Virus Elimination from Interspecific *Arachis* Hybrids

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ABSTRACT

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Some germ plasm collections of *Arachis* species and hybrids are maintained vegetatively. This study was conducted to develop protocols to eliminate virus from vegetatively maintained peanut. The culture of shoot meristems was effective for virus elimination from interspecific *Arachis* hybrids. Peanut mottle virus (PMV), peanut stripe virus (PStV), and tomato spotted wilt virus (TSWV) were not detected by DAS-ELISA in any plants regenerated from meristems treated with thermotherapy alone or combined with chemotherapy. Only 2.0% of the plants regenerated from untreated meristems contained PMV, and none contained PStV or TSWV. Shoot tip culture was not as effective as meristem culture for elimination of PMV. Plants regenerated from untreated shoot tips (1 cm long) of *Arachis* hybrids remained infected with PMV, whereas 38% of the plants regenerated from shoot tips treated with thermotherapy plus chemotherapy were infected with PMV. *Arachis* hybrids were more readily freed of TSWV and PStV than of PMV. Meristem culture, thermotherapy, and chemotherapy were not required for elimination of TSWV and PStV, and plants regenerated from untreated shoot tips were free of these viruses.

A number of wild peanuts (*Arachis* spp.) and interspecific *Arachis* hybrids are maintained vegetatively in germ plasm collections to conserve important economic traits, including resistance to diseases, mites, and insects (13). Once introduced, viruses can spread throughout these collections by insect vectors or routine handling of plants. With the introduction of germ plasm from around the world, exotic viruses that could threaten local crop production also may be introduced. Therefore, techniques for the detection and elimination of viruses are important parts of plant germ plasm introduction (11).

Techniques have been developed for detecting virus in individual peanut seeds without destroying seed viability (2,9). These techniques have been used to select virus-free seed of hundreds of introduced peanut cultivars, breeding lines, and commercial seed lots (8,22). However, techniques for virus elimination from peanut seed cannot be used with many wild peanut species and interspecific *Arachis* hybrids that are propagated and maintained vegetatively. Vegetative propagation is necessary because the plants do not flower, produce few or no seeds, or produce seeds that do not germinate after 1–2 yr in storage. The USDA Southern Regional Plant Introduction Station maintains over 440 accessions of wild peanuts, and 20–25% of these accessions were determined to be infected with one or more viruses.

Meristem and shoot tip cultures have been used to eliminate virus from a number of economically important species (24), but few studies of these techniques have been reported on *Arachis* species (1,5,18,23). These reports have included only two *Arachis* species, *A. hypogaea* L. and *A. villosulicarpa* Hoehne, and both of these species are easily propagated by seed. The meristems and shoot tips used in these studies were from 4-day-old seedlings to 4-wk-old plants of *A. hypogaea* and micropropagated shoots of *A. villosulicarpa*. Protocols are needed for plant regeneration from meristems of vegetatively maintained *Arachis* species and hybrids and for elimination of virus from *Arachis* germ plasm collections.

Meristem culture, thermotherapy, and chemotherapy have been used separately and in combination to eliminate viruses from plants (3,6,14–17,19). In the only report of virus elimination from *Arachis* plants, Chen and Sherwood (4) observed that a combination of shoot tip culture, thermotherapy, and chemotherapy was required to eliminate peanut mottle virus (PMV) from *A. hypogaea*. Our investigation was conducted to evaluate thermotherapy, chemotherapy, and meristem culture for elimination of virus from vegetatively propagated *Arachis*

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germ plasm lines maintained at the Southern Regional Plant Introduction Station.

MATERIALS AND METHODS

Shoot explants (3–6 cm long) from greenhouse-grown interspecific *Arachis* hybrids (Table 1) were washed in 70% ethanol for 1 min, then soaked for 10 min in a 0.1% (w/v) $HgCl_2$ solution with 10 drops per liter of Tween 20. Explants were then rinsed three times with sterile water and placed upright with the internode in MSB5 medium (Murashige and Skoog [MS] salts [21], B_5 vitamins [12], and 20 g/L of sucrose, adjusted to pH 5.8 and solidified with 8 g/L of gum agar, plant cell culture tested) that had been autoclaved for 15 min at 120 C and 105 Kg cm^{-2} in 25 × 150 mm culture tubes (15 ml medium per tube). Cultures were incubated at 25 C, with a 16-hr photoperiod, at 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lamps. Shoots were maintained in vitro by subculturing stem pieces (3–6 cm long) with one or more nodes to fresh MSB5 medium every 40–60 days.

