SHOOT-TIP CULTURE OF PEANUT FOR ELIMINATION OF PEANUT MOTTLE VIRUS

By

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ELIMINATION OF PEANUT

MOTTLE VIRUS

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PREFACE

The focus of this study was to develop improved techniques for the culture of peanut tissue and examine the applicability of these techniques for elimination of peanut mottle virus (PMV) from selected peanut germplasm. Methods for successful tip culture of peanut were developed, tip culture in conjunction with thermotherapy, chemotherapy, or both thermotherapy and chemotherapy were studied for elimination of PMV from peanut plants. Serology and indicator hosts were used to evaluate the effectiveness of the treatments for PMV elimination. The findings of this study should be useful for the control of PMV in vegetatively propagated peanut germplasm.

I wish to express my appreciation to all the people who have assisted me throughout my Master's program at Oklahoma State University. Special gratitude is extended to my major advisor, Dr. John L. Sherwood, for his guidance and assistance throughout this study and in the preparation of the manuscripts. Grateful appreciation is also extended to the other members of my committee: Drs. Becky Johnson and Hassan Melouk for their suggestions.

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CHAPTER I

INTRODUCTION

Several viruses belonging to different virus groups can infect the peanut plant (*Arachis* spp.). Some of the viruses that infect peanut are peanut mottle virus (PMV), peanut stunt virus (PSV), tomato spotted wilt virus (TSMV), groundnut rosette virus (GRV), peanut clump virus (PCV), cowpea mild mottle virus (CMMV), and peanut stripe virus (PStV). Since PMV was detected in peanut in 1965 (14), the virus has been reported to occur in all major peanut producing areas of the world (2, 3, 7, 8, 16, 20) and has become an important pathogen of peanut (15). PMV causes yield losses of 5-6% in peanut (15). PMV can also infect other legume species and causes economic loss on some crops (15). These crops include *Glycine max* L. Merr., *Lupinus albus* L., *L. angustifolius* L., *Phaseolus vulgaris* L., *Pisum sativum* L., *Trifolium substerraneum* L., *T. vesiculosum* Savi, and *Vigna unguiculata* L. Verde. The virus is localized in many cultivars of *P. vulgaris* and those in which necrotic lesions are produced are particularly useful as diagnostic indicators (15).

PMV is a member of the potyvirus group. Particles are flexuous rods (740-750 nm x 12 nm). They have a sedimentation coefficient of 155 S. They consist of one molecule of positive-sense, single-stranded RNA that has a molecular weight of 3.0×10^6 encapsidated by a single protein species with a molecular weight of 35,000 daltons. The thermal inactivation point of PMV is between 55 - 64 C, and the dilution end point is between 10^{-3} - 10^{-4} (5). There are numerous isolates of PMV. Biological properties, nucleic acid hybridization and serological relationships have been used to try to differentiate isolates. In one study, five strains of PMV were distinguished by symptomatology on peanut (19). Results from nucleic acid hybridization and serological tests indicated no difference among PMV isolates (4). Several serological methods, including monoclonal antibodies, have been used for the detection of peanut mottle virus (9, 23).

The spread of PMV is facilitated by infected peanut seed and aphids. PMV can be detected in 0.3-0.8% of seed harvested from an infected peanut plant. The amount of transmission is dependant on virus strain, peanut cultivar, and environment. PMV is not seed transmitted in _V_. _unguiculata_ (L.) Walp. Subs. _unguiculata_, soybean, _P_. _sativum_ L. and _Cassia obtusifolia_ L. PMV-infected peanut seed provides a primary source of inoculum of PMV for peanut and soybean in the field (14). PMV is transmitted by aphids between peanut plants and between peanut and other leguminous crops. PMV is transmitted in a nonpersistant

manner by several aphid species that include *Aphis craccivora* Koch, *A. gossypii* Glover, *Hyperomyzus lactucae* L., *Myzus persicae* Sulzer, *Rhopalosiphum padi*, and *R. maidis* (15). Because no resistance to PMV has been found in *A. hypogaea* (1, 14, 17), the use of PMV-free seed is one of the most effective ways to control this disease.

