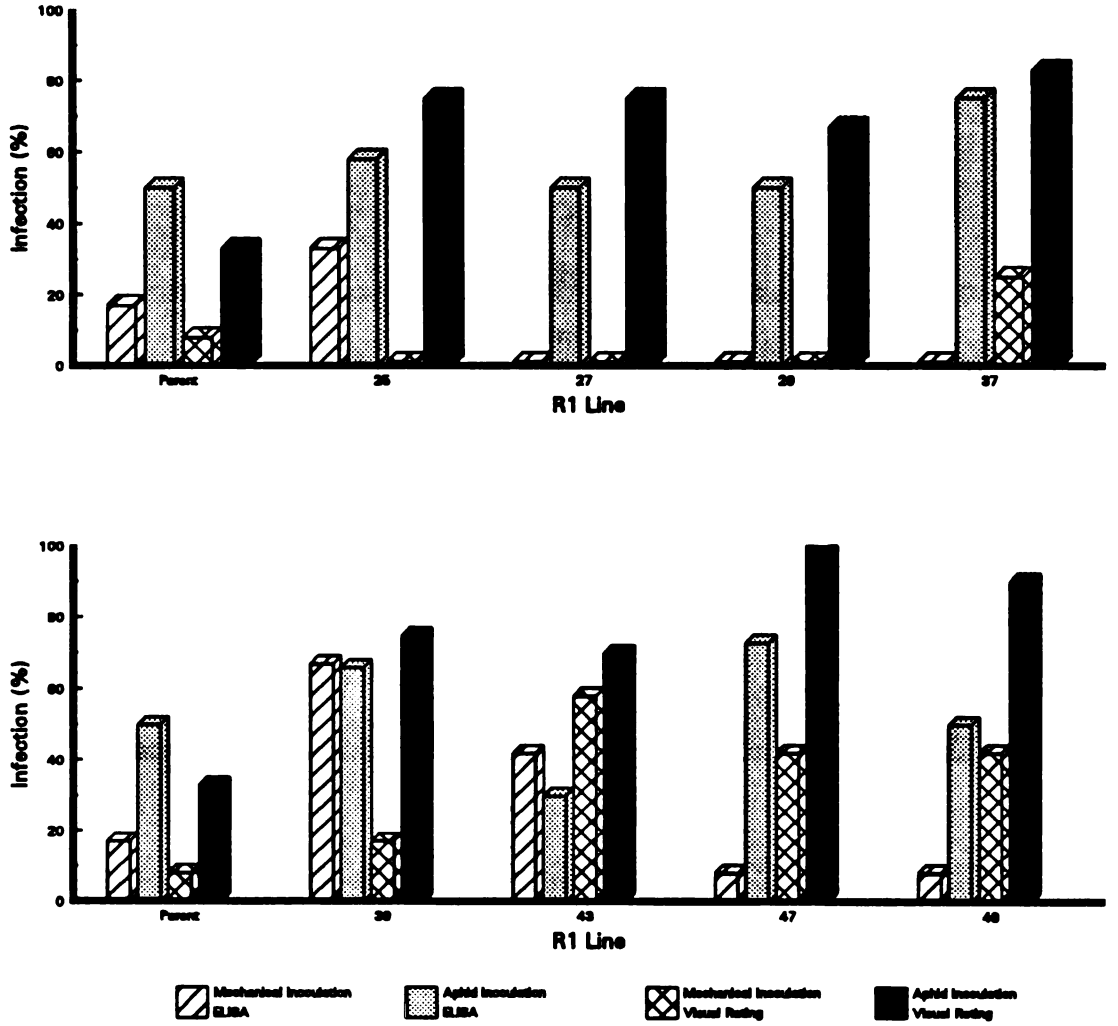


Figure 3.4 Experiment 4. A comparison of the percentage of infected plants of R1 generation lines of pepper (Capsicum annuum PI178849) obtained using mechanical or aphid-inoculation techniques and a comparison of visual rating or enzyme-linked immunosorbent assay (ELISA) analysis as detection methods for virus-infection.

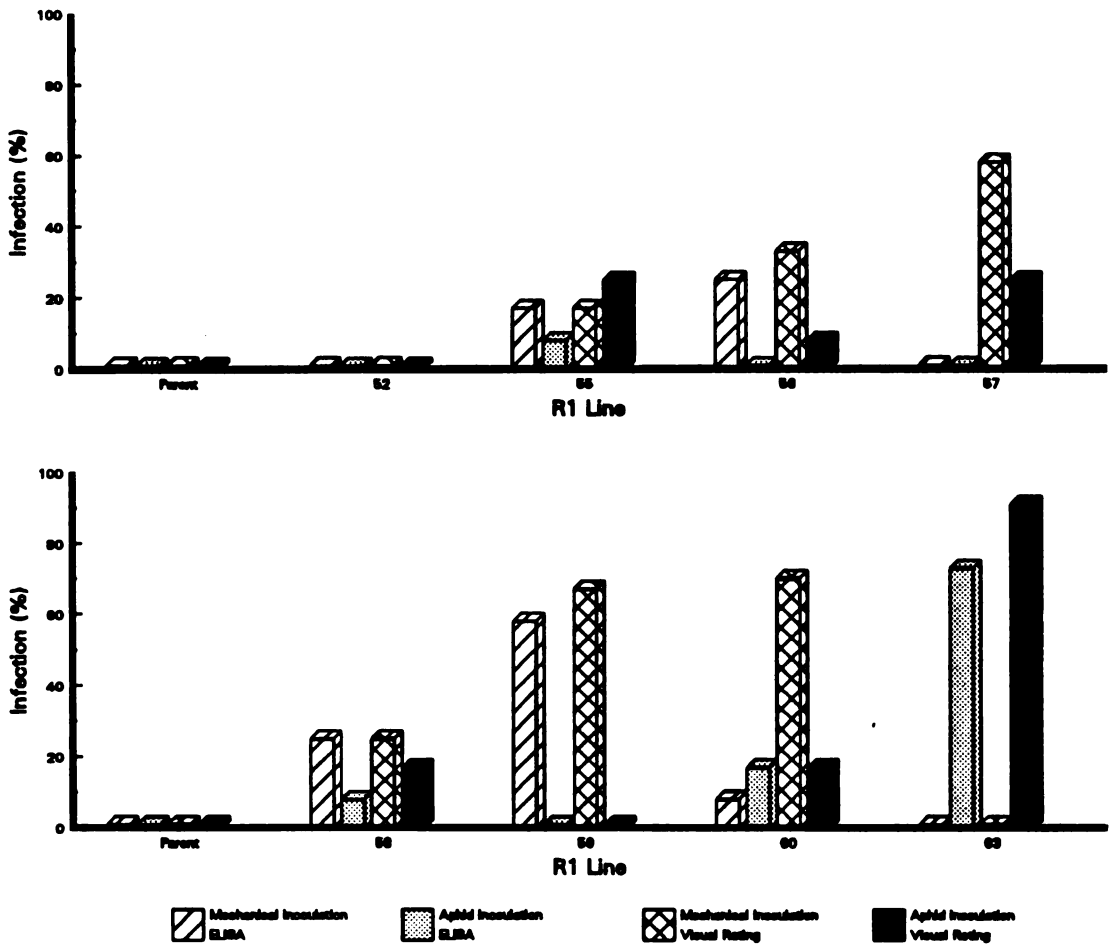


Figure 3.5 Experiment 5. A comparison of the percentage of infected plants of R1 generation lines of pepper (Capsicum annuum PI178849) obtained using mechanical or aphid-inoculation techniques and a comparison of visual rating or enzyme-linked immunosorbent assay (ELISA) analysis as detection methods for virus-infection.