Thermotherapy was applied to in vitro propagated shoots by incubating them at 35 C for 30–40 days. Apical meristems (meristem with zero or one leaf primordium) from micropropagated shoots were placed on 20 ml of an MSB5 medium supplemented with 1 mg/L each of α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) in 100 × 25 mm petri dishes (meristem media) and were incubated for 50 days at 25 C. Meristem cultures were transferred to MSB5 medium (15 ml per culture tube) supplemented with 1 mg/L of BAP to induce shoot elongation. After 30–60 days, shoots were transferred to MSB5 medium without phytohormones to induce rooting.

Nodal explants from greenhouse-grown *Arachis* hybrids 1–4 were cultured on MSB5 medium; MSB5 medium with 1 mg/L of BAP and 0.02 mg/L of NAA; MSB5 medium with 1 mg/L of BAP and 30 g/L of sucrose; MSB5 medium with 1 mg/L of BAP, 0.1 mg/L of NAA, and 30 g/L of sucrose; and MSB5 medium with 1 mg/L each of BAP and NAA and 30 g/L of sucrose to select a medium for micropropagation. The percentage of nodal explants producing shoots and roots was recorded after 30 days in culture. The experimental design was random and factorial, with *Arachis* hybrid and medium as independent variables. There were 10 single-explant replicates per treatment, and the experiment was performed twice.

Meristems from micropropagated shoots of *Arachis* hybrids were placed on meristem medium supplemented with a filter-sterilized antiviral compound to evaluate different antiviral agents for in vitro virus elimination. Compounds and dosages tested were ribavirin (RBV; Viratek), 20, 30, and 40 mg/L; 5-azacytidine (AZA), 10, 20, and 30 mg/L; 3-deazauridine (DZD), 5, 20, and 30 mg/L; and phosphonoacetic acid (PPAA), 5, 10, 25, and 50 mg/L. The experimental design was completely random, with six to eight single-meristem replicates per treatment. The experiment was performed three times with hybrid 1 and once each with hybrids 2, 3, and 5. After 30 days in culture, the diameter of culture tissue was measured, meristems producing shoot buds were counted, and the percentage producing shoots was calculated.

Shoot tips (1 cm long) from micropropagated shoots of *Arachis* hybrids 1, 2, 3, and 5 maintained at 25 or 35 C for 30–50 days were cultured for 50 days on meristem medium supplemented with 40 mg/L of RBV, 30 mg/L of AZA, 30 mg/L of DZD, or 50 mg/L of PPAA to test the effect of thermotherapy, chemotherapy, or thermotherapy plus chemotherapy on virus elimination. Shoot tips also were cultured on meristem medium without antiviral agents. Shoot buds and small shoots (1–2 cm long) that developed from the shoot tips were transferred to MSB5 medium with 1 mg/L of BAP to induce shoot elongation. Shoots (3–6 cm long) that developed from the shoot tips were transferred to MSB5 medium for rooting. Plants that developed from meristem and shoot tip cultures were transferred to the greenhouse 4–6 wk after shoots were placed into MSB5 medium. Culture medium was washed from the roots of the plants, and the plants were planted in 10-cm-diameter Jiffy pots in a soil mix. Plants were acclimated to greenhouse conditions by placing them on a mist bench for the first week. The plants were tested after 8–24 wk in the greenhouse by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (7). The IgGs for PMV and peanut stripe virus (PStV) were prepared from polyclonal antisera. A monoclonal antibody to tomato spotted wilt virus (TSWV) was used. The DAS-ELISA reaction was read 30 min after addition of substrate for PMV and 45–60 min for PStV and TSWV. Absorbance values were measured at 410 nm with a Dynatech model MR 700 microplate reader. Two wells on each ELISA plate contained aliquots of sap extracted from virus-free leaves of peanut cv. Florunner. The mean absorbance value of these wells was used as a negative check to correct for background absorbance for each plate. Positive threshold was considered to be an absorbance reading of 0.1 over the negative check.

