Dasoju, S. and B.E. Whipker. 1997. Efficacy of paclobutrazol drenches on growth of potted sunflowers grown in 16.5-cm pots. *HortScience* 32:438. (Abstr.)

Davis, T.D., F.L. Steffons, and N. Sankhla. 1988. Triazole plant growth regulators. *Hort. Rev.* 10:63-105.

Evans, M.R. and R.H. Stamps. 1996. Growth of bedding plants in sphagnum peat and coir dust-based substrates. *J. Environ. Hort.* 14:187-190.

Evans, M.R., S. Konduru, and R.H. Stamps. 1996. Source variation in physical and chemical properties of coconut coir dust. *HortScience* 31:965-967.

Lokesha, R., D.M. Mahishi, and G. Shivashankar. 1988. Studies on use of coconut coir dust as a rooting media. *Current Research Univ. of Agr. Sci. Bangalore* 17:157-158.

Million, J., J. Barrett, D. Clark, and T. Nell. 1997. Influence of several container media components on paclobutrazol efficacy. *HortScience* 32:436. (Abstr.)

Quarrels, J.R. and S.E. Newman. 1994. Pine bark levels and particle size influence the activity of paclobutrazol and uniconazole on 'Freedom' and 'Gutbier V-14 Glory' poinsettias. *HortScience* 29:737. (Abstr.)

Quinlan, J.D. 1982. An integrated approach to the chemical control of fruit tree growth and cropping. *Proc. Brit. Crop Protection Council* p. 575-583.

Talukdar, M. and S. Barooah. 1987. Effect of pot mixture on flowering in *Dendrobium densiflorum*. *Acta Hort.* 205:145-148.

Tschabold, E.E., W.C. Meredith, L.R. Guse, and E.V. Krumkalns. 1975. Ancymidol performance as altered by potting media composition. *J. Amer. Soc. Hort. Sci.* 100:142-144.

Walker, A. 1983. The fate and significance of herbicide residues in soil. *Sci. Hort.* 34:35-47.

Whipker, B.E. and P.A. Hammer. 1997. Efficacy of ancymidol, paclobutrazol, and uniconazole on growth of tuberous-rooted dahlias. *HortTechnology* 7:269-272.

Using Isothiazolone Biocides to Control Microbial and Fungal Contaminants in Plant Tissue Cultures

Randall P. Niedz

ADDITIONAL INDEX WORDS. bacteria, antibiotics, sterilization, antimicrobial

SUMMARY. Controlling bacterial and fungal contamination in plant tissue cultures is a serious problem. Antibiotics are currently used but are not always effective, can alter plant growth, and are costly, and resistant strains can result with extensive use. Plant preservative mixture (PPM) contains a mixture of two isothiazolones—methylchloroisothiazolinone and methylisothiazolinone, which are a class of broad-spectrum, widely used industrial biocides. The isothiazolones used in PPM are reported by the manufacturer to be nonphytotoxic at concentrations suitable for the prophylactic control of microbial contaminants in plant tissue cultures. Our results indicate that PPM can be routinely added to tissue culture medium to control air- and waterborne bacterial and fungal contaminants effectively.

Microbial and fungal contamination is one of the most persistent problems in plant cell and tissue culture. Contaminates enter the culture environment via

incompletely disinfested explants, explants harboring organisms internally where surface disinfection was unsuitable for their removal, or through a breakdown in the aseptic barriers (e.g., inadequate sterilization of culture medium, defective air flow filters, or poor aseptic techniques in manipulating the plant material).

Surface removal of contaminating organisms from plant tissue being prepared for culture is generally by treatment with oxidizing hypochlorite solutions, ethanol, mercuric chloride, or a combination of these or other treatments (George, 1993; Herman, 1996). The control of persistent or externally introduced contaminants from in vitro plant tissues has generally relied on antibiotics or fungicides (George, 1993; Herman, 1996). The routine use of antibiotics is not recommended for practical and ethical reasons, such as the expense, problems with phytotoxicity, and selection for resistant strains.