Tissue culture, thermotherapy and chemotherapy have been used to eliminate viruses from plants. The plant meristem was first established to be free of invading viruses by Limasset and other workers in the late 1940s (12). Meristem culture was first used to produce virus free stocks of dahlias (*Dahlia* spp.) and potatoes (*Solanum tuberosum* L.) in the 1950s (13, 24). Several other important vegetatively propagated food crops, e.g., cassava (*Manihot esculenta* Crantz.), sweet potato (*Ipomea batatas* Poir.), and taro (*Colocasia esculenta* L.), have been freed of viral pathogens by meristem culture (12). Success in eliminating viruses by meristem culture depends on the size of the meristem-tip and the host-virus combination. Shoot-tip explants (1-5 mm) are generally easier to excise and have a higher survival rate *in vitro* than meristem explants (less than 0.5 mm) (12). The application of these techniques to *A. hypogaea* has been limited. Kartha (11) and Russo (22) reported on the utilization of meristem and shoot-tip culture of peanut from plants grown *in vitro*. No reports were found on tip culture of greenhouse grown peanut for the elimination of peanut viruses.

One of the earliest records of the successful use of

thermotherapy, also called heat treatment, was the elimination of potato leaf curl from potato tubers by heating them to 37 C for 25 days (24). Hollings (1965) has listed about 90 plants diseases successfully treated by heat therapy (10). Nyland (18) first reported that viruses in carnation (Dianthus caryophyllus L.) stocks could be eliminated by growing at 40 C for 1 month before tips were isolated.

Since Commoner and Mercer in 1951 found that the nucleic acid base analogue thiouracil inhibited the multiplication of tobacco mosaic virus (TMV) in detached leaves (6), some reports about chemotherapy of plants infected with viruses have appeared. Unfortunately, many materials can only reduce virus concentration in plant meristems and do not eradicate virus (25). Recently, it was reported that chemotherapy of plants infected with TMV was less effective than thermotherapy and tissue culture (21).

No effective way to eliminate viruses from Arachis spp. has been demonstrated. It has been the purpose of this research to develop a system for regeneration of peanut plants from meristematic tissue; and evaluate tip culture, thermotherapy, and chemotherapy for elimination of PMV from infected plants.

This thesis includes two manuscripts, each representing an independent chapter written in a format that will facilitate their submission for publication to a national scientific journal. The approval of this format is in accordance with the Graduate College Policy of accepting a

thesis in manuscript form.

Chapter II, is entitled "Regeneration of Peanut (Arachis spp.) by Shoot-tip Culture". This chapter compares the regeneration of peanut meristem and peanut shoot-tip, and has been submitted to "Peanut Science". Chapter III, entitled "Elimination of Peanut Mottle Virus by Tip Culture, Thermotherapy and Chemotherapy", provides the first report of virus elimination from peanut germplasm, and will be submitted to "Plant Disease".

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CHAPTER II

Regeneration of Peanut (Arachis spp.) by Shoot-tip Culture

ABSTRACT

Shoot-tips of peanut (Arachis hypogaea L.) cultivars "Florunner" and "Pronto" grown in the greenhouse, and the tips of A. villosulicarpa from in vitro cultured plants were used for in vitro regeneration of peanut. The terminal and lateral buds excised from greenhouse grown plants were surface sterilized with 70% ethanol for 3 min followed by 0.525% sodium hypochlorite for 5 min. Shoot-tips 0.5-3 mm long were isolated from the buds, transferred to a modified Murashige-Skoog (MS) agar medium, and maintained at 26 C with a 16-h photoperiod. The modified MS medium contained MS mineral salts, B5 vitamins, and the hormones naphthaleneacetic acid (NAA) and 6-benzyladenine (BA). The best shoot growth of small shoot tips (0.5-1 mm) occurred with 5.0 uM of NAA and 5.0 uM of BA. Rooting occurred with 5.0 uM of NAA. Both shoot growth and rooting occurred on medium that contained 5.0 uM of NAA when large tips (1-3 mm) were cultured. Plantlets regenerated from the tips were successfully transferred to pots and grown to maturity.

INTRODUCTION

Shoot-tip culture and meristem culture have been used with many legumes for obtaining virus-free plants, for clonal propagation of plants, and for the conservation of germplasm. Meristem culture has been used for elimination of pea seed-borne mosaic virus from over 100 breeding lines of *Pisum sativum* L. (11, 12, 13), and for obtaining virus-free *Trifolium* plants (7). Many legumes have been clonally propagated from either root or shoot meristems. The species include *Trigonella foenum graecum* L., *Vigna unguiculata* L. (4), *Pisum sativum* L., *Cicer arietum* L., *Lens esculenta* Moench, *Phaseolus aureus* Roxb, and *P. mungo* L. (3). Cultured meristems and *in vitro* grown plantlets are being used for the exchange of and conservation of germplasm because they are generally pathogen free (4). Meristems or shoot-tips of about 20 species have been stored in liquid nitrogen and subsequently cultured (20). Cryopreservation of meristems may permit the long-term storage of germplasm in a genetically stable and pathogen-free condition (20).