Table 1. Descriptions of interspecific *Arachis* hybrids

Hybrid[a]	Parental number and section[b]	Virus[c]
1 (Grif 126)	88 × (8 × 19): A3 × (A1 × A1)	PMV, PStV
2 (Grif 101)	16 × (21 × 16): E2 × (E3 × E2)	PMV, TSWV
3 (Grif 95)	3 × 16: E2 × E2	PMV, PStV
4 (Grif 194)	52 × 3: E2 × E2	PMV, TSWV
5 (PI 338282)	PI 276255 × *A. repens*: E2 × C	PMV

[a] Grif = Local number for the Southern Regional Plant Introduction Station, Griffin, Georgia; PI = plant inventory number.
[b] Parental numbers: 88 = *A. hypogaea* L., NC2 ('Guaranian' × 'Bolivian'); 8 = *A. spegazzinii* Krap. & Greg. *nom. nud.*; 19 = *A. batizocoi* Krap. & Greg. *nom. nud.*; 16 = *Arachis* species; 21 = *A. rigonii* Krap. & Greg. *nom. nud.*, 3 = *A. paraguariensis* Chod. & Hassl.; 52 = *Arachis* species. Sections: A = Arachis, E = Erectoides, C = Caulorhizae. Classification follows that of Gregory and Gregory (13).
[c] Before meristem or shoot tip culture, plants tested positive by DAS-ELISA for peanut mottle virus (PMV), peanut stripe virus (PStV), and tomato spotted wilt virus (TSWV).

Table 2. Shoot regeneration from nodes of virus-infected *Arachis* hybrids

Medium[a]	Nodes with shoots[b] (%)	Nodes with roots (%)
MSB5	74	36
MSB5 with 1 mg/L BAP and 30 g/L sucrose	64	0
MSB5 with 1 mg/L BAP and 0.02 mg/L NAA	54	0
MSB5 with 1 mg/L BAP, 0.1 mg/L NAA, and 30 g/L sucrose	63	0
MSB5 with 1 mg/L BAP, 1 ml/L NAA, and 30 g/L sucrose	38	0
LSD	14	

[a] MSB5 = Murashige and Skoog (21) salts, B_5 vitamins (12), 2% sucrose, and 8 g/L of agar.
[b] Shoots were counted 30 days after nodes were placed in culture. Treatment means were averaged over *Arachis* hybrids 1, 2, 3, and 4 (see Table 1).

RESULTS

In preliminary experiments, 100% of the nodal explants from vegetatively maintained *Arachis* spp. treated 20 min with 1% NaOCl solution had bacterial contaminants, whereas 40–75% of explants treated 10 min with 0.1% $HgCl_2$ solution had contaminants. Mercuric chloride was used to surface-sterilize all

subsequent explant material before culturing.

The effects of *Arachis* hybrids and medium were highly significant ($P > 0.001$, F test) on shoot regeneration from nodal explants, but the *Arachis* hybrids/medium interaction was not significant. The highest percentage of shoot regeneration from nodal explants was on MSB5 medium without phytohormones (Table 2). Roots developed from 36% of the explants on MSB5 medium without phytohormones, but roots did not develop from explants cultured on other media. *Arachis* hybrid 1 had a significantly higher percentage of explants producing shoots than the other hybrids tested ($P = 0.05$, LSD).

The chemotherapy treatments applied to the meristem cultures of *Arachis* hybrids affected their growth diameter and shoot bud production (Table 3). The percentage of meristems producing shoot buds and the growth of meristem cultures were affected by the concentration of PPAA in the meristem culture medium ($P < 0.01$, F test). The percentage of meristems producing shoot buds also was affected by the concentration of AZA, DZD, and RBV in the meristem medium ($P > 0.01$, F test), but the growth of cultures was not significantly affected by these chemicals at the concentrations tested. Plants of *Arachis* hybrids 1, 2, 3, and 5 were regenerated from meristems cultured on medium with all concentrations of AZA, DZD, and RBV tested. Only five plants were regenerated from the 35 meristems cultured on medium with 5 mg/L of PPAA, and shoots did not regenerate from meristems cultured on the media with higher levels of PPAA.