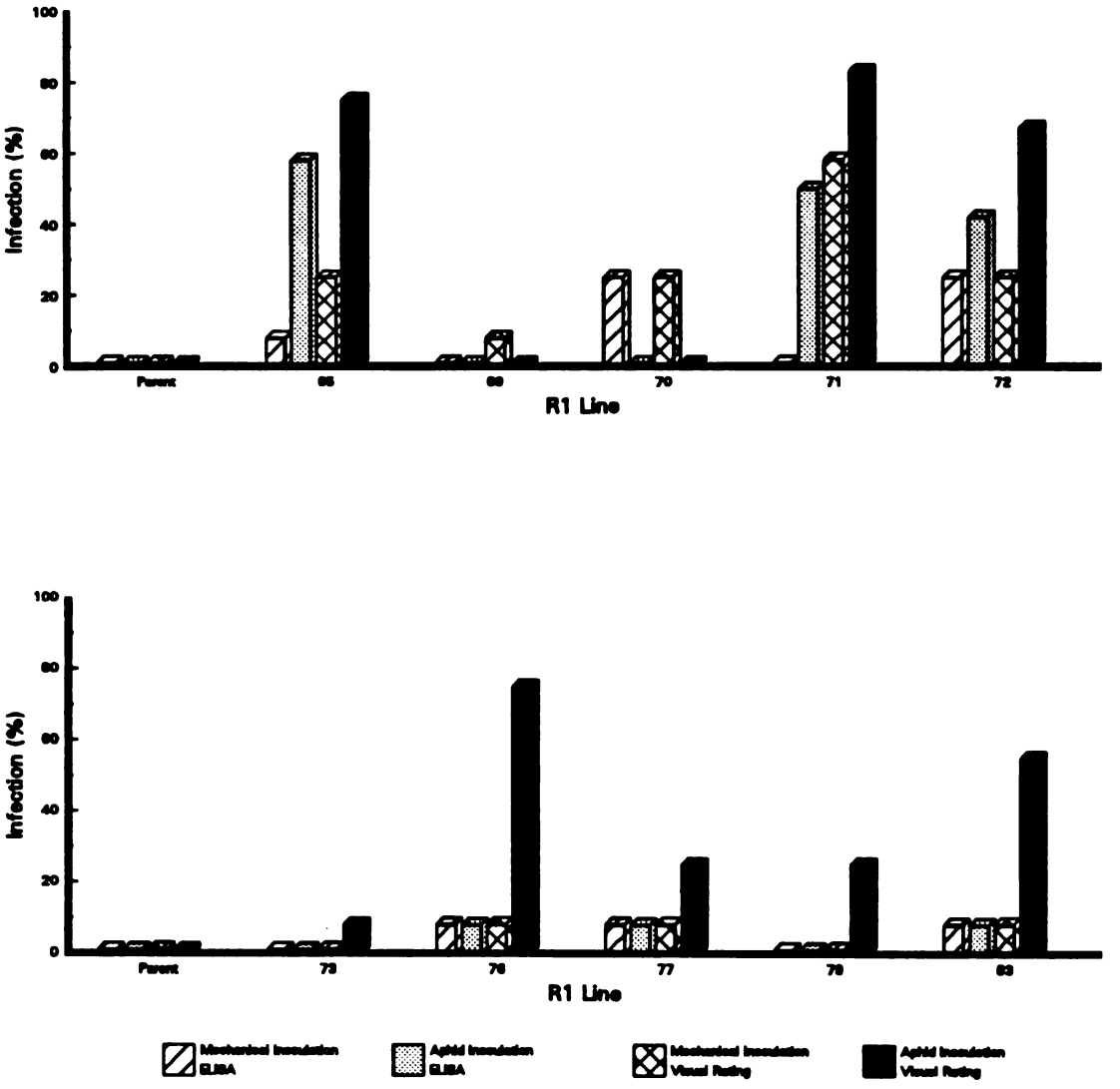
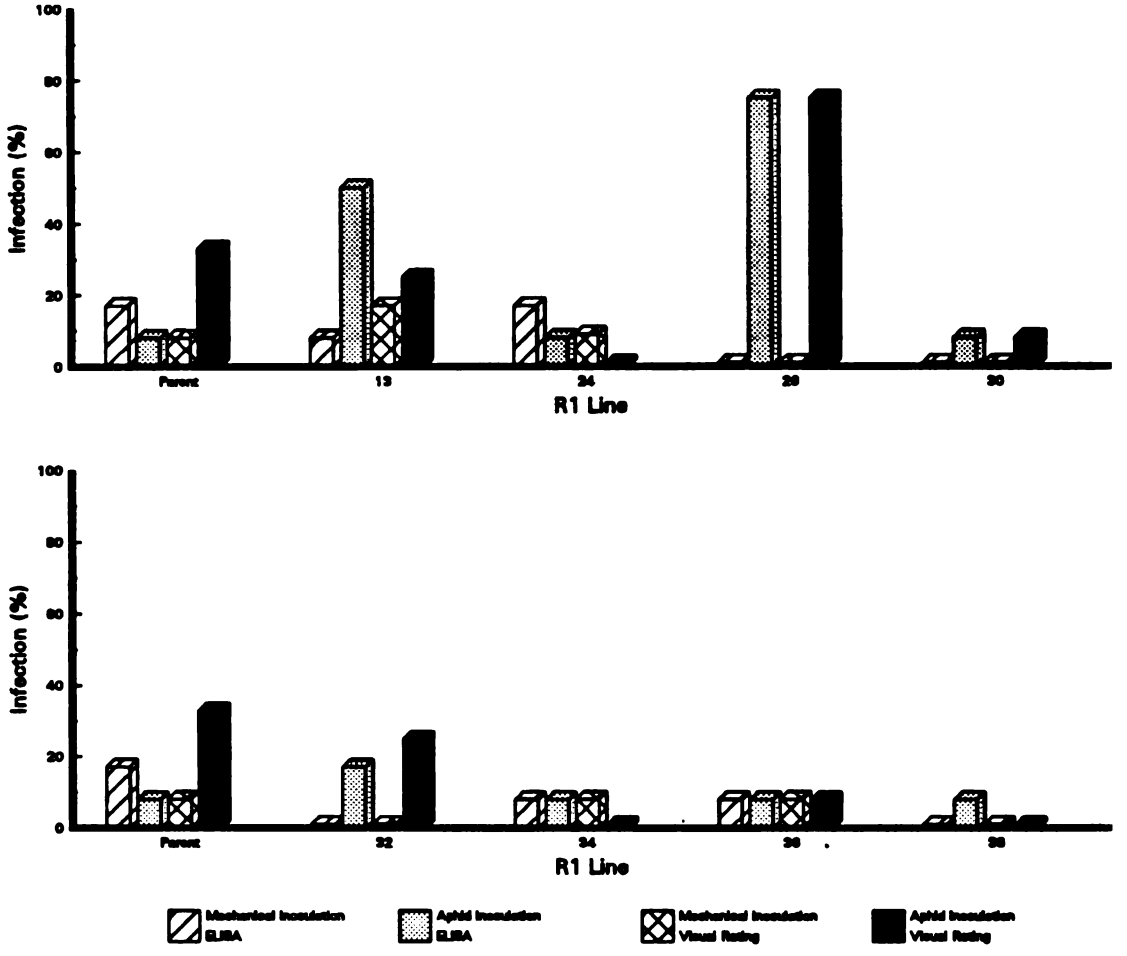
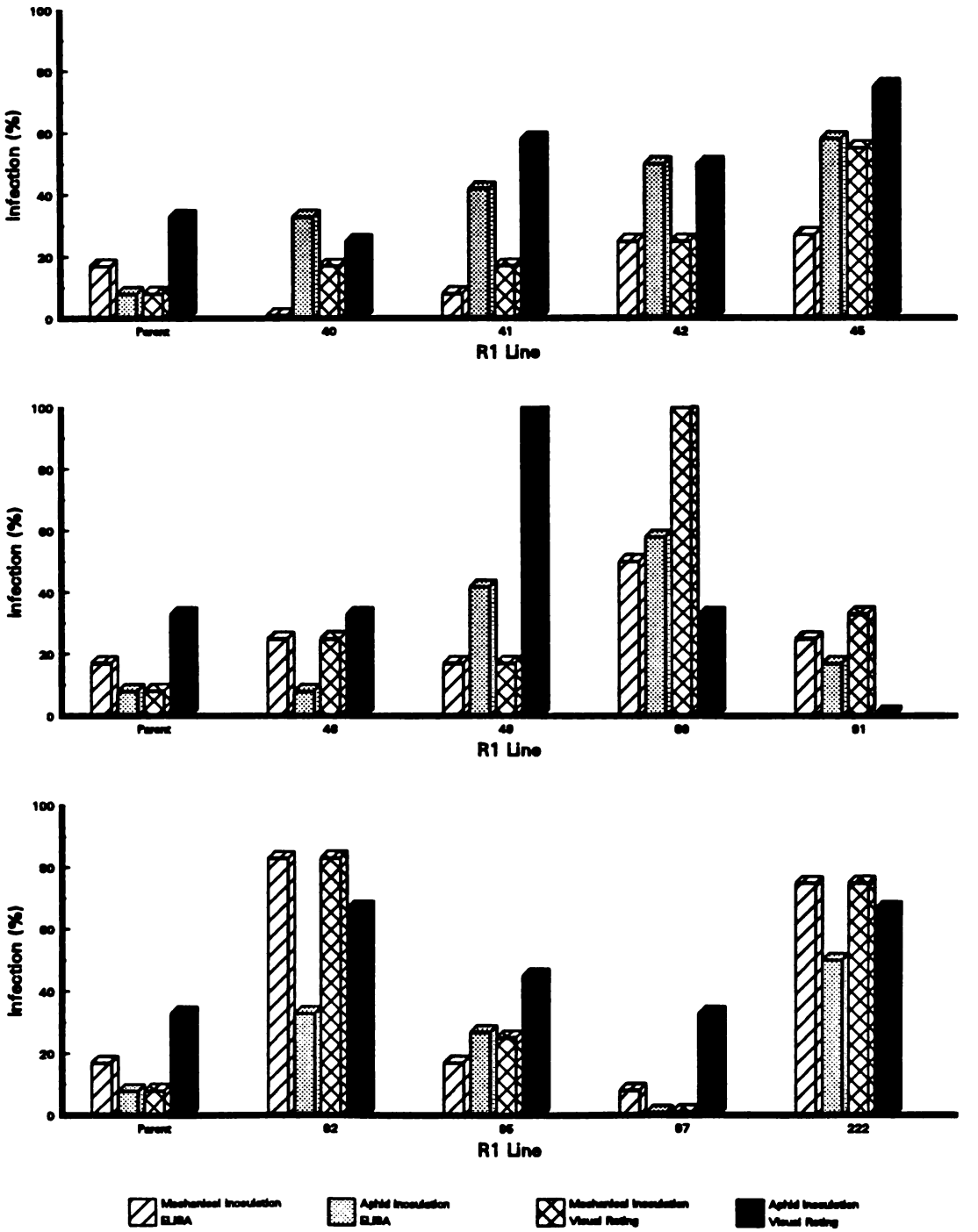