The use of industrial biocides has had limited use in plant tissue culture, and almost no applications exist for their use as media addenda for routine contamination control. These compounds include a wide range of materials used as preservatives, bactericides, fungicides, algacides, and slimicides (Lewis et al., 1978). Biocides are commonly used in water-cooling systems, swimming pools and spas, food and feed, paint, medical antiseptics, metalworking fluids, paper pulp processes, cosmetics, soaps, detergents, fabric, and petroleum products (Rossmore, 1995; Singer, 1976). Unlike antibiotics, biocides have a broad spectrum of activity against bacteria, fungi, algae, and slime molds (Rossmore, 1995). Activity against nematodes, mites, and insects has also been demonstrated (Lewis et al., 1978). Biocides generally cause extreme physical or chemical damage to cells (Chapman and Diehl, 1995), suggesting they would be extremely toxic to plant cells and tissues. For this reason they probably have not received consideration for use in plant tissue culture media.

Plant preservative mixture (PPM) is a mixture (patent no. 5,750,402; Plant Cell Technology, Washington, D.C.) of two isothiazolones—methylchloroisothiazolinone and methylisothiazolinone—used to prevent the growth of contaminating organisms in plant tissue culture media. PPM is reported by the manufacturer to be nonphytotoxic when used at 0.5 to 1.0

U.S. Department of Agriculture, Agricultural Research Service, 2120 Camden Road, Orlando, FL 32803.

Thanks to Delores Lomberg for her assistance in the preparation and maintenance of the protoplast and tissue cultures and Assaf Guri of Plant Cell Technology for generously providing PPM samples for testing. Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the USDA and does not imply its approval to the exclusion of other products or vendors that may also be suitable. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

mL·L⁻¹. Isothiazolones are broadly classed as electrophiles that react with cysteine and glutathione (Chapman and Diehl, 1995; Collier et al., 1990) and are widely used as industrial biocides and preservatives (Lewis et al., 1973, 1978). This research investigated the phytotoxicity and general microbial control by PPM of in vitro cultures of citrus seed, callus, protoplasts, and shoot regeneration from epicotyl explants.

Materials and methods

USING 3-ISOTHIAZOLONES IN NONSTERILE NUTRIENT BROTH AND MS MEDIUM. PPM was obtained from Plant Cell Technology. Nutrient broth (NB) is used in our laboratory for quality control monitoring of microbial contamination. It is a general culture medium for growing bacteria and fungi commonly associated with plant tissue culture. Liquid NB and seed germination medium (SGM) were used to determine the general effectiveness of PPM isothiazolones in controlling microbial growth.

NB was prepared with 17.2 g·L⁻¹ nutrient broth (Difco Laboratories, Detroit, Mich.), 10 g·L⁻¹ yeast extract, and 2.5 g·L⁻¹ glucose. SGM was prepared with revised Murashige and Skoog (MS) medium without growth regulators (Murashige and Skoog, 1962) and solidified with 0.8% (w/v) agar. All other experiments to determine effective PPM levels were conducted with 10 mL NB or SGM in 70 × 21-mm glass vials. Autoclaved or untreated PPM was added to nonautoclaved NB or SGM at concentrations of 0 to 20 mL·L⁻¹ and incubated in the dark at 37 °C for 90 d, and the number of days for the media to become cloudy was recorded. Ten vials were used per treatment and the experiments were repeated once.

Initial inoculum densities of nonsterile NB and SGM with and without agar were calculated from a serial dilution spread onto agar-solidified NB medium. Autoclaving of all culture media and PPM was for 20 min at 1.05 kg·cm⁻² at 121 °C. Filter-sterilized PPM was passed through a 0.22-μm cellulose acetate syringe filter.