As determined by DAS-ELISA, 98% of the plants regenerated from meristems of *Arachis* hybrids 1, 2, 3, 4, and 5 were free of PMV and 100% were free of PStV and TSWV. All plants regenerated from meristems treated with thermotherapy or thermotherapy plus chemotherapy were free of PMV, TSWV, and PStV (Table 4). All plants regenerated from shoot tip culture alone or in combination with thermotherapy, chemotherapy, or thermotherapy plus chemotherapy tested negative for PStV and TSWV. Shoot tip culture with thermotherapy eliminated PMV from 45% of the plants tested. Shoot tip culture combined with both thermotherapy and chemotherapy eliminated PMV from 64% of the plants from the RBV treatment, 50% from the AZA treatment, and 76% from the DZD treatment. All plants regenerated from the shoot tips cultured without thermotherapy tested positive for PMV. Plants were not recovered from shoot tips treated with 50 mg/L of PPAA.

DISCUSSION

The culture of shoot meristems was effective for virus elimination from interspecific *Arachis* hybrids. All plants regenerated from meristems treated with thermotherapy or thermotherapy plus chemotherapy were free of PMV, PStV, and TSWV as determined by DAS-ELISA. Only 2.0% of the plants regenerated from untreated meristems contained PMV, and none contained PStV or TSWV. Shoot tip culture was not as effective as meristem culture for elimination of PMV. All plants regenerated from untreated shoot tips (1 cm long) of *Arachis* hybrids remained infected with PMV, whereas 55% of the plants regenerated from shoot tips treated with thermotherapy and 35% treated with thermotherapy plus chemotherapy were infected with PMV. Chen and Sherwood (5) observed that PMV was not eliminated from *A. hypogaea* by the culture of shoot tips (0.1–0.3 cm long) alone or

Table 3. Growth and regeneration from shoot meristems of *Arachis* hybrid 1 on medium supplemented with antiviral agents[a]

Chemical[b]	Concentration (mg/L)	Shoot bud production (%)	Culture diameter[c] (mm)
Control	...	71	9.6
AZA	10	81	2.7
	20	69	4.2
	30	50	2.3
DZD	5	76	7.6
	10	86	6.0
	30	66	5.7
RBV	20	79	7.7
	30	73	4.4
	40	86	4.5
PPAA	5	5	1.9
	10	5	0.9
	25	0	0.7
	50	0	0.6
LSD		28	0.4

[a] Meristems were evaluated after 30 days in culture on an MSB5 medium with 1 mg/L each of NAA and BAP.
[b] Control = no antiviral agent, AZA = 5-azacytidine, DZD = 3-deazauridine, RBV = ribavirin, PPAA = phosphonoacetic acid.
[c] Recorded 30 days after shoot meristems were placed on culture medium.

Table 4. Virus elimination from interspecific *Arachis* hybrids

Culture[a]	Thermotherapy[b]	Chemotherapy[c]	Percent virus infection[d] PMV	PStV	TSWV
Micropropagated shoots	−	−	100	100	100
Shoot tips	−	−	100	0	0
	+	−	55	0	0
	−	40 mg/L RBV	100	0	0
	−	30 mg/L DZD	100	0	0
	−	30 mg/L AZA	100	0	0
	+	40 mg/L RBV	36	0	0
	+	30 mg/L DZD	24	0	0
	+	30 mg/L AZA	50	0	0
Shoot meristems	−	−	2	0	0
	+	−	0	0	0
	+	20 mg/L RBV	0	0	0
	+	30 mg/L RBV	0	0	0
	+	40 mg/L RBV	0	0	0
	+	5 mg/L DZD	0	0	0
	+	10 mg/L DZD	0	0	0
	+	30 mg/L DZD	0	0	0
	+	10 mg/L AZA	0	0	0
	+	20 mg/L AZA	0	0	0
	+	30 mg/L AZA	0	0	0
	+	5 mg/L PPAA	0	0	0

