We suggest on the basis of this study that due to the bitter flavonoids levels are especially influenced by the parents and should be considered when conducting crosses. This study shows that ploidy level influences flavonoid content.

Literature Cited


A DISCUSSION OF IN VITRO CONTAMINATION CONTROL OF EXPLANTS FROM GREENHOUSE AND FIELD GROWN TREES

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Abstract. Controlling fungal and bacterial contamination of woody plant material is important and extremely difficult from field sources. Greenhouse grown material has less decontamination problems with the field material. Isothiazolones biocides and sodium dichloroisocyanurate (NaDCC) were used singly and in combination to reduce microbial contamination to less than 5% in bud explants derived from field-grown citrus trees. Coating the explant with a slurry containing 20 mL-1 Plant Preservative Mixture (PPM), a mixture of two isothiazolones, and culturing on medium containing 5 mL-1 PPM resulted in 63% clean explants compared to >90% contamination with standard disinfection procedures. Explants treated for 48 hr with 100 or 300 ppm NaDCC resulted in 83% and 96% clean explants, respectively. Phytoxicity problems were present at the 20 mL-1.

In vitro contamination by fungi, bacteria, or yeast is one of the most serious problems of commercial and research plant tissue culture laboratories (Leifert et al., 1994). The establishment of an in vitro culture requires the removal of culturable fungal and bacterial contaminants. Contamination can be especially troublesome form field obtained materials. The inability to adequately control contamination levels is the primary reason for failure of commercial laboratories (Leifert and Woodward, 1997). Chemical methods used include antibiotics and fungicides, alcohols, mercuric chloride, and oxidizing biocides such as halogen compounds (e.g., chlorine, bromine, and iodine) and hydrogen peroxide. Each method used balances factors such as the plant species, type of explant, phytotoxicity, type of contaminant(s), and cost to obtain desired results.

Isothiazolones are a class of industrial biocides that have been used prophylactically in the form of Plant Preservative Mixture (PPM) in tissue culture medium to control microbial contamination (Niedz, 1998). PPM contains a mixture of two isothiazolones, methylchloroisothiazolinone and methylisothiazolinone (Guri and Patel, 1998). Adequate control of microbial contamination was achieved as long as inoculum levels were low. Also, little phytotoxicity was observed even at the highest concentrations tested of 2 mL-1, twice the highest recommended rate by the manufacturer (Niedz, 1998). In this study, significantly higher isothiazolone levels are used to control the inoculum types and levels associated with greenhouse and field-grown plant material.

References


Some of the most common sterilants used in plant tissue culture are chlorine derivatives, generally in the form of inorganic sodium or calcium hypochlorite. Regardless of the form of chlorine used when diluted in water hypochlorous acid (HOCl) the primary sterilant is formed. Sodium dichloroisocyanurate (NaDCC) is an organic form of chlorine with a pH of about 7 in solution. At this pH HOCl ions make up 80% of the ionic constitution. NaDCC has been used to sterilize plant material for tissue culture (Parkinson et al., 1996; Pink and Walkey, 1984) and to control bacterial growth in cut flower vase water (Marousky, 1977). We report preliminary studies of the effective use of NaDCC singly and in combination with PPM in removing culturable contaminants from greenhouse and field grown citrus buds.

Materials and Methods

Plant Material. Three- to four-year-old greenhouse grown sweet orange trees (Citrus sinensis L. Osbeck cv. Madame Venus) and six- to eight-year-old field grown sweet orange trees (C. sinensis cv. Valencia) were used as bud explant sources for decontamination experiments. Other experiments used explants from field grown Grapefruit trees (C. paradisi cv. Marsh). All bud sticks from greenhouse or field trees were taken from fully expanded flush of previously pruned trees. Buds were excised as 2-3 cm segments, disinfested, and cultured singly in 60 x 15 mm polystyrene culture dishes containing MS (Murashige and Skoog, 1962) salts and vitamins plus 3% (w/v) sucrose. The medium was adjusted to pH 5.8, 8 g-L¹ agar added, and then autoclaved for 20 min at 120 C and 104 kPa. Cultures were maintained at a constant 27°C under cool-white fluorescent lamps (30 µmol-s⁻¹m⁻²) for a 16 hr photoperiod.