Success in obtaining plants from meristems or shoot-tips usually depends on the size of the starting material and the culture conditions. Shoot-tip explants (1-5 mm) are generally easier to excise and have a higher survival rate *in vitro* than meristem explants. The addition of NAA will induce complete plant formation from shoot pieces of several legumes (3). Although meristem and shoot-tip culture has been used in a variety of species (21), the application of

these techniques to peanut (Arachis hypogeae L.) has been limited. Kartha (13) and Russo (19) reported on the utilization of meristem and shoot-tip culture of peanut from plants grown in vitro. No reports appear to have been published on tip culture of greenhouse grown peanuts. Shoot tip cultures could be an important source of material as many germplasm collections are maintained in greenhouses or under non-aseptic conditions. In addition, information on the response of shoot-tips of peanut to BA and NAA is lacking. This study was undertaken to investigate the requirements for culture and regeneration of plants from meristems and shoot-tips of two cultivars of peanut and A. villosulicarpa L. A preliminary report has been published (6).

MATERIALS AND METHODS

Seeds of the peanut cvs. "Florunner" (a runner type) and "Pronto" (a spanish type) were planted in soil (80% commercial soil mix, 20% sand) in the greenhouse and the shoots were collected from 3 week-old plants. After removing the leaves, the shoots were rinsed in tap water with Tween 20 (1%) for 5 min, 70% ethanol for 3 min, and 0.525% sodium hypochlorite for 5 min. This was followed by three rinses with sterile water. The buds were excised from the shoots under aseptic conditions. Meristems (0.5-1 mm) and shoot tips (1-3 mm) were isolated from the buds and cultured in small test tubes (1.8 x 15 cm) containing 10 ml of nutrient medium. Shoot-tips and meristems of A.

villosulicarpa were isolated from plants maintained as previously described in vitro (18).

The medium consisted of MS mineral salts (15), 3% sucrose, vitamins (9), 0.8% agar, and BA and/or NAA were added in various concentrations. The pH of the medium was adjusted to 5.7 with KOH or HCl prior to adding the agar. The medium was then autoclaved at 120 C for 15 min. After autoclaving, filter sterilized ampicillin (25 mg/mL) was added to the medium to a final concentration of 80 mg/L.

All cultures were maintained at a constant temperature of 26 C with a 16-h photoperiod, and at a light intensity of 67 uE M^{-2} sec^{-1}. Plantlets were transferred to a pot containing a sandy loam soil and covered with transparent plastic for one week to maintain a high humidity.

RESULTS

Ten meristems from each cultivated genotypes were placed on one of six media supplemented with different concentrations of BA or NAA. The 120 meristems were maintained as indicated and results recorded after 50 days (Table 1). Large shoots (1-3 cm) from both cultivated genotypes were produced from meristems on the medium containing 5.0 uM NAA and 5.0 uM BA. However, no roots formed on these shoots on this medium. Roots, but not shoots, were formed from meristems on a medium containing 10 uM NAA and 0.1 uM BA. No single medium induced both roots and shoots.

Because shoots regenerated from meristems failed to

produce roots, they were transferred to other media with different concentrations of NAA and BA for the induction of roots (Table 2). Initially there were 3 replicates for each genotypes on each of 25 different media. Based on observations after 50 days of the 225 explants, the best root formation and shoot enlargement occurred on the medium that contained only 5.0 uM NAA. Roots formed 20 days earlier for the shoots of "Florunner" and "Pronto" than for the shoots of _A_. _villosulicarpa_. In order to assure that induction of roots occurred on media supplemented only with NAA, another 30 explants were cultured on each of the four different concentrations of NAA. Again, the best growth was obtained in the 30 explants of each genotype cultured on the medium that contained 5.0 uM NAA. A greater percentage of cultivated genotypes rooted than _A_. _villosulicarpa_. In an additional experiment on rooting of shoots on media with 5.0 uM NAA, all 150 cultivated shoots of each cultivated genotype rooted, but only 80 out of 100 cultured shoots of _A_. _villosulicarpa_ rooted.

Based on the results from the culture of meristems, different NAA and BA combinations were used to develop a medium for the development of shoots and roots from shoot tips (1-3 mm). Three terminal buds and three lateral buds from each cultivar were placed on each of 25 different media. After 50 days, the medium containing NAA proved more satisfactory for shoot-tip culture (Table 3). The best growth of shoot tips was found in the medium containing 5.0 uM NAA. There were minimal differences in the growth of

terminal buds and lateral buds from either cultivar. A similar experiment was conducted with terminal buds from _A. villosulicarpa_ on each of the five media containing different concentrations of NAA. Five shoot tips were cultured on each of the five media. As with the cultivated genotypes, the medium containing 5.0 uM of NAA was the most effective. There were growth differences between the genotypes. Within one month the shoot tips of "Florunner" and "Pronto" had grown larger than the shoot tips of _A. villosulicarpa_.