[a] In vitro propagated shoots (3–6 cm long) were cultured on MSB5 medium. Shoot meristems (0.3 mm in diameter) and shoot tips (1 cm long) from micropropagated shoots were cultured on MSB5 medium with 1 mg/L each of NAA and BAP.
[b] In vitro propagated shoots were cultured at 35 C for 30–40 days before culturing meristems or shoot tips.
[c] Antiviral agents were filter-sterilized and added to the meristem and shoot tip culture medium. RBV = ribavirin, DZD = 3-deazauridine, AZA = 5-azacytidine, PPAA = phosphonoacetic acid.
[d] Plants were tested by DAS-ELISA 8–24 wk after becoming acclimated to greenhouse conditions. Percent infection was based on the number of plants regenerated from hybrids 1, 2, 3, 4, and 5 (see Table 1) for peanut mottle virus (PMV); hybrids 1 and 3 for peanut stripe virus (PStV); and hybrids 2 and 4 for tomato spotted wilt virus (TSWV).

with thermotherapy or chemotherapy but was eliminated by shoot tip culture, thermotherapy, and chemotherapy combined. We found that chemotherapy and thermotherapy were not required to eliminate PMV from interspecific *Arachis* hybrids regenerated from meristems.

This is the first report of elimination of TSWV and PStV from vegetatively maintained *Arachis* plants. TSWV and PStV were easier to eliminate than PMV. Plants regenerated from untreated shoot tips were free from TSWV and PStV. Meristem culture, thermotherapy, and chemotherapy were not required for elimination of TSWV and PStV from the *Arachis* hybrids in this study. If the viruses infecting *Arachis* accessions are identified as TSWV or PStV, shoot tip culture alone may be sufficient for virus elimination. However, since the plants infected with TSWV and PStV in this study were also infected with PMV, additional research needs to be done to determine if the presence of PMV affects the ability to eliminate TSWV and PStV in *Arachis* species.

Previous investigations reporting plant regeneration from meristems or shoot tips of *Arachis* species have used explants from seedlings or young plants (1,5, 18,23). Shoot tip and meristem explants from *Arachis* plants maintained vegetatively in the greenhouse were difficult to rid of bacterial contaminants. In vitro propagation of plants prior to meristem culture allowed selection of meristems free from bacterial and fungal contaminants. This selection saved the labor and materials needed to culture meristems from the high percentage of contaminated shoot tips of the vegetatively maintained *Arachis* plants. In vitro propagation of *Arachis* shoots also allowed for easy application of thermotherapy in a small space.

A number of antiviral agents have been evaluated for use in virus elimination in plants, and some of these chemicals have been observed to be toxic to plant tissue (3,10,16,19). Potato nodal explants died when cultured on media supplemented with AZA or DZD at 25 mg/L (14), and RBV in culture media at concentrations higher than 20 mg/L was toxic to shoot tips of *A. hypogaea* (4). We regenerated plants of *Arachis* hybrids from meristems cultured on media with 30 mg/L of AZA or DZD or 40 mg/L of RBV. Although these chemical treatments were not necessary for elimination of PMV, TSWV, and PStV from the *Arachis* hybrids evaluated in our investigation, such chemotherapy treatments may be needed to eliminate other viruses from *Arachis* plants. Plants did not regenerate from meristems cultured on media with 10, 25, or 50 mg/L of PPAA, and few were regenerated from meristems cultured with 5 mg/L of PPAA. Lower concentrations of PPAA need to be evaluated if this chemical is to be used for chemotherapy with meristems of *Arachis* species.

Meristem culture requires more labor than shoot tip culture, plant regeneration is slower, and meristems do not survive as well as shoot tips in tissue culture (4). However, meristem culture alone avoids use of antiviral chemicals that are toxic to plant tissue and are potential mutagens (20). Meristem culture alone also eliminates the time and space required for thermotherapy of shoot tip cultures. Meristem culture alone or combined with thermotherapy and chemotherapy is being applied to the other virus-infected *Arachis* accessions in the USDA-ARS Southern Regional Plant Introduction Station germ plasm collection to free these accessions of virus so they can be distributed.

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