Figure 3.5 Experiment 5. A comparison of the percentage of infected plants of R1 generation lines of pepper (Capsicum annuum PI178849) obtained using mechanical or aphid-inoculation techniques and a comparison of visual rating or enzyme-linked immunosorbent assay (ELISA) analysis as detection methods for virus-infection.

Figure 3.6 Experiment 6. A comparison of the percentage of infected plants of R1 generation lines of pepper (Capsicum annuum PI178849) obtained using mechanical or aphid-inoculation techniques and a comparison of visual rating or enzyme-linked immunosorbent assay (ELISA) analysis as detection methods for virus-infection.


Infection (%)
100
80
60
40
20
0
Parent
13
24
28
30
R1 Line
Infection (%)
100
80
60
40
20
0
Parent
22
24
28
30
R1 Line
Mechanical Inoculation ELISA
Aphid Inoculation ELISA
Mechanical Inoculation Visual Rating
Aphid Inoculation Visual Rating


Infection (%)
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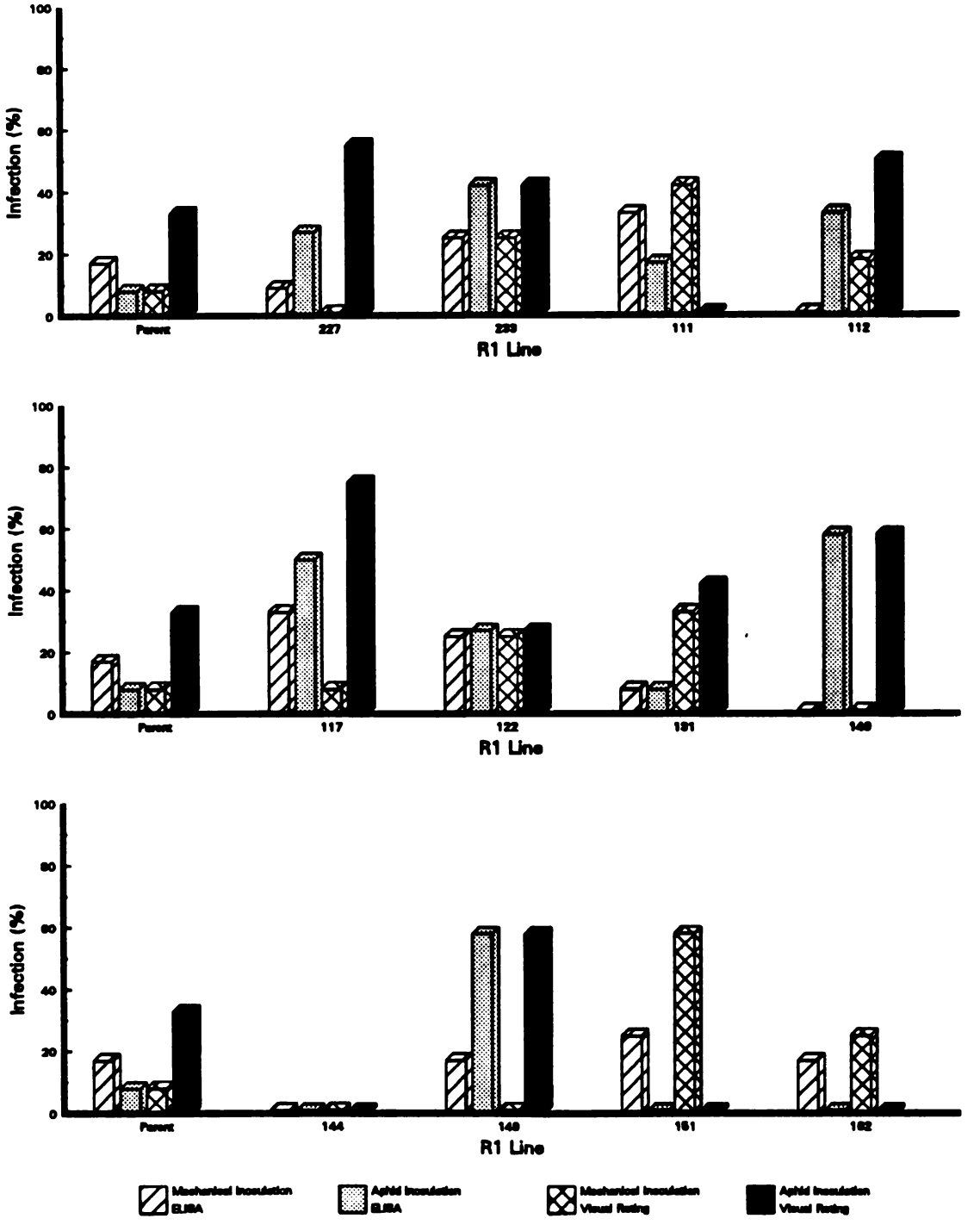
40
20
0
Parent
40
41
42
45
R1 Line
Infection (%)
100
80
60
40
20
0
Parent
48
49
89
91
R1 Line
Infection (%)
100
80
60
40
20
0
Parent
82
85
87
222
R1 Line
Mechanical Inoculation ELISA
Aphid Inoculation ELISA
Mechanical Inoculation Visual Rating
Aphid Inoculation Visual Rating


