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INFLUENCE OF PLANT PRESERVATIVE MIXTURE (PPM)™ ON ADVENTITIOUS ORGANOGENESIS IN MELON, PETUNIA, AND TOBACCO

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Summary

The influence of PPM™ on somatic embryogenesis in melon, adventitious shoot organogenesis in petunia, and androgenesis in tobacco was studied by culturing explants in regeneration media supplemented with 0, 2, 5 or 10 ml l^{-1} PPM for 8–12 wk. The percentage of melon cotyledon explants that produced callus and somatic embryos and the number of embryos per explant were reduced when incubated in embryo initiation and embryo development media containing more than 5 ml l^{-1} PPM. Less PPM was required to inhibit petunia shoot organogenesis. The number of shoots and number of buds per Petri dish were reduced 3–6.9-fold when leaf explants were incubated in shoot regeneration medium containing more than 2 ml l^{-1} PPM. In contrast, the addition of up to 10 ml l^{-1} PPM to tobacco anther culture medium had no effect on androgenesis. Our results suggest that the influence of PPM on plant regeneration depends on the plant species. We recommend that experimenters examine a range of PPM concentrations when using it for the first time on an untested plant species.

Key words: tissue culture; somatic embryogenesis; androgenesis; *Cucumis melo* L; *Petunia hybrida* Hort.; *Nicotiana tabacum* L.

Introduction

Contamination of tissue culture media and explants by bacteria and fungi can be a major obstacle in the establishment and maintenance of plant tissue cultures. A myriad of methods have been examined to eliminate fungal and bacterial contamination in tissue cultures including antibiotic (Kneifel and Leonhardt, 1992; Leifert et al., 1992) and fungicide (Haldeman et al., 1987) treatments. While these methods can be effective, phytotoxicity (Reed and Tanpraset, 1995), inactivation by heat or light (Seckinger, 1995), and reduced sensitivity of microorganisms to antimicrobial treatments *in vitro* (Barrett and Cassells, 1994) are common problems.

Plant Preservative Mixture (PPM™) is a heat-stable, broad-spectrum biocide that reduces microbial contamination in plant tissue cultures (Guri and Patel, 1998). The active ingredients in PPM include methylisothiazolinone, magnesium chloride, magnesium nitrate, sodium benzoate and potassium sorbate. Together these inorganic salts act by targeting specific fundamental enzymes in the Krebs cycle and electron transport chain. PPM has been shown to inhibit growth of microorganisms while having a minimal effect on *in vitro* seed germination of most plant species tested at concentration ranges between 0.5 and 4 ml l^{-1} (Guri and Patel, 1998).

Little information is available regarding the effect of PPM on adventitious organ regeneration in plant tissues. Niedz (1998) observed that 0–2 ml l^{-1} PPM had no effect on the growth of nonembryogenic callus (Val88-1) of sweet orange [*Citrus sinensis* (L.) Osbeck 'Valencia'], and adventitious shoot regeneration from epicotyl explants of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and rough lemon (*Citrus jambhiri* Lush.). In contrast, similar concentrations of PPM were toxic to freshly isolated protoplasts of the sweet orange embryogenic line H89 (Niedz, 1998). However, protoplast sensitivity to PPM disappeared once the cells regenerated a new wall and divided. The purpose of this study was to test the influence of various concentrations of PPM on the establishment and organ regeneration of somatic embryogenic cultures of melon, shoot organogenic cultures of petunia, and androgenic cultures of tobacco.