Each treatment contained at least 10 dishes, and the experiment was repeated one to three times. Contamination data was compiled from 30 days after culture and 15 days incubation in nutrient broth (NB) except when noted. NB was prepared with 17.2 g-L¹ nutrient broth (Difco Laboratories, Detroit, MI), 10 g-L¹ yeast extract, and 2.5 g-L¹ glucose.

PPM Treatments. PPM (Guri and Patel, 1998) was obtained from Plant Cell Technology (Washington, DC) and was added to the medium after autoclaving. PPM was added to MS culture medium containing 0.2% agar or 0.8% agar at a concentration of 0 or 20 mL-L¹, or 0, 5, 10, or 20 mL-L¹, respectively. A standard disinfestation procedure was developed as a baseline to determine the relative effectiveness of PPM. Explants were first treated with the standard disinfestation procedure as follows: bud nodes were cut and air-dried for 1 hr, a 1 min dip in 1 N sulfuric acid immediately followed by a dip in 1 M sodium bicarbonate, and a second one min dip in 1 M sodium bicarbonate 30 min soak in 20% commercial bleach (5.25% sodium hypochlorite) with 0.1% Silwet L-77 (OSI Industries, Terrytown, NY) as a wetting agent, and 3 rinses with sterile distilled water. All disinfestation agents were added to a 500 mL jar and agitated with gentle stirring except where noted. After the standard disinfestation, explants were dipped into 0.2% agar slurry and placed onto culture medium. The slurry dip treatment included two concentrations of PPM and the medium contained four concentrations of PPM. This resulted in a factorial arrangement of explants on PPM-containing slurry and medium. Data was collected as percent contamination from two experiments with 12 replications each, transformed by arcsine, and analyzed by standard ANOVA.

Incubation of explants in a suspension culture containing PPM was also examined. Explants were prepared as before with the standard disinfestation treatment, but were placed into flasks containing liquid MS medium containing 0, 5, 10, or 20 mL-L¹ PPM for five days. Explants were then removed and cultured on medium containing no PPM.

Sodium dichloroisocyanurate treatments. Dichloroisocyanuric acid (NaDCC) was obtained from Sigma Chemical (St. Louis, MO) as the sodium salt. Explants were treated with the standard disinfestation treatment listed using sodium hypochlorite (NaOCl) or NaDCC (0, 100, 300 ppm, or 500 ppm for 24 or 48 hr) as the sources of chlorine. Explants were treated and placed into vials containing NB without rinsing, and scored for visible contamination in 15 days. Treatments with little contamination were repeated but the explants were either coated or not coated with slurry (20 mL-L¹ PPM) and placed onto MS medium containing 0 or 5 mL-L¹ PPM.

Results and Discussion

A baseline sterilization procedure was developed that included an initial detergent wash, this step was eliminated in later tests. Predrying of the cut buds explants, an alcohol and acid dip, followed by treatment with a NaOCl solution and organosilica wetting agent were used. The initial use of the sulfuric acid treatment was used to reduce gross contaminant levels. Predrying of the cut stems was done to allow the ends to seal and minimize damage to the vascular tissue. Phloem sieve elements contain a P-protein that polymerizes rapidly on exposure to oxygen (Sjolund et al., 1983). It is believed P-protein functions in wound sealing on the plant. A quick ethanol and acid dip were added to further reduce inoculum levels. This process of using polar and non-polar solutions attempted to dislodge organisms of different cell wall or membrane encapsulating materials. Preliminary experiments comparing hydrochloric and sulfuric acids indicated a similar efficacy (data not shown). The NaOCl soak is a common chlorine treatment used to disinfect plant tissue of contaminants. In preliminary experiments NaOCl was compared with hydrogen peroxide using bud explants from greenhouse-grown trees. The standard disinfestation treatment with NaOCl resulted in 85% contamination, whereas 100% contamination resulted when hydrogen peroxide was used. Peroxide may not be as effective due as chlorine because of a different mode of action, and did not have any antimicrobial action. Silwet L-77, an organosilicone adjuvant with little phytotoxicity to plants, was added to enhance coverage (surface tension) of the biocide solutions.

The degree of contamination was determined by culturing the explants on MS-based medium followed by a subsequent transfer of the explants with no visible contamination after 30 days to nutrient broth. Bacteria and fungi will generally multiply much more rapidly in nutrient broth than MS-based medium. Nutrient broth, both liquid and semi-solid, is used in our laboratory for quality control, and to identify inoculum sources when contamination problems arise.