Discussion

A previous study (13) found that a medium containing 0.1 uM BA and 10 uM NAA was good for meristem culture of peanut. However, in this study no whole plantlets were formed when meristems from greenhouse grown cultivated peanuts and _in vitro_ cultured _A. villosulicarpa_ were transferred to a medium supplemented with 0.1 uM BA and 10 uM NAA.

The results of this study indicate that plant regeneration from peanut meristems from two cultivated and a wild genotype is best achieved in two steps. The first step, shoot growth, is induced on medium supplemented with 5.0 uM NAA and 5.0 uM BA. The second step, root formatin, is then induced on a medium supplemented with 5.0 uM NAA. Rooting of larger shoot tips from the three genotypes required medium supplemented with only 5.0 uM NAA. Other researchers (2, 5, 16, 17, 18) have found a medium

supplemented with NAA alone stimulates root production of explants from peanut. This is true of both cultivated (2, 5, 16, 17) and wild (5, 17, 18) genotypes. This is the first report of growth of shoot-tips of peanut on a medium which is supplemented with only NAA.

There are several advantages to using shoot-tip culture instead of meristem culture to propagate peanut. First, shoot tip culture is less laborous than meristem culture. No subculture is required when larger tips are cultured *in vitro*. Secondly, plants regenerate more rapidly. Once root production is stimulated in cultured shoot-tips, shoot enlargement and growth rapidly follow. This response has also been observed in other species (3). Finally plants regenerated from meristems are generally more true-to-type than plants regenerated from callus or cell suspensions (10).

Peanut is an important source of protein for food and livestock feed production, and peanut production can suffer from diseases. Some of these diseases, e.g. peanut mottle virus (PMV), peanut stripe virus (PStV), and *Sclerotinia*, may be seed-transmitted or spread by germplasm exchange (1, 8, 14, 22). Shoot-tip culture has been reported to be useful in virus elimination, clonal propagation and the conservation of germplasm. The application of this technique to peanut may contribute to the control of virus disease and improvement of peanuts.

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Table 1. Effects of different combinations of regulators on meristem culture of peanut [a]

Medium[b]		
BA (uM)	NAA (uM)	Results[c]
0.1	10	Callus and roots
1	10	Callus
5	10	Callus and multiple shoots
5	5	Multiple shoots
1	5	Multiple shoots
1	1	Single small shoots

[a] Meristems were from peanut cultivars "Florunner" and "Pronto".

[b] Medium contained MS mineral salts (14), B5 vitamins (9), 3% sucrose and 0.8% agar.

[c] Results were recorded after 50 days of meristem culture.

Table 2. Effects of different combinations of growth regulators on development and differentiation of shoots of different genotypes of peanuts[a]

Medium[b] (uM)		Genotype		
BA	NAA	cv. Florunner	cv. Pronto	_A. villosulicarpa_
0	0	Small shoots	No response[c]	Small roots
0	2.5	Strong roots	Long roots	Short roots
0	5.0	Strong roots	Short roots	Callus, fine roots
0	7.5	Strong roots	Short roots	Strong root, callus
0	10	Fine roots	Callus	Small fine roots
0.05	0	No response	No response	Shoots, little callus
0.05	2.5	No response	No response	Shoots, callus
0.05	5.0	No response	No response	Shoots, callus, roots
0.05	7.5	Callus	Little callus	Strong roots, callus
0.05	10	Little callus	Little callus	Callus
0.5	0	No response	Shoots	Shoots, callus
0.5	2.5	Small root	Shoots, callus	Small shoots, callus
0.5	5.0	Callus	Callus	Callus
0.5	7.5	Callus	Callus	Shoots, callus
0.5	10	Callus	Callus	Shoots, much callus
1.0	0	No response	One new shoot	Shoots, callus
1.0	2.5	Callus	Multiple shoots	Much callus
1.0	5.0	Callus	Shoots	Shoots, much callus
1.0	7.5	Callus	Callus	Much callus
1.0	10	Shoot, callus	Shoots, callus	Little callus
5.0	0	No response	No response[c]	Shoots, much callus
5.0	2.5	Shoots	Shoots	Much callus
5.0	5.0	Callus	Shoots	Much callus
5.0	7.5	Callus	Callus	Callus
5.0	10	Shoots, callus	Shoots, callus	Callus

a. Each treatment consisted of three replicates. They all had the same results. The results were from 50 days of culture.

b. Medium contained MS mineral salts (14), B5 vitamins (9), 3% sucrose and 0.8% agar.

c. No root, callus and shoot enlargement occurred.