Materials and Methods

Somatic embryogenesis in melon. Quiescent seeds of *Cucumis melo* L. 'Eden Gem' were decoated and allowed to imbibe sterile distilled water for 3 h. Embryos were surface disinfected by agitation for 15 min in 100 ml of a 15% aqueous bleach solution (0.75% NaOCl plus 1 ml l^{-1} 'Tween 20') followed by three rinses with sterile distilled water. Cotyledonary base explants were dissected following the procedure outlined by Gray et al. (1993). Explants (four) were cultured in 15 × 100 mm Petri plates containing 25 ml of melon embryo induction (EI) medium [Murashige and Skoog (MS; 1962) basal salts with (per liter) 30 g sucrose, 1 g *myo*-inositol, 10 mg thiamine–HCl, 1 mg nicotinic acid, 0.5 mg pyridoxine, 22.5 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma Chemical Co, St. Louis, MO), 0.45 μ*M* thidiazuron (TDZ) and 7 g TC agar (JRH Biosciences, Lenexa, KS) at pH 5.4] containing 0, 2, 5 or 10 ml l^{-1} PPM (Plant Cell Technology, Inc.). TDZ was purified from Dropp 50WP (AgrEvo USA Company, Collierville, TN) using acetone. PPM was added to cooled media after autoclaving for 20 min at 121°C and 1 kg cm^{-2}. Explants were cultured in darkness at

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25°C for 2 wk before transfer to embryo development (ED) medium (similar to EI but without 2,4-D and TDZ) containing 0, 2, 5 or 10 ml l^{-1} PPM. Explants were incubated in a 16-h light photoperiod (50 μmol m^{-2} s^{-1} from cool-white fluorescent lamps) and 25°C during embryo development. There were 10 plates per treatment with four explants per plate. The number of explants with callus, number of explants with somatic embryos and number of somatic embryos per plate were recorded after 8 wk on ED medium.

Shoot organogenesis in petunia. Plants of *Petunia hybrida* Hort. 'Blue Picotee' were grown in the greenhouse in 15-cm pots containing soilless growing medium (1 peat: 1 perlite: 1 vermiculite). Plants were fertilized weekly with 200 ppm of 20-20-20 water-soluble fertilizer. The youngest fully expanded leaves were harvested from 2-4-mo.-old plants for explant preparation. Leaves were surface-disinfected in a 10% aqueous bleach solution (0.525% NaOCl plus 1 ml l^{-1} 'Tween 20') for 10 min before three rinses in sterile distilled water. Disinfected leaves were trimmed to 0.5 × 0.5 cm explants, with each explant containing a portion of the midvein. Explants (two) were transferred to 15 × 60 mm Petri dishes containing 25 ml of petunia shoot regeneration medium [MS with (per liter) 30 g sucrose, 100 mg *myo*-inositol, 2 mg glycine, 0.1 mg thiamine–HCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine, 2.5 μ*M* benzyladenine (Sigma Chemical Co.) and 7 g TC agar (JRH Biosciences) at pH 5.7 (Preece, 2000)] containing 0, 2, 5 or 10 ml l^{-1} PPM. Explants were transferred to fresh medium of the same composition 4 wk after culture initiation. Cultures were incubated in a 16-h light photoperiod (50 μmol m^{-2} s^{-1} from cool-white fluorescent lamps) at 25°C. There were 10 plates per treatment with two explants per plate. The number of explants with harvestable shoots and the number of harvestable shoots per plate plus the number of explants with buds and the number of buds per plate were recorded after 8 wk. Harvestable shoots were defined as those having a vertical axis (≥1.0 cm) with a discernible apex and at least two nodes. Growths not fitting this description were classified as buds.

Androgenesis in tobacco. Plants of *Nicotiana tabacum* L. 'Samsun' were grown in the greenhouse in 25-cm pots containing soilless growing medium (1 peat: 1 perlite: 1 vermiculite). Plants were fertilized weekly with 200 ppm of 20-20-20 water-soluble fertilizer. Flower buds were harvested from mature plants when calyx and corolla were of similar length and stored in moist paper towel in a zipper-type plastic bag at 5°C for 3–7 d (Reed, 2000). Buds were surface-disinfected in 70% ethanol for 1 min followed by 20 min in a 20% aqueous bleach solution (1% NaOCl plus 1 ml l^{-1} 'Tween 20') followed by three rinses with sterile distilled water. Anthers were removed from flower buds and cultured in 15 × 30 mm Petri dishes containing 15 ml of anther culture medium [Nitsch and Nitsch (1969) salts with (per liter) 20 g sucrose, 100 mg *myo*-inositol, 2 mg glycine, 5 mg nicotinic acid, 0.5 mg pyridoxine, 0.5 mg thiamine–HCl, 0.5 mg folic acid, 0.05 mg biotin, 2 g activated charcoal and 3 g Gelrite (Sigma Chemical Co.) at pH 5.5] supplemented with 0, 2, 5 or 10 ml l^{-1} PPM. Explants were transferred to fresh medium of the same composition 4 wk after culture initiation. Cultures were incubated in a 16-h light photoperiod (50 μmol m^{-2} s^{-1} from cool-white fluorescent lamps) at 25°C. There were seven plates per treatment with 10 anthers per plate. The number of anthers that formed plantlets and the number of plantlets per plate were recorded after 8 wk.