PLANT MATERIAL—SEED. Seed from trifoliate orange [*Poncirus trifoliata* (L.) Raf.] (TO) and rough lemon (*Citrus jambhiri* Lush.) (RL) were used in germination tests. Seeds were peeled, immersed for 20 min in 50% commercial bleach (5.25% sodium hypochlorite w/v) containing a few drops of the detergent Tween 20 as a wetting agent, and

washed three times in sterile distilled water, then six seeds were sown into each of 10 polycarbonate GA-7 vessel (Magenta Corp., Chicago) containing 50 mL of SGM. The experiment was repeated once. Filtered or autoclaved PPM was added to autoclaved or unautoclaved (but heated to melt the agar) SGM at concentrations of 0 to 2 mL·L⁻¹. Seed germination and seedling growth were at 30 μmol·s⁻¹·m⁻² with a 4-h photoperiod provided by cool-white fluorescent lamps and a constant 27 °C temperature. To determine PPM's effectiveness in inhibiting microbial growth in nonsterile tissue culture medium, prepared SGM with agar was microwaved to melt the agar but not autoclaved, 2 mL·L⁻¹ untreated PPM was added, the medium was poured into autoclaved polycarbonate GA-7 vessels, and RL were seeds sown.

PLANT MATERIAL—NONEMBRYOGENIC CALLUS. A nonembryogenic cell line Val88-1 derived from immature juice vesicles of 'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck] was initiated and later maintained in GA-7 vessels with a polypropylene closure (Magenta Corp.) as previously described (Osswald et al., 1992). Filtered or autoclaved PPM was added to autoclaved culture medium at concentrations ranging from 0 to 2 mL·L⁻¹. Six GA-7 vessels were used per treatment and the experiment was repeated once.

PLANT MATERIAL—EPICOTYL EXPLANTS.

TO and RL seeds were germinated as described above. Seedlings that measured 5 cm from the base to the growing tip were used as a source of epicotyl explants. Six epicotyl explants 1 cm long were placed into each of six 100 × 15-mm polystyrene dishes containing shoot regeneration medium composed of revised MS salts and vitamins and supplemented with 20 μM 6-benzylaminopurine (BAP) and 3% sucrose (w/v) to initiate shoot regeneration. Dishes were sealed with Parafilm tape and incubated under cool-white fluorescent lamps (80 μmol·s⁻¹·m⁻², 16-h photoperiod). Filtered or autoclaved PPM was added to autoclaved shoot regeneration medium at concentrations of 0 to 2 mL·L⁻¹. The experiment was repeated once.

PLANT MATERIAL—PROTOPLASTS.

Protoplasts were isolated and cultured from sweet orange embryogenic cell line H89 as previously described (Niedz, 1993). Untreated PPM was added to autoclaved citrus protoplast medium (CPM) medium (Niedz, 1993) 0, 1, 5, or 10 d after protoplast isolation and at concentrations of 0 to 1 mL·L⁻¹. Five 60 × 15-mm culture dishes were used per treatment and the experiment was repeated once.

Results and discussion

Isothiazolones were supplied as PPM, a commercial formulation con-

Table 1. Effect of untreated and autoclaved plant preservative mixture (PPM) on microbial growth in seed germination medium (SGM) and nutrient broth (NB).

[PPM] mL·L ⁻¹	PPM sterilization	SGM ^z	Days SGM remained clear		Days NB remained clear	
			(no.)	NB ^z	(no.)	(no.)
0		+	0	+	0	0
0.5	Untreated	—	Clear	+	0	0
	Autoclaved	—	Clear	+	0	0
0.75	Untreated	—	Clear	+	0	0
	Autoclaved	—	Clear	+	0	0
1	Untreated	—	Clear	+	1	1
	Autoclaved	—	Clear	+	1	1
1.5	Untreated	—	Clear	+	1	1
	Autoclaved	—	Clear	+	1	1
2	Untreated	—	Clear	+	1	1
	Autoclaved	—	Clear	+	1	1
2.5	Untreated	—	Clear	+	3	3
	Autoclaved	—	Clear	+	2	2
5	Untreated	—	Clear	+	6	6
	Autoclaved	—	Clear	+	7	7
10	Untreated	—	Clear	50% clear	12	12
	Autoclaved	—	Clear	40% clear	11	11
20	Untreated	—	Clear	—	Clear	Clear
	Autoclaved	—	Clear	—	Clear	Clear