Infection (%)
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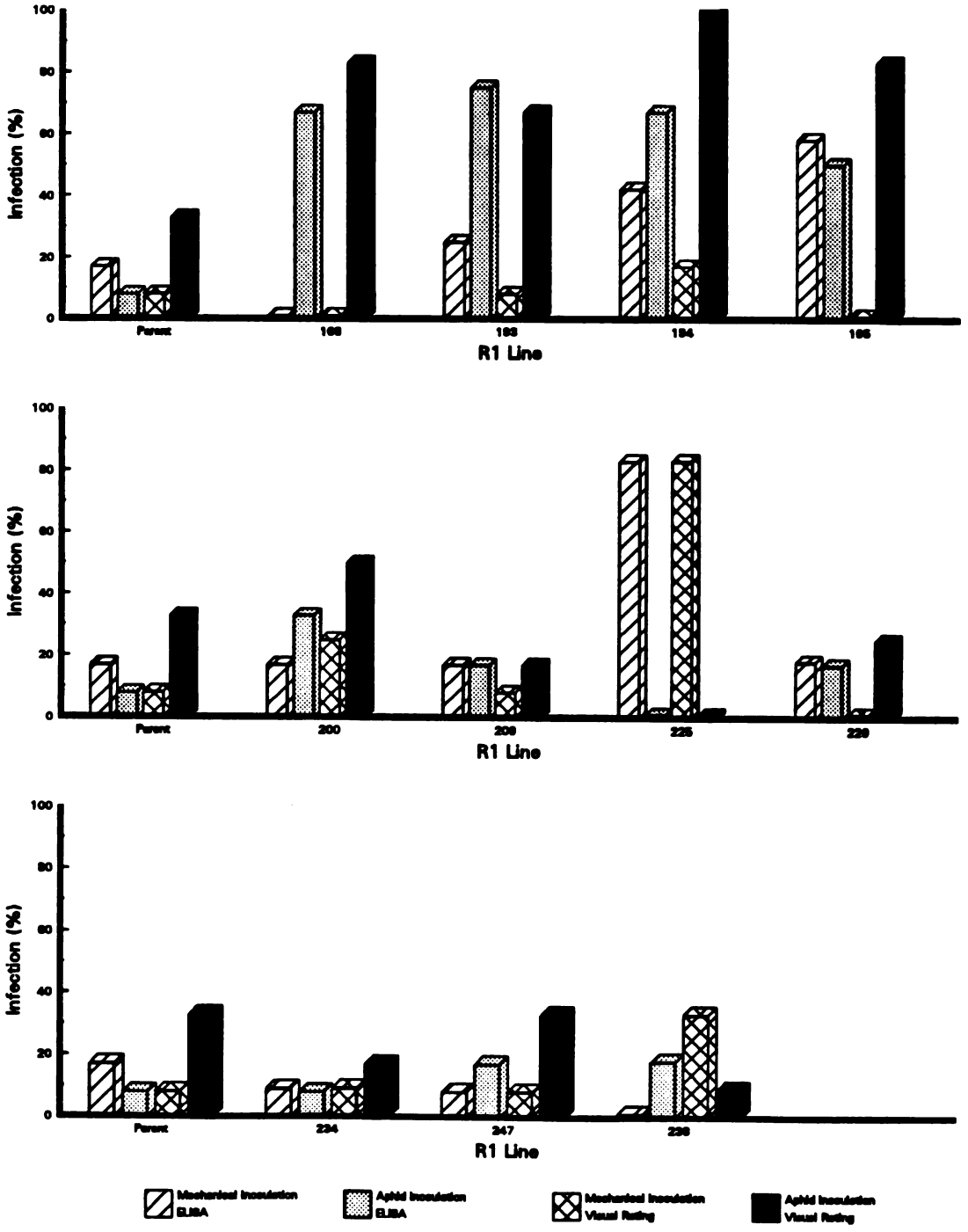
60
40
20
0
Parent
227
239
111
112
R1 Line
Infection (%)
100
80
60
40
20
0
Parent
117
122
121
149
R1 Line
Infection (%)
100
80
60
40
20
0
Parent
144
148
151
162
R1 Line


Infection (%)
100
80
60
40
20
0
Parent
R1 Line
Infection (%)
100
80
60
40
20
0
Parent
200
225
R1 Line
Infection (%)
100
80
60
40
20
0
Parent
234
247
R1 Line
Mechanical Inoculation ELISA
Aphid Inoculation ELISA
Mechanical Inoculation Visual Rating
Aphid Inoculation Visual Rating

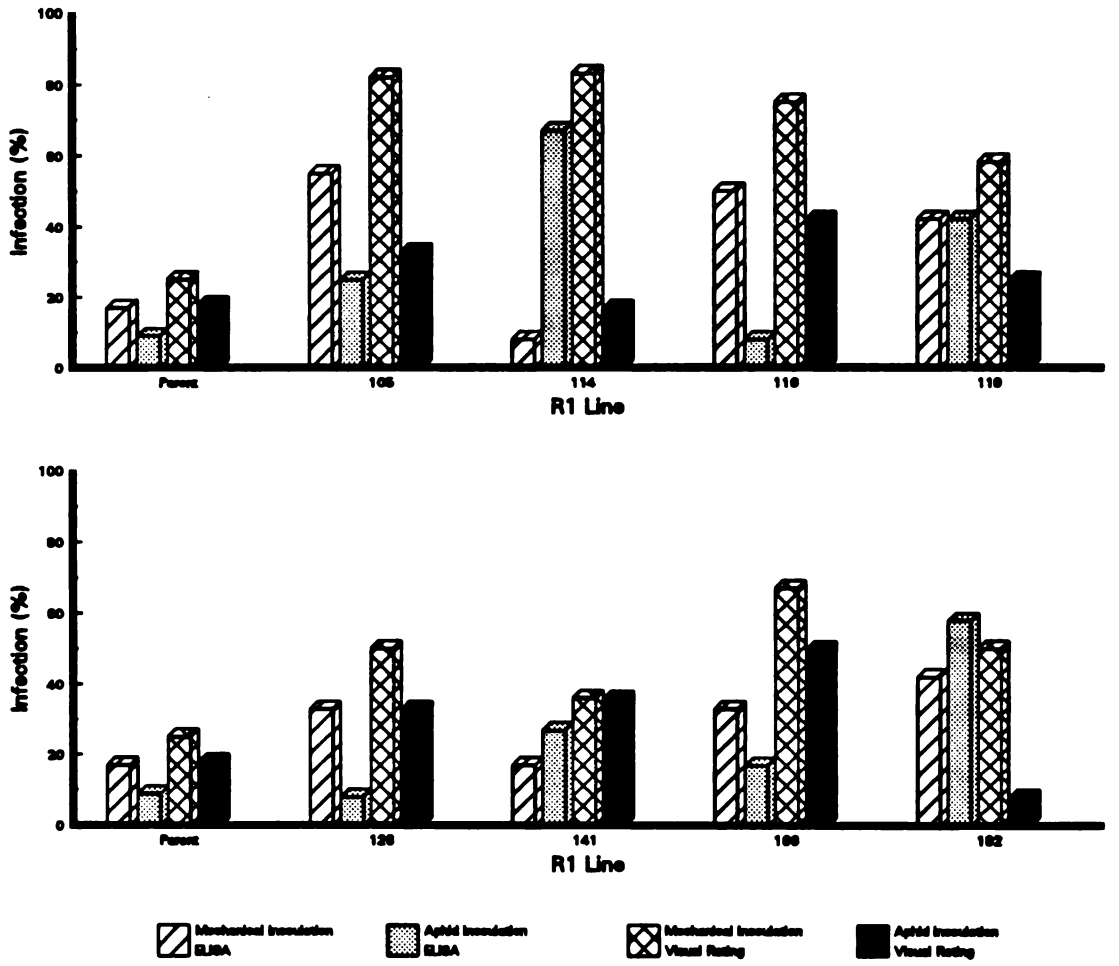


Figure 3.7 Experiment 7. A comparison of the percentage of infected plants of R1 generation lines of pepper (Capsicum annuum PI178849) obtained using mechanical or aphid-inoculation techniques and a comparison of visual rating or enzyme-linked immunosorbent assay (ELISA) analysis as detection methods for virus-infection.

were considered susceptible to CMV because at least 10% of the plants of these lines were CMV-positive using one of the inoculation techniques. Only 2/95 lines were rated CMV-negative during the initial experiment. However, reinoculation of these two lines resulted in >40% plants visually rated as CMV-positive.

DISCUSSION

The screening of R1 generation lines for resistance to CMV revealed extensive variation in response among the lines and also within the parental line. Variation was also observed in the same line using rub and aphid inoculation techniques when infection was assessed using a visual rating system for symptom development or ELISA. In one experiment, none of the plants in the parental line were determined to be infected using a visual rating system and ELISA. Variability among parents such as this was also observed by Brown et al. (1986) when screening lettuce R1 lines for resistance to lettuce mosaic virus.

Most of these uninfected plants are believed to be escapes, i.e. inoculated plants in which infection was unsuccessful. Because escapes occurred in both parental and R1 generation lines within the same experiment, these were not viewed as being significant. Unsuccessful infection of pepper leading to escapes can be caused by a number of factors. Pepper leaves contain a protein and a phenolic substance which act as natural inhibitors against subsequent mechanical inoculation of virus. In a study by Horvath and

Nienhaus (1982), pepper plant extracts reduced the activity of CMV up to 100% in cowpea. In our experiments, attempts to mechanically transmit CMV from infected pepper to local lesion hosts (Chenopodium and cowpea) were repeatedly unsuccessful probably due to these inhibitors. This may also explain the variation in infection of the parent and R1 generation lines which were inoculated mechanically.