Slurry Experiments. Initial experiments indicated that field-grown trees were much more difficult to free of culturable contaminants than greenhouse-grown trees. Contaminants on bud explants from greenhouse-grown trees were controlled (>95% clean) with the standard disinfestation procedure followed by culture on medium containing 5 mL-L¹ PPM. Dipping into a PPM slurry was not necessary. Phytotoxicity was minimal and buds grew normally. Using buds from field-grown trees, preliminary experiments with PPM (with-
out standard disinfestation treatment) observed that fungi on contaminated explants would grow only on the upper surface of the explant. The hyphae would grow up and over the PPM-contaminated explants would grow only on the upper surface (out standard disinfestation treatment) observed that fungi on 

ANOVA with a highly significant 'Medium' main effect (Tab. 2) confirmed the observation that PPM in the medium was more effective than the slurry.

Table 1. Contamination levels of citrus bud explants from field grown trees when treated with PPM-containing slurry and culture medium.

<table>
<thead>
<tr>
<th>Slurry PPM (mls/L)</th>
<th>Medium PPM (mls/L)</th>
<th>Percent of explants with visible contamination²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>92</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
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</tr>
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<td>0</td>
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<td>10</td>
<td>33</td>
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<tr>
<td>20</td>
<td>20</td>
<td>17</td>
</tr>
</tbody>
</table>

²The combined results from two experiments with 12 replications each for a total of 24. Explants showing no detectable contamination at 30 days were placed into nutrient broth and incubated for 14 days at 37°C.

The effectiveness of NaDCC compared to commercial bleach is due to the greater proportion of HOCl. More HOCl is present because the ratio of HOCl to OCl⁻ ion is dependent solely on the pH of the solution (Fig. 1) (White, 1986). The pH of a 300 ppm NaDCC solution is about 6.8, and 10 for a 20% commercial bleach solution (1% NaOCl). HOCl constitutes 84.54% of the species at pH 6.8, but only 0.34% at pH 10. At pH 10 the primary species is the OCl⁻ ion. The germicidal efficiency of HOCl is 20 to 300 times more effective, depending on the target organism, than the OCl⁻ ion (White, 1986), indicating that NaDCC should be more effective than commercial bleach in controlling contaminants. However, in
a study by Coates(1985) a comparison of NaDCC and NaOCl found them to be equally effective in control Pseudomonas aeruginosa, but that the commercial NaOCl solutions varied greatly in their concentration. Commercial bleach has a problem in that their av.Cl varies greatly with the age of the solution (Coates, 1985). The age of a particular container of bleach is generally unknown. Conversely, because NaDCC is a powder with a shelf life of years, the chlorine content of freshly made solutions is precise and consistent. NaDCC appears to offer some distinct advantages over commercial bleach preparations.

Citrus appears to have mostly external contaminants. The reasons are: 1) in almost all treatments explants that appeared ‘clean’ on medium almost always tested ‘clean’ in NB, and 2) contamination was effectively controlled by surface sterilization with NaDCC. Some types of contaminants are more effectively controlled by NaDCC than NaOCl. A combination of NaDCC surface sterilization followed by culture in PPM-containing medium was not any more effective than NaDCC singly. However, including PPM in the medium may provide control in species where explants have internal contaminant. Further work is underway to determine the extent of its efficacy.

**Literature Cited**


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**EVALUATION OF TEBUCONAZOLE FOR CONTROL OF POSTHARVEST DISEASES OF FLORIDA CITRUS**

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Abstract. Tebuconazole (Elite 45 DF), a fungicide registered preharvest on banana and stone fruits, is being considered for postharvest registration of citrus to control stem-end rot (SER) (Diplodia natalensis, Phomopsis citri), green mold (Penicillium digitatum), and sour rot (Geotrichum candidum). Aqueous suspensions of tebuconazole at 1000-3000 ppm were applied to 'Hamlin' or 'Temple' oranges, 'Sunburst' tangerines, or 'Orlando' tangelos naturally affected by SER and green mold or artificially inoculated with G. candidum. Control of SER was obtained with tebuconazole but the efficacy was usually inferior to that of the commercial fungicides thiabendazole or imazafl. However, control of green mold with tebuconazole was comparable to control with these fungicides. Control of sour rot was significantly better with tebuconazole than with sodium orthophenylphenate, the only commercial citrus fungicide with some activity against this disease.