^z(+)Indicates the medium became cloudy (>1 × 10⁷ cfu/mL), (—)indicates the medium remained clear.

taining a mixture of two isothiazolones. The specific combination of the two isothiazolone compounds contained in PPM are reported by the manufacturer to control bacterial and fungal contamination effectively in vitro at a level (0.5 to 1.0 mL·L⁻¹) not inhibitory to the culture of plant tissue.

PPM ADDITION TO NONSTERILE NUTRIENT BROTH AND MS MEDIUM. The results of these experiments are presented in Table 1. Cloudy samples had $>1 \times 10^7$ colony forming units (cfu)/mL as determined by serial dilution and plating on NB plates. The initial bacterial inoculum density in NB was estimated at 10,000 cfu/mL. Determination of initial inoculum density provides information on how much bacterial inoculum was present in the nonsterile media used in these tests. Four colonies with different morphologies were observed on the NB plates. None of the four colony types were yeast as determined by microscopic examination. Occasionally, vials with NB that remained clear (bacteria free) would show a single spherical fungal colony. This was observed in the 5- and 10-mL·L⁻¹ PPM but never in the 20-mL·L⁻¹ PPM vials. No further attempts were made to identify these bacteria or fungi. In contrast to NB, SGM had an initial inoculum density of 6000 cfu/mL with or without agar as estimated on NB plates.

There were no differences between autoclaved and untreated PPM, indicating that PPM is heat stable and can be sterilized by autoclaving. For NB, 1 mL·L⁻¹ PPM was the lowest level where contaminant growth inhibition was observed (1 d), at 10 mL·L⁻¹ the results were mixed with some vials remaining clear for 90 d, and at 20 mL·L⁻¹ all vials remained clear. For MS-based SGM, 0.5 mL·L⁻¹ PPM, the lowest level tested, inhibited growth. To determine if PPM had inhibited or killed the bacteria and fungi in the clear NB and SGM treatment vials, 50 μ L of solution was removed by pipet, injected into sterile NB, and incubated at 37 °C for 30 d. No growth was observed in these tests, indicating that PPM had sterilized the media.

These results demonstrated that PPM does have biocidal properties in NB and SGM. However, there was a substantial difference in PPM's effect of controlling microbial growth in NB and SGM medium. No microbial growth was observed for 90 d in the

SGM at the lowest PPM concentration used (0.5 mL·L⁻¹), but 20 mL·L⁻¹ PPM was required to inhibit microbial growth fully in the NB medium. NB is a richer medium than SGM and more suitable for microbial growth.

PLANT MATERIAL—SEED. To determine PPM's phytotoxicity on citrus seed it was added to SGM at 0, 0.5, 1, 1.5, or 2 mL·L⁻¹. The highest level (2 mL·L⁻¹) is twice the highest level recommended by the manufacturer. The results for TO and RL are summarized in Table 2. For seed germination, the only significant difference was better germination of RL, a variable unrelated to PPM. For seedling growth, as measured by seedling height, seedlings of both species had greater growth in PPM-containing medium. For example, TO seedling height was 1.3 and 3.5 cm, respectively, for 0 and 2 mL·L⁻¹ PPM and 2.1 and 3.5 cm, respectively, for RL seedling height. Whether the effect relates to inhibition of latent microbes, altered seedling metabolic activity, or is artifactual is unknown. No phytotoxicity was observed by adding PPM up to 2 mL·L⁻¹. For all PPM treatments for TO and RL, an average of 2 and 4.8 seeds per container (6 seeds per con-

tainer), respectively, germinated. The average height of TO and RL seedlings across all PPM treatments was 2.5 and 3.5 cm, respectively. Again, this was not related to PPM, but was because RL was more vigorous and had germinating seed than TO.