Infected plants used as inoculum in experiments may have contained a low virus titer or the titer of the virus may have differed among the experiments, leading to variation in infection.

Variability among aphid-inoculated plants may have been caused by differences in virus titer among experiments or uncontrollable variation among aphid feeding.

In a study of resistance to TMV in tomato somaclones, Barden et al. observed a delay in symptom development of 46 days in 18/376 somaclones. Among the 93 lines studied in this experiment, only two remained uninfected after 30 days. Plants from these two lines were re-inoculated and visual assessment revealed >40% infection. Plants from those two lines were most likely uninfected escapes.

If somaclonal lines were to be rated as resistant to CMV, a delay in symptom development and virus detection must be observed. Therefore, lines in which as little as 10% of the plants were infected would likely be discarded as susceptible and the unsuccessful infection attributed to escapes.

Somaclonal variation may hold promise as a means for generating virus resistance. The screening regimen, however, is labor-intensive, and tremendous variability may be observed.

LIST OF REFERENCES

Barden, K.A., Smith, S.S., and Murakishi, H.H. 1986. Regeneration and screening of tomato somaclones for resistance to tobacco mosaic virus. Plant Sci. 45:209-213.

Barrios, E.P., Mosakar, H.I., and Black, L.L. 1971. The inheritance of resistance to tobacco etch and cucumber mosaic viruses in Capsicum frutescens. Phytopathology 61:1318.

Brown, C., Lucas, J.A., Crute, I.R., Walkey, D.G.A., and Power, J.B. 1986. An assessmant of genetic variability in somacloned lettuce plants (Lactuca sativa) and their offspring. Ann. Appl. Biol. 109:391-407.

Krishnamurthi, M., and Tlaskal, J. 1974. Fiji disease resistant Saccharum officianarum var. Pindar subclones from tissue cultures. Proc. Int. Soc. Sugarcane Technol. 15:130-137.

Larkin, P.J., and Scowcroft, W.R. 1981. Somaclonal variation -- a new source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60:197-214.

CHAPTER 4

PROTOPLAST INFECTION WITH VIRUS PARTICLES

ABSTRACT

Protoplasts were consistently isolated from pepper (Capsicum annuum) with an average viability of 85%. Variables studied among electroporation experiments included virus concentration, protoplast density, and power levels. Infection of protoplasts was achieved in two experiments, one using a protoplast density of 5 x 10^5/ml, virus concentration of 50 ug/ml, and power levels of 250, 300, 350, or 400 volts/cm, and the other using a protoplast density of 6.5 x 10^5, virus concentration of 125 ug/ml, and power level of 250 volts/cm. Subsequently, voltage was shown to sharply reduce protoplast viability above 100 volts/cm.

INTRODUCTION

Disease-resistant plants have traditionally been produced by identifying resistant genotypes and then crossing these individuals with cultivars that possess traits which are agriculturally valuable. Although traditional breeding has been highly successful in many cases, there are limitations to classical plant breeding

methods. Some of the limitations include: 1) the desired disease resistance is not always available, 2) the resistance may only exist in a species that is not interfertile with the crop, 3) the resistance genes may be tightly linked to other undesirable traits or 4) the resistance may be multigenic and difficult to transfer (Knott and Dvorak, 1976; Wenzel, 1985).

Tissue culture techniques may be used to overcome some of the factors limiting traditional plant breeding. Protoplasts are currently being used as a source of plant material for a range of *in vitro* genetic manipulations aimed at studying and developing disease-resistant crop plants. One technique, protoplast fusion, involves the transfer of resistance genes from a resistant to a susceptible host, resulting in the production of interspecific plant hybrids (Glimelius, 1988). Virus resistant transgenic plants containing cloned viral genes have been produced using plant transformation and molecular genetic technologies (Powell-Abel et al., 1986). The most significant contribution of tissue culture technologies has been as a tool to study basic mechanisms of pathogen virulence and host defense. The most extensive studies used protoplasts to study the mechanism of infection and replication of plant viruses (Zaitlin and Hull, 1987).

Utilization of plant protoplasts in an *in vitro* selection system may offer a means of selecting plant genotypes with increased resistance to disease. Screening

for resistance to plant pathogens at the single cell level offers unique advantages. Millions of cells can be screened in a short time, the technique holds promise for selection of resistance from susceptible cultivars (Shepard, 1981), and may allow for the recovery of increased levels of disease resistance (Murakishi and Carlson, 1982).

To date, viral pathogens offer the most promise as direct selection agents in the use of _in vitro_ screening systems because protoplasts can be synchronously infected with viruses (Hibi et al., 1986; Maule et al., 1980; Motoyoshi and Oshima, 1975; Nishiguchi et al., 1987). Infection of protoplasts has been demonstrated in a variety of plant species such as barley, _Brassica_, cowpea, cucumber, tobacco, and tomato (Okuno and Furusawa, 1978; Maule, 1983; Koike et al., 1977; Maule et al., 1980; Hibi et al., 1986; Nishiguchi et al., 1987; Motoyoshi and Oshima, 1975). Also, studies using protoplasts isolated from resistant host plants of tomato and cucumber infected with TMV and CMV respectively, revealed that resistance to the pathogen functions at the single cell level (Maule et al., 1980; Motoyoshi and Oshima, 1975). In addition, Murakishi and Carlson (1982), working with protoplasts isolated from systemically infected tobacco plants, demonstrated that viral pathogens can be used as selection agents at the single cell level to isolate variant cells, if conditions are manipulated to allow for uniform infection and for the preferential growth and selection of virus-resistant cells.

For selections to be successful at the single cell level, each cell must be exposed to the selection agent, otherwise there will be too many escapes. Therefore, the ability to achieve a high efficiency of infection by the virus is a key concern. Electroporation has been adapted as a genetic technique for simultaneous and uniform infection of plant protoplasts with viruses. Viral infectivity greater than 90-95% has been demonstrated with the use of electroporation (Hibi et al., 1986; Nishiguchi et al., 1987; Watts et al., 1987).

In order to obtain resistant plants from this selection, shoot regeneration of the crop plant after genetic manipulation is necessary. Shoot regeneration from protoplants has been successful in solanaceous crops with tobacco, potato and tomato included in the first crop species regenerated from protoplasts. Pepper (Capsicum annuum) a member of the solanaceous family, was used as the host plant in this study. Protoplasts of pepper have been isolated and regenerated into whole plants (Diaz et al., 1988; Saxena et al., 1981.).

A number of plant viruses have been used in host-pathogen-interaction studies. Pepper mild mottle virus (PMMV), a more virulent strain of tobacco mosaic virus (TMV), that has been isolated from TMV- and tomato mosaic virus-resistant pepper in Italy and Spain was used in this study (Wetter et al., 1984). The interaction of TMV and

protoplasts of various hosts has been studied extensively (Murakishi et al., 1984).

The research performed in this study was aimed at identifying a method for the development of virus-resistant germplasm in C. annuum. The development of a system for uniform, simultaneous infection of cells with virus also offers a means of studying basic methods of pathogen virulence and host defense, in the area of viral infection and replication in plant cells.

The goal of this study was to assess the use of virus-infected protoplasts as a means of selecting for virus-resistant plants at the single cell level. To achieve this goal, those variables (power level, virus concentration, and protoplast density) which influence the uptake of particles of PMMV into protoplasts of pepper by electroporation were investigated. Also the cultural conditions (light intensity, temperature, and culture medium) which would preferentially permit the growth of uninfected cells or of variant cells less affected by virus-infection were investigated.

MATERIALS AND METHODS

Protoplast Isolation

Seeds of C. annuum were surface sterilized by immersion in 95% ethanol for 2 min followed by 30 min in 50% aqueous (v/v) commercial bleach (2.62% sodium hypochlorite) containing 1-2 drops/250 ml Tween 20 (Sigma Chemical Co., St. Louis, MO). The seeds were thoroughly rinsed three

times with sterile distilled water, and soaked in sterile distilled water for 30 min. Nine seeds per container were sown in GA-7 vessels (Magenta Corp., Chicago, IL) containing 50 ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) which was amended with 165 mg NH_4NO_3, 100 mg myo-inositol, 2 mg glycine, 0.5 mg nicotinic acid, 0.5 mg pyroxidine·HCl, 0.2 mg thiamine·HCl, 30 g sucrose and 8 g Bacto agar (Difco Laboratories, Detroit, MI) per liter. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl. Pepper seedlings were grown in a growth room at 25 C under cool-white fluorescent lights supplying 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on a 16-hr photoperiod.