Table 3. Comparison of bud source and different combinations of growth regulators on shoot tip culture of peanut[a]

Medium[b] (uM) BA	NAA	Terminal bud	Lateral bud
0	0	Small shoot	Small shoot
0	0.05	Large shoot	Large shoot
0	0.5	Large shoot	Small shoot
0	1.0	Large shoot, root	Large shoot, root
0	5	Strong root, shoot	Shoot, root
0.05	0	Large shoot	Shoot, callus
0.05	0.05	Small shoot	Shoot, callus
0.05	0.5	Small shoot	Large shoot, callus
0.05	1.0	Large shoot, callus	Large shoot, little callus
0.05	5.0	Much callus	Root
0.5	0	Little callus, shoot	Large shoot
0.5	0.05	Large shoot, callus	Small shoot
0.5	0.5	Shoot, little callus	Shoot, callus
0.5	1.0	Large shoot, callus	Large shoot
0.5	5.0	Callus, one shoot	Small shoot
1.0	0	Large shoot, much callus	Big shoot
1.0	0.05	Shoot, little callus	Much callus
1.0	0.5	Large shoot, callus	Shoot, much callus
1.0	1.0	Large shoot, callus	Callus
1.0	5.0	Shoot, callus	Shoot, callus
5.0	0	Large shoot	Small shoot
5.0	0.05	Shoots	Large shoot, callus
5.0	0.5	Small shoots, callus	Small shoot
5.0	1.0	Small shoots, callus	Callus
5.0	5.0	Small shoots, callus	Callus, shoot

a. Each treatment has three replicates. The shoot tips were from peanut cultivars "Florunner" and "Pronto".
b. Medium contained MS mineral salts (14), B5 vitamin (9), 3% sucrose and 0.8% agar.

CHAPTER III

ELIMINATION OF PEANUT MOTTLE VIRUS BY TIP CULTURE, THERMOTHERAPY AND CHEMOTHERAPY

ABSTRACT

Peanut (*Arachis hypogaea* L.) plants (cvs. "Florunner" and "Pronto") were inoculated at the 2 leaf stage with peanut mottle virus (PMV) to obtain PMV-infected plants. Shoot tips from plants grown in the greenhouse (approximately 27 C) or from plants maintained at 35 C were used for tip culture. In experiments when ribavirin was used, it was added to the culture medium at 5 mg/l, 10 mg/l, 15 mg/l or 20 mg/l. No plants regenerated from shoot-tips taken from virus infected plants were found virus-free. After 45 days at 35 C, 93% of "Florunner" and 95% of "Pronto" tested negative for PMV by ELISA of foliar tissue. When shoot tips from the plants that tested negative by ELISA were used for tip culture, no virus-free plants were obtained. No virus-free plants were obtained from tips cultured on medium supplemented with ribavirin. However, combining tip culture, thermotherapy and chemotherapy, 80% of "Florunner" and 100% of "Pronto" were negative for PMV.

INTRODUCTION

Tissue culture, thermotherapy and chemotherapy have been used to eliminate viruses from plants. Meristem culture has been used to obtain virus-free plants (17,26, 36, 37). Several viruses have been eliminated from legume crops using meristem culture. These include elimination of pea seed-borne mosaic virus from Pisum sativum L.(24); and clover yellow vein virus, white clover mosaic virus, alfalfa mosaic virus and peanut stunt virus from Trifolium repens L.(3, 8).

Heat treatment is also an effective method for freeing plant material of viruses. Over 100 viruses have been eliminated from at least one host plant by heat treatment (25). However, the results vary with which part of the plant is treated, the virus, and the method of heat treatment. Sugarcane (Saccharum officinale L.) has been successfully freed of ratoon stunting disease by treating sugarcane sets in hot water (7). Prunus necrotic ringspot virus can be eliminated by growing infected cherry (Prunus spp.) for more than three weeks at 40 C (21).

Chemotherapy has also been reported to be effective in elimination of virus from infected tissue. A synthetic riboside, ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), also known as virazole, has been reported to have activity against several plant viruses (6, 11, 23). Virus-free Prunus serrulata Cult. were obtained after spraying plants weekly with 500 ppm ribavirin (10).