To determine if PPM could substitute for autoclaving, RL seed were sown into microwaved (to melt the agar) SGM supplemented with 0 or 2 mL·L⁻¹ PPM. At the end of 30 d no visible bacterial or fungal growth were present in the microwaved SGM + 2 mL·L⁻¹ PPM, but bacterial and fungal contamination were present in the SGM without PPM. PPM's effectiveness in totally inhibiting microbial growth occurred for two possible reasons. First, 2 mL·L⁻¹ PPM was used compared to the first experiments with NB and SGM where PPM effectively sterilized SGM at only 0.5 mL·L⁻¹. Second, the inoculum density was probably less than what was measured during the first experiment since it was necessary to melt the agar by microwaving the SGM medium. These results suggest that the combination of relatively high levels PPM (2 mL·L⁻¹) combined with microwaving can substitute for autoclave sterilization. Under conditions

Table 2. Treatment means for seed germination and seedling height of trifoliolate orange and rough lemon grown in vitro containing plant preservative mixture (PPM) sterilized by autoclaving² or filtration³.

PPM concn (mL·L ⁻¹)	Mean seeds germinated (no.) per container	Mean seedling ht (cm) per container
Trifoliolate orange		
Control (0)	1.6	1.3
Autoclaved		
0.5	3.4	2.8
1.0	1.4	4.0
1.5	2.2	1.4
2.0	1.8	3.5
Filtered		
0.5	2.0	3.5
1.0	3.0	1.5
1.5	1.8	2.2
2.0	2.0	3.6
Rough lemon		
Control (0)	4.4	2.1
Autoclaved		
0.5	5.2	5.7
1.0	5.2	5.1
1.5	4.6	2.7
2.0	4.8	3.5
Filtered		
0.5	5.2	5.0
1.0	5.6	2.9
1.5	4.2	2.8
2.0	5.0	3.4

²20 min at 1.05 kg·cm⁻² at 121 °C.

³An 0.22- μ m cellulose acetate syringe filter.

of low microbial inoculum density, PPM can provide significant control.

PLANT MATERIAL—NONEMBRYOGENIC CALLUS. To determine PPM's phytotoxicity on citrus nonembryogenic callus (Val88-1), it was added to callus maintenance medium at concentrations of 0, 1, and 2 mL·L⁻¹. About 3 g of callus was inoculated into each container. Fresh weight was recorded for two 30-d growth cycles. ANOVA was used to analyze the fresh weight gain data. Data were analyzed as total absolute fresh weight increase as well as total percentage of fresh weight increase over the two growth cycles. Analysis of absolute or percentage fresh weight increase detected no significant differences among any of the variables or contrasts. No phytotoxicity was observed by adding PPM up to 2 mL·L⁻¹. An average of 24 g or 803% increase in fresh weight was recorded for these cultures over the two 30-d growth cycles. The inclusion of PPM into the culture medium over two growth cycles with no differences in fresh weight accumulation suggests that PPM can be added prophylactically to these cultures without phytotoxicity.

PLANT MATERIAL—EPICOTYL EXPLANTS. To determine the effect of PPM on organogenesis, it was added to shoot regeneration medium at 0, 0.5, or 1 mL·L⁻¹. Epicotyl explants from TO were cultured on this medium, and after 30 d the total number of shoots per replication, average shoot number per explant per replication, and the number of explants with buds that had not yet developed into shoots were recorded. ANOVA was used to determine if PPM had any influence on shoot regeneration. No phytotoxicity was observed. For each of the three measured variables, no significant differences were detected. An average of 2.2 explants with shoots per container, 2.3 shoots per explant, and 3.9 explants with buds but no shoots per container were recorded for these cultures.