Experimental design and statistical analysis. Treatments in all experiments were arranged in a completely randomized design with subsampling. Each experiment was conducted twice. Continuous data were analyzed using ANOVA and polynomial contrast statements (Little and Hills, 1978). Percentage data were analyzed using Poisson regression (Mize et al., 1999). Treatment means were compared using standard error of the mean. Statistix® for Windows analytical software was used for data analyses (Anonymous, 1998).

Results and Discussion

Response of explant tissue to PPM varied depending on the plant species and concentration of PPM in the medium. Therefore, test results of each species will be discussed separately.

The percentage of melon explants that produced callus and percentage of explants that produced embryos were influenced by PPM concentration ($P < 0.0001$ and $P = 0.033$, respectively). Low concentrations (0–5 ml l^{-1}) of PPM had no effect on callus formation and embryo production whereas both were drastically reduced at the highest (10 ml l^{-1}) level (Table 1). The number of embryos per plate at 10 ml l^{-1} PPM was about 25% the amount produced at lower concentrations ($P = 0.047$). Our results are similar to the response of cucumber seedlings to PPM. Guri and Patel (1998) observed that germination of cucumber seeds *in vitro*

TABLE 1

INFLUENCE OF VARIOUS CONCENTRATIONS OF PLANT PRESERVATIVE MIXTURE (PPM) ON ADVENTITIOUS ORGANOGENESIS IN MELON, PETUNIA AND TOBACCO

	Melon[a]			Petunia[b]				Tobacco[c]	
PPM conc. (ml l^{-1})	Explants with callus (%)	Explants with embryos (%)	Embryos per dish	Explants with shoots (%)	Shoots per dish	Explants with buds (%)	Buds per dish	Anthers with plantlets (%)	Plantlets per dish
0	98 ± 3	23 ± 6	2.0 ± 0.8	55 ± 10	6.1 ± 1.3	79 ± 10	12.3 ± 2.9	32 ± 6	23.3 ± 6.2
2	100 ± 0	20 ± 7	1.0 ± 0.4	42 ± 10	4.0 ± 1.3	63 ± 11	10.7 ± 2.4	30 ± 4	19.1 ± 2.8
5	96 ± 2	31 ± 7	1.6 ± 0.4	18 ± 8	0.8 ± 0.5	33 ± 9	2.7 ± 1.0	35 ± 6	24.8 ± 5.0
10	39 ± 8	8 ± 3	0.3 ± 0.1	8 ± 4	0.2 ± 0.1	28 ± 9	2.7 ± 1.1	33 ± 6	23.1 ± 4.2
				P values					
ANOVA	<0.0001	0.033	0.047	0.02	<0.0001	<0.0001	0.0002	0.923	0.797
Polynomial contrasts									
Linear	<0.0001	0.083	0.036	<0.0001	<0.0001	<0.0001	<0.0001	0.743	0.764
Quadratic	<0.0001	0.043	0.767	0.189	0.047	0.037	0.077	0.782	0.984
Cubic	0.492	0.173	0.122	0.598	0.5831	0.393	0.151	0.592	0.341

±Values represent the standard error of the mean.

[a] Melon cotyledon base explants (four per dish) were cultured in 15 × 100 ml Petri dishes containing 25 ml of embryo development medium (Gray et al., 1993) for 2 wk before transfer to Petri dishes containing 25 ml of embryo development (ED) medium. Data recorded after 8 wk in ED included the percentage of explants with callus per dish, percentage of explants with embryos per dish and number of embryos per dish.