Raspberry bushy dwarf virus (RBDV) was eliminated from red raspberry (Rubus ideaus L.) in tip culture on medium containing 0.4 uM ribavirin (19).

In cases where viruses are difficult to eliminate by meristem culture alone, or where viruses are resistant to thermotherapy or chemotherapy; a combination of thermotherapy, chemotherapy and tip culture has proven beneficial. Tip culture alone, or tip culture with thermotherapy has been used to free 136 potato (Solanum tuberosum L.) cultivars of viruses (26). Other viruses have also been eliminated by a combination of tip culture and thermotherapy (2, 5, 16, 18). A combination of chemotherapy and tip culture has been used for the elimination of RBDV from red raspberry, potato virus Y (PVY) and S (PVS) from potato, and tobacco mosaic virus (TMV) from tobacco (Nicotiana tobacum L.)(19, 22, 35).

Since peanut mottle virus (PMV) was first detected in peanut (Arachis hypogaea L.) in 1965 (20), PMV has become an important pathogen in all major peanut producing areas of the world (2, 4, 9, 12, 14, 24, 30). PMV causes yield losses of 5-6% and may alter the chemistry of the peanut seed (20). No resistence to PMV has been found in cultivated peanut (1, 20, 27). PMV-infected peanut seed provides a primary source of inoculum for infection of peanut in the field. PMV can be found in 0.3-8.5% of the seed from an infected plant. The use of PMV-free seed is probably the most effective way to control this disease.

Germplasm collections of Arachis spp. are an important

source for new genetic resistance for use in peanut breeding programs. Some wild species of _Arachis_ maintained in collections are susceptible to PMV and may harbor different strains of the virus (31, 34). Some of this germplasm material can only be vegetatively propagated. Therefore, methods for elimination of PMV from peanut and related species are needed. The objective of this research was to investigate the applicability of tip culture alone, or in conjunction with thermotherapy and/or chemotherapy in the elimination fo PMV from peanut.

MATERIAL AND METHODS

Source of infected material. PMV-infected _A. hypogeae_ cv. Florunner and Pronto were obtained by inoculating plants at the 2-leaf stage with PMV. Inoculum was made by grinding infected pea tissue (1 g/ml) in 0.01 M phosphate buffer (pH 8.0). Host plants were inoculated by rubbing leaves previously dusted with 225 um corundum. Approximately two weeks after inoculation, when plants expressed symptoms of infection, the plants were tested by ELISA as outlined below. From the plants tested positive by ELISA, some were subjected to thermotherapy as outlined below before tips were isolated, and some were used for tip culture directly. Uninoculated plants were used for tip culture for comparison with the diseased plants. Both cultivars were used in each experiment.

Virus detection. An ELISA using both a polyclonal antibody (PAB) and a monoclonal antibody (MAB) was used for

detection of PMV as previously reported (32). Plates were coated with 1 ug/ml of IgG from rabbit anti-PMV polyclonal serum. Tested samples (0.2 g/ml) were ground in PBS-Tween (phosphate buffered saline with 0.05% Tween 20) containing 2% polyvinyl pyrrolidone (PVP). Samples were added to the plate for incubation overnight at 4 C. For detection of bound virus, MAB culture supernatant diluted with PBS-Tween containing 2% PVP and 0.2% ovalbumin was added, followed by alkaline phosphatase labeled goat anti-mouse antibody at the manufacturer's recommended working dilution. P-nitrophenyl phosphate in diethanolamine substrate buffer was added to detect the bound virus. Plates were incubated 1 hour before reading in a Bio-Tek EIA plate reader (Bio-Tek Instruments, Inc., Burlington, VT). A sample was considered positive if the ELISA reading was higher than the average reading of three healthy control samples plus three standard deviations. In addition to ELISA, bean (Phaseolus vulgaris L. cv. Topcrop) was used as an indicator host for detection of PMV in the regenerated plants.

Tip culture. Under aseptic conditions, leaves were removed from shoots and the shoots were then surface sterilized as follows. Shoots were washed sequentially in 500 ml sterile distilled water containing a few drops of Tween-20 for 5 minutes, 70% ethanol for 3 minutes, 0.525% sodium hypochlorite for 5 minutes, and finally rinsed 3 times in sterile distilled water. Tips approximately 0.5 or 1-3 mm in length were dissected from the surface sterilized shoots and transferred to culture medium. The medium

contained MS mineral salts (29), B5 vitamins (13), 3% sucrose, 1 mg/L NAA and 8 g/L agar. The medium was adjusted to pH 5.6 and then autoclaved at 120 C for 15 min. Once tips were transferred to the medium, they were placed in incubators at 27 C with a 16-h photoperiod and a light intensity of 67 uE $m^{-2}sec^{-1}$.