Fully expanded cotyledons from 3 wk-old seedlings were used for isolation of protoplasts. The following steps were performed using sterile technique. The cotyledons were removed from the seedlings with a scalpel, allowed to become flaccid, and the lower epidermis of the cotyledon was removed using fine forceps. The cotyledons were placed peeled-side down in a petri dish containing 20 ml of a filter-sterilized enzyme solution containing 1.0% (w/v) Cellulysin, 0.5% (w/v) Driselase, 0.1% (w/v) Macerase (Calbiochem, Behring Diagnostics, San Diego, CA; Kyowa Hakko Kogyo Co., Japan; Calbiochem), CPW salts (consisted of:), and 0.4 M sorbitol at pH 6.0. Cotyledons were incubated at 25 C in the dark on a gyrotory shaker at 45 rev/min for 4-5 hours. The petri dish containing the enzyme solution was

weighed before and after the addition of cotyledons to determine the amount of plant tissue.

The enzyme solution containing protoplasts and cotyledon debris was filtered through a 60 μm nylon sieve into a petri dish, and the remaining cotyledon debris was gently rinsed with W5 salts (consisted of: 0.11 M $CaCl_2$, 0.13 M NaCl, 5 mM KCl, 5 mM glucose, 5 mM MES) at pH 5.8. The protoplasts were transferred to 15 ml round-bottomed centrifuge tubes which were centrifuged at 35 x g or 400 rpm in an HNS-II IEC No. 2355 tabletop centrifuge for 10 min. The supernatant was gently removed by suction using a disposable pipette, and the pellet was resuspended by rolling in a small amount of W5 salts solution. The protoplasts were rinsed in 10 ml of W5 salts solution, centrifuged again at 35 x g and the supernatant removed by suction using a disposable pipette. The protoplasts were rinsed a total of three times in this manner. The final step in isolation of the protoplasts was to float the intact protoplasts on a density buffer made of Lymphoprep (9.6% w/v sodium metrizoate and 5.6% w/v ficoll; Accurate Chemical and Sci Corp, Westbury, NY):0.5 M sucrose-10 mM $CaCl_2$ in W5 salts at a 1:2 ratio. The intact protoplasts formed a band at the top of the solution which was removed by suction and placed in a clean centrifuge tube.

Protoplast viability and yield

The viability of protoplasts after isolation was determined using an inverted microscope and rating the protoplasts for their general appearance. Viable protoplasts remained spherical with evenly distributed chloroplasts. Non-viable protoplasts were not spherical and were misshapen. The percent viability was determined by dividing the number of viable protoplasts by the total number of protoplasts.

The yield of protoplasts was determined per gram of plant tissue using a hemocytometer to count the viable-looking protoplasts. The concentration of protoplasts was then adjusted to the proper amount for each experiment.

Purification of virus

A virus solution of 20 μg/ml in 0.2 M phosphate buffer (pH 7.2) was mechanically inoculated onto leaves of 6-8 week-old Nicotiana clevelandii plants and allowed to multiply for 3 wks. The tobacco leaves were removed and weighed. The leaf tissue was frozen and stored at -20 C until further use. For purification, the method of Gooding and Herbert (1967) was followed with slight modifications. The frozen tissue was thawed and homogenized (1 g plant tissue/2 ml 0.1 M phosphate buffer, pH 7.0) in a blender for 3 min. The homogenate was filtered through four layers of cheesecloth and the filtrate distributed into 250 ml centrifuge bottles. The filtrate was centrifuged at 10,000 rpm for 15 min at 4 C in a Sorvall GSA rotor (16,400 x g) to

remove cellular debris. The supernatant was collected, adjusted to 8% w/v polyethylene glycol (PEG) MW 8000 and to 0.2 M NaCl, and stirred at room temperature for 30 min to precipitate the virus. The entire mixture was centrifuged at 10,000 rpm in a Sorvall SS-34 rotor (9400 x g) for 10 min at 4 C, and the virus precipitate resuspended in 0.01 M phosphate buffer, pH 7.0 at 1/5 the volume used in the PEG precipitation.

Three cycles of high (108,000 x g) and low speed (12,000 x g) centrifugations were performed to further purify the virus. Ultracentrifugation of the virus solution was performed at 25,000 rpm for 2.5 h at 4 C in a Beckman Ti70 rotor to pellet the virus. The pellets were resuspended in 0.01 M phosphate buffer, pH 7.0 after overnight incubation at 4 C and centrifuged for 10 min at 10,000 rpm (SS-34 rotor) to remove more cell debris.

Concentration of the virus was estimated based on absorbance at 260 nm. At this wavelength, the extinction coefficient (1 cm pathlength) for purified PMMV absorbance (1 mg/ml) is 3.18 (Wetter, 1975). The purified virus was filtered through a Morton ultra-fine fritted disc filter (0.9-1.4 μ pore diameter) to remove microorganisms, and stored in 0.01 M phosphate buffer. After filtering, the absorbance at 260 nm was measured and the virus was diluted to a concentration of 1 mg/ml and stored in 1 ml aliquots at 4 C.

Inoculation of protoplasts

Protoplasts of pepper susceptible to PMMV were inoculated by electroporation using the method of Nishiguchi et al., (1987) with slight modifications. Immediately before inoculation, the protoplast pellet was resuspended in a solution of 0.7 M mannitol, followed by centrifugation at 35 x g for 10 min. The supernatant was removed and an ice cold solution of 0.7 M mannitol containing PMMV particles was added to the protoplast pellet to give a concentration between 1-6.5 x 10^5 protoplasts/ml. The mixture was then transferred into a microcuvette tube and incubated on ice for 10 min.

An exponential pulse was applied to the solution of protoplasts in the microcuvette using an electroporation system (Prototype Design Services, Biotechnology Equipment, Madison, WI 53705). The distance between the electrodes was approximately 0.3 cm. The voltage varied in experiments. After electroporation, the protoplasts were transferred to a 15 ml culture tube and kept on ice for at least 10 min. Protoplasts were resuspended in several ml of 0.7 M mannitol and centrifuged at 35 x g for 10 min to remove any excess virus particles. The protoplasts were resuspended in 1.0 ml of KMT medium and were cultured in a petri dish at 28 C under continuous cool-white fluorescent illumination at 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Protoplast viability

Protoplasts were harvested 24 hr after electroporation and used to determine viability and infection. Protoplast viability was determined using Evan's blue, a non-permeating pigment which penetrates dead or damaged protoplasts and is excluded by live intact protoplasts (Gaff and Okong'O-Ogola, 1971). Protoplasts were incubated in Evan's blue dye (0.25% w/v Evan's blue in W5 salts, pH 6.0) solution for at least 5 min to allow penetration of the pigment before being viewed using an inverted microscope. The percentage of viability was determined using the above described method.

Preparation of the FITC-conjugate

Conjugation of immunoglobulin g (IgG) with fluorescein isothiocyanate (FITC) was carried out by using modifications of a published technique (Spendlove, 1967). Serum was collected from the rabbit following three virus injections at 2-wk intervals. The IgG fraction was precipitated by the dropwise addition of 25 ml $(NH_4)_2SO_4$ (saturated solution) to 50 ml of serum with stirring. This was mixed for 3 h at 0 C. The precipitate was collected by centrifugation at 5000 rpm in a SS-34 rotor for 15 min. The pellet was then dissolved in 40 ml of phosphate buffered saline (PBS, which was 0.01 M K- phosphate buffer, pH 7.0, containing 0.85% NaCl), and precipitated a second time by the dropwise addition of 20 ml saturated $(NH_4)_2SO_4$ while stirring for 1 h at 0 C. After centrifugation at 6000 rpm for 15 min, the pellet was dissolved in 6 ml PBS. The IgG was then dialyzed

against five changes of PBS over a period of 24 h. The solution was then clarified by centrifugation at 14,000 x g for 30 min and the supernatant containing the IgG was collected. The protein concentration was determined by measuring the absorption at 280 nm, using the extinction coefficient of 1.8 for rabbit IgG (McGuigan and Eisen, 1968).