[b] Petunia leaf explants (two per dish) were cultured in 15 × 100 ml Petri dishes containing 25 ml of petunia shoot regeneration medium (Preece, 2000). Explants were transferred to dishes with fresh medium 4 wk after culture initiation. Data recorded after 8 wk included the percentage of explants with shoots per dish, number of shoots per dish, percentage of explants with buds per dish and number of buds per dish.

[c] Tobacco anthers were cultured in 15 × 60 ml Petri dishes containing 15 ml of Nitsch and Nitsch anther culture medium (Nitsch, 1969). Anthers were transferred to plates of fresh medium after 4 wk. Data recorded after 8 wk included the percentage of anthers with plantlets per dish and the number of plantlets per dish.

was reduced over the controls when seeds were incubated in medium containing more than 4 ml l^{-1} PPM.

Petunia leaf explants were more sensitive to PPM than melon cotyledons. The percentages of explants with shoots and buds decreased with increasing levels of PPM ($P < 0.0001$). Over 40% of explants produced shoots and more than 60% of explants produced buds when incubated on medium with 0–2 ml l^{-1} PPM (Table 1). The percentage of explants that produced shoots was reduced 3–6.9-fold at 5 and 10 ml l^{-1} PPM, respectively, over the controls while the percentage of explants that produced buds was reduced 2.4–2.8-fold at 5 and 10 ml l^{-1}, respectively. The number of shoots and the number of buds per plate were also reduced when more than 2 ml l^{-1} PPM was added to regeneration medium ($P < 0.0001$). The number of shoots per plate was reduced 7.6- and 30.5-fold at 5 and 10 ml l^{-1} PPM, respectively, while the number of buds per plate declined 4.6-fold at 5 and 10 ml l^{-1}. Previous studies testing the influence of PPM on other members of the Solanaceae demonstrated that pepper (*Capsicum annuum* L.) and eggplant (*Solanum melongena* L.) seed germination *in vitro* was inhibited at 1.2 and 4 ml l^{-1} PPM, respectively (Guri and Patel, 1998). According to our results, it appears that petunia and pepper have similar susceptibility to PPM.

Androgenesis from tobacco anthers was not influenced by PPM (Table 1). The percentage of anthers that produced plantlets ($P = 0.923$) and the number of plantlets per plate ($P = 0.797$) were similar at all concentrations of PPM tested. These results were surprising as other solanaceous species have demonstrated reduced root production when seeds were incubated in medium containing PPM at concentrations ranging from 1.4 to 1.6 ml l^{-1} (Guri and Patel, 1998).

Information regarding the influence of more than 2 ml l^{-1} PPM on adventitious organogenesis has not been published previously. Niedz (1998) demonstrated that low concentrations (0.5–2 ml l^{-1}) had no influence on the growth of nonembryogenic callus (Val88-1) of sweet orange [*Citrus sinensis* (L.) Osbeck 'Valencia'], and shoot organogenesis from epicotyl segments of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and rough lemon (*Citrus jambhiri* Lush.) but inhibited cell wall regeneration and division of freshly isolated protoplasts from embryogenic sweet orange line H89. This study demonstrates that PPM can safely be used for adventitious organ regeneration. However, plant species, even species in the same family, react differently to PPM. For example, within the Solanaceae, petunia leaf explants were very sensitive to PPM, demonstrating an inhibition of adventitious shoot and bud regeneration at concentrations above 2 ml l^{-1}, whereas tobacco anthers were insensitive to PPM, failing to demonstrate an inhibitory effect on androgenesis at concentrations as high as 10 ml l^{-1}. In another plant family, the Cucurbitaceae, melon cotyledon explants were less sensitive to PPM than petunia, tolerating concentrations up to 5 ml l^{-1}, but more sensitive than tobacco. Therefore, it is necessary to examine a range of PPM concentrations when using it for the first time on an untested plant species.

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