Thermotherapy. PMV-Infected plants were grown at 35 C with a 15-h photoperiod and a light intensity of 67 uE m^{-2} sec^{-1}. After 30 days, leaves were tested for PMV by ELISA, and the tips next to the negative leaves were isolated and cultured as outlined above.

Chemotherapy. The culture medium used for tip culture was autoclaved and cooled before adding filter-sterilized ribavirin (Sigma Chemical Co. St. Louis, MO.) to a final concentration of 0, 5, 10, 15, or 20 mg/l. Shoot tips from either uninfected or PMV-infected plants were placed on the medium and incubated as outlined above.

RESULTS

Tip culture. Plants regenerated from cultured tips were not found to be free of PMV (Table 1). The plants regenerated from small tips (0.5 mm) did not show symptoms of infection by PMV, but tested positive for PMV in ELISA. Plants regenerated from large tips (1-3 mm) showed symptoms of infection by PMV, but they regenerated more rapidly than those from small tips.

Thermotherapy. The newly formed leaves from heat-treated plants were tested for PMV by ELISA. The longer the

plants were subjected to the heat treatment the greater the percentage of plants that were negative for PMV by ELISA (Table 2). Plants that had leaves that were negative for PMV were taller than plants that had leaves that tested positive for PMV. The results from ELISA indicated that the percentage of leaves positive for PMV varied with the age of the leaves (Table 3). The oldest leaves in the treated plants had the lowest percentage of leaves positive for PMV. The youngest leaves had a lower percentage of PMV positive leaves than the middle leaves. This indicated that perhaps virus-free plants could be obtained by culturing buds next to the leaves that did not test positive for PMV.

After the leaves of treated plants were indexed for PMV, the buds by the leaves that were negative for PMV were used for tip culture. Although the plants regenerated from the selected buds did not show symptoms of PMV infection, they were positive for PMV in indicator host tests and ELISA (Table 4).

Chemotherapy. Leaves from plants regenerated on medium containing ribavirin were tested for PMV by ELISA (Table 5). Plants from tips cultured on medium with 5, 10, 15 mg/l ribavirin showed symptoms of PMV infection. Plants from tips cultured on medium with 20 mg/l ribavirin showed no symptoms of PMV infection, but they were positive for PMV in indicator host test and ELISA. No virus-free plants were obtained from tips subjected to chemotherapy.

Combination of tip culture, thermotherapy, and chemotherapy. Since no virus-free plants were obtained by

tip culture alone, or a combination of tip culture and thermotherapy or chemotherapy; a combination of tip culture, thermotherapy and chemotherapy was tested. The shoot-tips from plants that had been heat treated for 30 days and that indexed negative for PMV were subsequently cultured on medium containing 20 mg/l ribavirin. Regenerated plants did not show any symptoms of PMV infection and most of the plants were negative for PMV in indicator host tests and ELISA (Table 6). From the plants obtained, 80% of "Florunner" and 100% of "Pronto" were negative for PMV.

DISCUSSION

The results indicate that tip culture, or a combination of tip culture and thermotherapy or chemotherapy, was not effective in eliminating PMV from peanut plants. It has been reported that viruses might, to a varying extent, invade meristematic cells. This depends on the virus and host species (28). Viruses which are present in the meristem at relative high concentrations, e.g. TMV, cherry leaf roll virus and cucumber mosaic virus, are not eradicated by meristem culture alone (33). PMV could not be eliminated by meristem culture or tip culture alone.

Although thermotherapy has been an effective method for elimination of some viruses, the results vary from different parts of plants, kinds of viruses, and methods of heat treatments. It was reported that growing tissue subjected to a prolonged hot air treatment at 30-40 C was the most effective method of eliminating viruses by thermotherapy

(15). In this study, the percentage of leaves testing positive for PMV by ELISA decreased after the plants were grown at 35 C for 30-45 days. However, plants, regenerated from tips next to leaves that tested negative for PMV, were positive for PMV by ELISA. This may indicate that the leaves negative for PMV after thermotherapy contained virus, but below the level of detection by ELISA. The virus then increased in the plantlet after tip culture, and no virus-free plants were obtained.