To label the IgG with FITC (ICN Immunobiologicals, Lisle, IL) 2 ml of the purified IgG was stirred while adding 1.2 ml of FITC solution dropwise. For the FITC solution, 15 mg FITC was mixed with 1 g protein, made in freshly prepared 0.1 M Na_2HPO_4. An additional 0.4 ml of freshly prepared Na_2PO_4 was added, followed by the addition of 0.4 ml 0.04 M NaOH (pH 9.4-9.6). The solution was loaded onto a Sephadex G-25 column (2.5 x 15 cm) which was previously equilibrated with PBS to free the conjugated IgG from uncoupled dye. The conjugate was then eluted with PBS and the first visible (greenish-yellow) band was collected in fractions. The eluate was centrifuged at 15,000 rpm for 30 min to clarify. The supernatant containing the conjugate was adjusted to 0.1% NaN_3 and stored at 4 C.

Cross absorption of FITC-conjugate with acetone powder of healthy tobacco leaves.

To minimize non-specific staining, the conjugated IgG was adsorbed with acetone-extracted powder of healthy tobacco leaves. Ten g of healthy leaves were picked, placed in 100 ml cold acetone (0 C) and homogenized at maximum

speed in a Waring blender for 5 min. More acetone was added to make a volume of 300 ml. The homogenate was washed on a Buchner funnel lined with Whatman No. 1 filter paper with cold acetone until the filtrate was nearly colorless. The homogenate was further pulverized by grinding in a dry mortar, resulting in an almost powder-like substance. This was dried under vacuum overnight and stored in a desiccator at room temperature.

The acetone powder was prepared for cross-absorption by several washes with PBS, pH 7.0. To 40 ml PBS, 0.2 g of powder was added and stirred for 10 min. The suspension of powder was centrifuged at 3000 rpm in an SS-34 rotor for 7 min, and the pellet was resuspended in 40 ml PBS, and centrifuged again as before. The pellet containing the acetone powder was saved, resuspended in 35 ml PBS and divided into two tubes for cross-absorption of the different FITC-conjugate bands collected from the Sephadex column. To determine the optimum amount of cross-absorption necessary, one tube contained approximately twice the amount of the pellet as the second tube. Each fraction was centrifuged again and 2 ml of FITC-conjugate solution was added to each pellet. This was stirred with the aid of a glass rod and the mixture was incubated for 30 min at 37 C. After unwanted antibodies were adsorbed to the tobacco acetone powder, a centrifugation at 3000 rpm for 7 min removed the solids, and the supernatant fraction containing the FITC-conjugate was saved. Tests for the optimum amount of

acetone powder needed for cross-absorption were made by trial and error method using identical sets of isolated infected protoplasts. The various fractions were labelled and stored at 4 C.

Preparation and staining of infected protoplasts

The percentage of virus-infected protoplasts was quantified by sampling the protoplasts after specified post-inoculation periods and staining with viral coat-protein specific antibodies conjugated to FITC using the procedure outlined by Otsuki and Takebe (1969). Infected protoplasts from approximately 0.5 ml of the cultured suspension were pelleted by centrifugation at 100 x g for 2 min and the pellet was resuspended in a small volume of KMT medium. Duplicated drops of this concentrated preparation were placed on a microscope slide previously coated with a thin film of Mayer's albumin (egg white + phenol) and allowed to dry. Cells were fixed by immersion in 95% ethanol for 5 min, followed by a rinse for 5 min in PBS. Slides were carefully blotted with filter paper and protoplast spots were stained with one drop of 1:6 dilution of FITC-conjugate in PBS. These slides were incubated at 100% relative humidity at 37 C for 1 hr in a water bath, following which the unbound FITC-antibody conjugate was removed by rinsing the slides in a large volume of PBS for 10 min. The specimen was mounted for viewing by carefully blotting away excess PBS, adding a drop of PBS:glycerol (9:1) and placing a coverslip over the cells.

Detection of virus infection by fluorescence microscopy

FITC-labelled cells were viewed with a Zeiss-GFl epiluminescent microscope equipped with exciter filters KP-490 and LP-445, dichroic reflector 510 and barrier filter 520. Infected cells contained specks which were a bright fluorescent yellow-green in color. Uninfected cells exhibited slight light green fluorescence, and dead cells appeared dull orange-brown in color. When chlorophyll was present, cells fluoresced red. The percentage of infected cells was determined by rating at least 300 viable protoplasts in the sample as fluorescent or non-fluorescent. The ratio of fluorescent to total viable protoplasts counted gave the percent fluorescent protoplasts.

Photography

Photographs of the fluorescing cells were taken with ASA 400 daylight film. Exposures were between 10 and 30 min.

RESULTS

Inoculation of protoplasts

Protoplasts were consistently isolated using the protocol with average yields of 5.1 x 10^6/g of tissue. The average viability of the isolated protoplasts was 85%.

A total of 26 electroporation experiments were performed using the following varying parameters: virus concentration 50 - 300 µg/treatment; protoplast density 1 x 10^5 - 6.5 x 10^5/ml; and power levels of 100 - 400 volts/cm. Infection was achieved in two experiments with the treatment

conditions outlined in Table 4.1. Infected protoplasts were detected using antibodies conjugated to FITC (Fig. 4.1).

Because positive results were obtained in only two experiments and only a low percentage (1-2%) of protoplasts were infected in these experiments, the effect of voltage on protoplast viability was studied. As the voltage increased above 100 volts, viability of the protoplasts was reduced sharply (Fig. 4.2). In fact, in the successful experiments, total protoplast viability after electroporation was probably <10% which would explain why the total percentage of infection was low.

Also, the infectivity of the virus inoculum was retested in local lesion assays on tobacco and Chenopodium and the virus was infective at a level of 0.01 μg/ml.

DISCUSSION

A requirement in the development of an in vitro tissue culture selection system is efficient regeneration of the source material of the crop plant in study. There have been two reports of regeneration of pepper from protoplasts, however, whole plant regeneration was limited to C. annuum cv. California Wonder and cv. Dulce (Diaz, 1988; Saxena, 1981). Using the protocols presented, our laboratory was unable to replicate whole plant regeneration from protoplasts; extensive efforts resulted in only the formation of callus tissue (S. Linderman, personal communication). Although there have been many virus-host-interaction investigations, only a few crop plants have been

Table 4.1. Treatment conditions of successful experiments infecting pepper protoplasts with pepper mild mottle virus using electroporation.

Protoplast density	Virus concentration[a]	volts/cm
5.0×10^5	50	250
		300
		350
		400
6.5×10^5	125	250

[a] Concentration of virus in ug/ml.

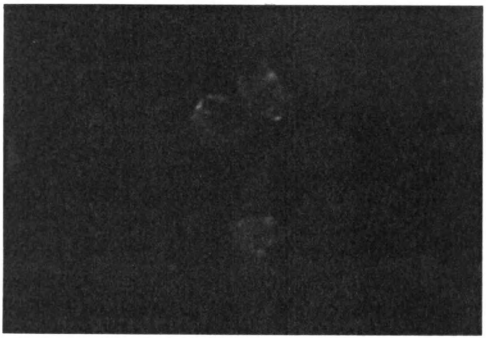


Figure 4.1 Fluorescence microscopy detection of protoplasts of pepper (Capsicum annuum) infected with pepper mild mottle virus by electroporation.