PLANT MATERIAL—PROTOPLASTS. Freshly isolated protoplasts were cultured in CPM containing PPM at 0, 0.25, 0.5, 0.75, or 1 mL·L⁻¹. Protoplasts died within 1 d in all PPM-

containing media. PPM was then added at the same concentrations but from 1 to 10 d after protoplast isolation. At all PPM concentrations protoplasts survived and divided when PPM was added 1, 5, or 10 d after isolation. The fresh weight of protoplast-derived colonies was recorded 30 d after protoplast isolation and the data were analyzed by ANOVA.

PPM was toxic to freshly isolated protoplasts, but the protoplasts could tolerate PPM within 24 h. Growth of protoplast-derived callus was slightly affected when PPM was added to day-1 protoplast cultures, but this phytotoxic effect disappeared by day 5. H89 protoplasts begin active cell division on day 5 (Niedz, 1993), and whether the sensitivity of protoplasts to PPM is related to cell wall formation or to concomitant metabolic changes that are occurring is yet unknown.

Conclusions

The results of these experiments demonstrate that the nonselective industrial biocide PPM can be used effectively to control the growth of bacteria and fungi in plant tissue cultures. The isothiazolones present in PPM exhibit little phytotoxicity at the levels recommended by the manufacturer. The only significant phytotoxicity observed was to freshly isolated protoplasts that were initially very sensitive to PPM in the protoplast culture medium. However, this sensitivity decreased markedly within 24 h and disappeared by day 5.

PPM appears most effective in inhibiting the growth of air- and waterborne bacteria and fungi at low inoculum densities. Since it can be autoclaved with the medium it may be best suited as a preservative agent in culture medium to control low-level contamination. Using PPM to control higher inoculum levels of bacteria and fungi commonly encountered for explant surface sterilization may require significantly higher levels than the levels used in these experiments. Higher PPM levels might be applied in the medium or a short exposure to higher PPM levels to reduce the inoculum level followed by culture on lower PPM levels.

Literature cited

- Chapman, J.S. and M.A. Diehl. 1995. Methylchloroisothiazolone-induced growth inhibition and lethality in *Escherichia coli*. J. Appl. Bacteriol. 78:134-141.
- Collier, P.J., A.J. Ramsey, P. Austin, and P. Gilbert. 1990. Growth inhibitory and biocidal activity of some isothiazolone biocides. J. Appl. Bacteriol. 69:569-577.
- George, E.F. 1993. Plant propagation by tissue culture. Part 1. The technology. 2nd ed. Exegetics Limited, England.
- Herman, E.B. 1996. Recent advances in plant tissue culture. IV: Microbial contamination of plant tissue cultures. Agritech Consultants, Mohegan Lake, N.Y.
- Lewis, S.N., G.A. Miller, and A.B. Law. 1973. 3-Isothiazolones. U.S. Patent 3,761,488.
- Lewis, S.N., G.A. Miller, and A.B. Law. 1978. 3-Isothiazolones as biocides. U.S. Patent 4,105,431.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
- Niedz, R.P. 1993. Culturing embryogenic protoplasts of 'Hamlin' sweet orange in calcium alginate beads. Plant Cell Tiss. Org. Cult. 34:19-25.
- Osswald, W.F., R.E. McDonald, R.P. Niedz, J.P. Shapiro, and R.T. Mayer. 1992. Quantitative fluorometric analysis of plant and microbial chitosanases. Anal. Biochem. 204:40-46.
- Rossmore, H.W. 1995. Handbook of biocide and preservative use. Blackie Academic & Professional, Glasgow, U.K.
- Singer, M. 1976. Laboratory procedures for assessing the potential of antimicrobial agents as industrial biocides. Process Biochem. (July/August)30-35.