Ribavirin has been shown to have activity against RBDV, PVY and PVS. Solid media containing different concentrations of ribavirin were used in this study. Although the concentration of PMV decreased in the plants regenerated on medium containing ribavirin, PMV was not completely eradicated. Virus eradication may not be obtained when shoot-tips are placed on solid media containing ribavirin (35). This could be due to the inability of the chemical to diffuse rapidly enough into the developing tissues. Virus-free plants might be obtained by culturing tips in liquid medium containing ribavirin, but this was not done in this study. Equal concentrations of ribavirin were found to be more phytotoxic to tips from infected peanut plants than tips from uninfected peanut plants. There may be several factors that control the efficacy of ribavirin for virus elimination. These could include the concentration of ribavirin, the effects of ribavirin on PMV, and the application method. Higher concentrations of ribavirin may be useful for elimination of

PMV if phytoxicity problems can be circumvented. Addition of ribavirin as a spray may be one possible approach.

It has been reported that a combination of tip culture, thermotherapy and chemotherapy can be beneficial in the elimination of viurses (35). Although PMV was not eradicated by tip culture alone, or a combination of tip culture and thermotherapy or chemotherapy; these treatments resulted in lower amount of PMV in plants according to ELISA readings. Virus-free plants were obtained when a combination of tip culture, thermotherapy and chemotherapy were used. Methods for the elimination of PMV in peanut seed or for the control of PMV in vegetatively propagated peanut germplasm has not been reported. The methods used in this study may be useful for eliminating PMV and other viruses from peanut.

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Table 1. Detection of peanut mottle virus (PMV) by ELISA in *Arachis hypogaea* L. regenerated by tip culture of previously infected plants[a] .

Cultivar	Size of tips	Number of plants	Plants positive for PMV
Florunner	0.5 mm	10	10
Pronto	0.5 mm	10	10
Florunner	1-3 mm	10	10
Pronto	1-3 mm	10	10

[a] The results from the culture of tips (0.5 mm) were obtained 1 month later than the results of tips (1-3 mm).

Table 2. Detection of peanut mottle virus (PMV) by ELISA in *Arachis hypogaea* L. subjected to thermotherapy[a].

Cultivar	Duration of treatment (day)	Number of plants	Number of plants and percentage negative for PMV
Florunner	30	30	15 (50%)
Florunner	45	45	42 (93%)
Pronto	30	20	11 (55%)
Pronto	45	62	59 (95%)

[a] PMV-infected peanut plants were grown in the chamber (35 C) for different duration time. The youngest leaves of these plants were tested by ELISA.

Table 3. Detection of peanut mottle virus (PMV) by ELISA in different leaves of *Arachis hypogaea* L. subjected to thermotherapy[e].

Cultivar	Number of plants	Percentage of leaves negative for PMV (%)[a]		
		Old[b]	Middle[c]	Young[d]
Florunner	10	90	0	40
Pronto	10	50	0	25

[a] Between 10 and 30 leaves were tested for each age.
[b] The first leaves produced at the beginning of thermotherapy.
[c] The leaves produced in the middle of thermotherapy.
[d] The last leaves produced at the end of thermotherapy.
[e] PMV-infected peanut plants were grown in the chamber (35 C) for 1 month. The leaves from different parts of the plants were tested by ELISA.

Table 4. Detection of peanut mottle virus (PMV) in *Arachis hypogaea* L. subjected to thermotherapy at 35 C and regenerated by tip culture.

Cultivar	Number of plants	Number of plants negative for PMV		
		Symptom	Indicator host[a]	ELISA
Florunner	5	5	0	0
Pronto	5	5	0	0

a. Bean (*Phaseolus vulgaris* L. cv. Topcrop).

Table 5. Detection of peanut mottle virus (PMV) in Arachis hypogaea L. regenerated by tip culture on medium supplemented with ribavirin.

Variety	Number of plants	Ribavirin (mg/l)	Number of plants negative for PMV		
			Symptom	Indicator host[a]	ELISA
Florunner	5	5	0	0	0
Florunner	5	10	0	0	0
Florunner	5	15	2	0	0
Florunner	5	20	5	0	0
Pronto	5	5	0	0	0
Pronto	5	10	0	0	0
Pronto	5	15	0	0	0
Pronto	5	20	5	0	0

a. Bean (Phaseolus vulgaris L. cv. Topcrop).

Table 6. Detection of peanut mottle virus (PMV) in *Arachis hypogaea* L. regenerated by tip culture and subjected to chemotherapy[a] and thermotherapy[b].

Variety	Number of plants	Number of plants negative for PMV		
		Symptom	Indicator host[c]	ELISA
Florunner	5	5	4	4
Pronto	5	5	5	5

[a] Ribavirin at 20 mg/l.
[b] Grown at 35 C for 30 days.
[c] Bean (*Phaseolus vulgaris* L. cv. Topcrop).

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