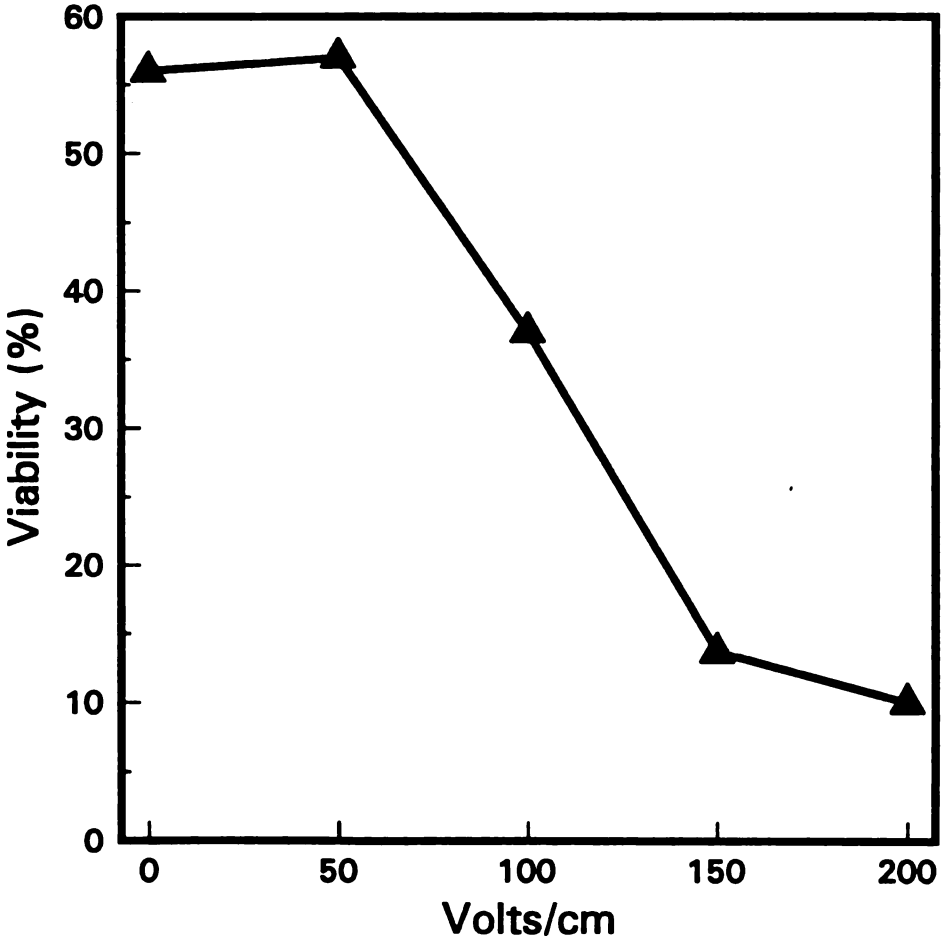


Figure 4.2 Viability of protoplasts of pepper (Capsicum annuum) after exposure to various voltages during electroporation experiments.

used. In these protoplast/virus studies, crop plants such as potato, tobacco and tomato, in which the culture and regeneration requirements have been worked out and a number of successful genetic manipulations have been employed, were chosen.

Obviously, for selections to be successful at the single cell level, each cell must be exposed to the selection agent. Of the plant pathogens, viruses appear to be the selection agent of choice, since simultaneous infection as great as 95% has been achieved (Watts et al., 1987). However, based on our current understanding of virus infection and replication processes, it has proven unobtainable to achieve 100% infection of protoplasts in culture.

Selection for disease resistance would be most efficient at the single cell level, but this requires that the specific resistance genes be expressed during the culture process. The selection agent would have to kill or inhibit growth of the cell to the point that the resistant cells could divide, form callus and regenerate faster than the susceptible cells and thus be identified. Infection of protoplasts with viral pathogens rarely leads to death of the cell. Also, the percentage of possible resistant variants is so much lower than the 5-10% virus-free protoplasts that escape infection, the method is unsuitable for selecting variants during culture. This type of

selection system would require a large number of protoclones to be indexed to identify the variant.

In vitro single cell selection systems could be successfully used to isolate variants if the discussed conditions could be manipulated to allow uniform infection, preferential growth of the variant cells, and regeneration of the variant crop plant.

LIST OF REFERENCES

Diaz, I., Moreno, R. and Power, J.B. 1988. Plant regeneration from protoplasts of Capsicum annuum. Plant Cell Rep. 7:210-212.

Gaff, D.F. and Okong'O-Ogola, O. 1971. The use of non-permeating pigments for testing the survival of cells. J. Expt. Bot. 22:756-758.

Gooding, G.V. and Herbert, T.T. 1967. A simple technique for purification of tobacco mosaic virus in large quantities. Phytopathology 57:1285.

Glimelius, K. 1988. Potentials of protoplast fusion in plant breeding programmes. Plant Cell Tiss. Org. Cult. 12:163-172.

Hibi, T., Kano, H., Sugiura, M., Kazami, T. and Kimura, S. 1986. High efficiency electro-transfection of tobacco mesophyll protoplasts with tobacco mosaic virus RNA. J. Gen Virol. 67:2037-2042.

Knott, D.R. and Dvorak, J. 1976. Alien germplasm as a source of resistance to disease. Annu. Rev. Phytopathol. 14:211-235.

Koike, M., Hibi, T., and Yora, K. 1977. Infection of cowpea mesophyll protoplasts with cucumber mosaic virus. Virology 83:413-416.

Marte, M. and Wetter C. 1986. Occurence of pepper mild mottle virus in pepper cultivars from Italy and Spain. J. Plant Dis. Prot. 93:37-43.

Maule, A.J., Boulton, M.I., Edmunds, C. and Wood, K.R. 1980. Polyethylene glycol-mediated infection of cucumber protoplasts by cucumber mosaic virus and virus RNA. J. Gen. Virol. 47:199-203.

Maule, A.J. 1983. Infection of protoplasts from several Brassica species with cauliflower mosaic virus following inoculation using polyethylene glycol. J. Gen. Virol. 64:2655-2660.

McGuigan, J. E. and Eisen, H. N. 1968. Differences in spectral properties and tryptophan content among rabbit anti-2,4- dinitrophenyl antibodies of the gamma-G-immunoglobulin class. Biochemistry 7:1919-1928.

Motoyoshi, F., and Oshima, N. 1975. Infection with tobacco mosaic virus of leaf mesophyll protoplasts from susceptible and resistant lines of tomato. J. Gen. Virol. 29:81-91.

Motoyoshi, F., and Oshima, N. 1975. Expression of genetically controlled resistance to tobacco mosaic virus infection in isolated tomato leaf mesophyll protoplasts. J. Gen. Virol. 34:499-502.

Murakishi, H. and Carlson, P. 1982. In vitro selection of Nicotiana sylvestris variants with limited resistance to tobacco mosaic virus. Plant Cell Rep. 1:94-97.

Murakishi, H., Lesney, M., and Carlson, P. 1984. Protoplasts and plant viruses. Adv. Cell Cult. 3:1-55.

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

Niedz, R.P., Stephens, C.T. and Murakishi, H.H. 1987. Isolation of pepper (Capsicum annuum) protoplasts. Phytopathology 77:1747. (abst.)

Nishiguchi, M., Sato, T. and Motoyoshi F. 1987. An improved method for electroporation in plant protoplasts: infection of tobacco protoplasts by tobacco mosaic virus particles. Plant Cell Reps. 6:90-93.

Okuno, T., and Furusawa, I. 1978. The use of osmotic shock for the inoculation of barley protoplasts with brome mosaic virus. J. Gen. Virol. 39:187-190.

Otsuki, Y., and Takebe, I. 1969. Fluorescent antibody staining of tobacco mosaic virus antigen in tobacco mesophyll protoplasts. Virology 38:497-499.

Powell-Abel, R., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T., and Beachy, R.N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Saxena, P.K., Gill, R., Rashid, A., and Maheshwari, S.C. 1981. Isolation and culture of protoplasts of Capsicum annuum L. and their regeneration into plants flowering in vitro. Protoplasma 108:357-360.

Shepard, J. F. 1981. Protoplasts as sources of disease resistance in plants. Ann. Rev. Phytopath. 19:145-155.

Spendlove, R. S. 1967. Microscopic Techniques. In Methods in Virology, Vol III. pp. 475-520. (Edited by K. Maramorosch and H. Koprowski), Academic Press, New York.

Watts, J.W., King, J.M., and Stacey, N.J. 1987. Inoculation of protoplasts with viruses by electroporation. Virology 157:40-46.

Wenzel, G. 1985. Strategies in unconventional breeding for disease resistance. Ann. Rev. Phytopath. 23:149-172.

Wetter, C. and Conti, M. 1988. Pepper mild mottle virus. Descriptions of Plant Viruses, No. 330. Commonwealth Mycological Institute, Association of Applied Biologists, Kew, Surrey, England.

Wetter, C., Conti, M., Altschuh, D., Tabillion R., and van Regenmortel, M.H.V. 1984. Pepper mild mottle virus, a tobamovirus infecting pepper cultivars in Sicily. Phytopathlology 74:405-410.

Zaitlin, M. and Hull, R. 1987. Plant virus-host interactions. Ann. Rev. Physiol. 38:291-315.