Effect of biocide addition on plantlet growth and contamination occurrence during the in vitro culture of blueberry

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Abstract Interest and great demand for blueberry (Vaccinium corymbosum) have increased, as V. corymbosum is now one of the most economically important crops in Korea. It is expected that blueberry production and the area planted for cultivation will increase consistently in the years ahead because of high profitability and the consumer’s demand for healthy ingredients. Effective mass production of blueberry is urgently needed for commercial cultivation establishment, but a main limitation is lack of a propagation system that produces a disease-free plant material for commercial plantation. A large amount of research has focused entirely on developing tissue culture techniques for blueberry propagation. However, controlling fungal and bacterial contamination of woody plant material is extremely difficult. Our study was conducted to investigate the effect of biocide addition during the in vitro culture of blueberry on plantlet growth and contamination occurrence. Four biocides, including Plant Preservative Mixture (PPM™), vancomycin, nystatin and penicillin G, were used in varying concentrations during the in vitro propagation of blueberry. When nystatin was added into the medium at low concentrations, the overall growth of blueberry plantlets was retarded. Addition of vancomycin and penicillin G in high concentrations decreased contamination but induced plantlet mortality. On the other hand, when 1ml/L PPM™ was added, the growth characteristics of blueberry plantlets did not significantly differ from non-treatment (control), and the contamination occurrence rate was very low. From these results, we found that the addition of the appropriate biocide could provide an effective method to reduce contamination in the culture process, thereby raising in vitro production efficiency.

Keywords Contaminant, In vitro culture, Nystatin, Penicillin G, PPM, Vancomycin

Introduction

Vaccinium fruits are now considered as a health food because they exhibit relatively high antioxidant and anti-inflammatory properties (Prior et al. 1998; Ehlenfeldt and Prior 2001; Zheng and Wang 2003). Blueberry (Vaccinium corymbosum) is by far the most important commercial crop worldwide, as well as in Korea. Cultivars of these crops are vegetatively propagated by stem cuttings to maintain their hereditary characteristics (Douglas 1966). This propagation method is slow and many genotypes do not properly respond to root-inducing growth regulators. As an effective alternative, tissue culture techniques have been used for rapid mass propagation of superior genotypes (regardless of season) and the production of virus-free plants. In addition, these in vitro culture techniques are broadly applied in conventional breeding programs (Meiners et al. 2007).

However, microbial contamination usually occurs during the plant tissue culture process, causing many serious problems. Contamination by different sources, such as bacteria and fungi, reduces productivity and prevents successful culture procedures. Therefore, various methods are used to eliminate bacterial and fungal contamination, including the application of sterilizing agents and antibiotics and fungicides, and inactivation by heat and light (Kneifel and Leonhardt 1992; Leifert et al. 1992; Salehi et al. 1997; Haldeman et al. 1987; Reed and Tanprasert 1995; Seckinger 1995). After surface sterilization, explants are either submerged in an antibiotic-antimycotic solution, or these agents are added
to the culture medium to hinder the growth of microorganisms on the explants (Colgecen et al. 2011).

Contaminants are often difficult to detect because they predominantly remain within the plant tissue (Viss et al. 1991). Contaminated plants may exhibit no visible symptoms, reduced multiplication and rooting rates, or may die (Leifert et al. 1989). Introduction of microorganisms results from poor aseptic technique or inadequately sterilized equipment, but can be overcome with improvements in training or equipment handling. However, elimination of internal contaminants is much more serious and problematic (Buckley et al. 1995). Ideal antimicrobial agents should be soluble, stable, unaffected by pH and media, lacking in side effects, broadly bactericidal and fungicidal, suitable in combinations, nonresistance-inducing, inexpensive and nontoxic to human health (Falkiner 1990). Therefore, many biocides have been evaluated on the bacterial and fungal contaminants of various plants.

Plant Preservative Mixture (PPM™) is a combination of two broad-spectrum industrial isothiazolone biocides, chloromethylisothiazolone and methylisothiazolone. The active ingredients in PPM™ also include magnesium chloride, magnesium nitrate, potassium sorbate and sodium benzoate. This agent is heat stable and can be autoclaved with culture medium (Lunghusen 1998). Mutants resistant to PPM™ rarely develop because it targets specific multiple enzymatic sites in the Krebs cycle and electron transport chain of microorganisms (Chapman and Diehl 1995). Niedz (1998) tested PPM™ with many types of citrus tissue culture and demonstrated that it could be used with culture media to prevent bacterial and fungal contamination. The positive effects of PPM™ have been reported in a number of plants, including the non-embryogenic callus of sweet orange, shoot regeneration of rough lemon, adventitious melon, petunia, tobacco and pepper (Compton and Koch 2001; Guri and Patel 1998).

Nystatin is a fungistatic and fungicidal polyene antibiotic that increases the permeability of the cell membrane of sensitive fungi by binding to sterols, chiefly ergosterol (Brezzis et al. 1984). Its main action is against Candida species. It is also effective against Aspergillus, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, Blastomyces dermatidis and other yeasts and fungi (Garrod et al. 1981; Medoff and Kobayashi 1980).

Vancomycin is an amphoteric glycopeptide antibiotic produced by Streptomyces orientalis. It inhibits bacterial cell wall synthesis by binding to peptidoglycans. This antibiotic has a relatively narrow spectrum of activity and prevents the growth of Gram-positive bacteria. All Gram-negative bacteria are known to be resistant (Pollock et al. 1983).

Penicillin G acts by inhibiting cell wall synthesis through binding to penicillin binding proteins (PBPs), inhibiting peptidoglycan chain cross-linking. This antibiotic is active against Gram-positive bacteria but much less effective against Gram-negative organisms (Pollock et al. 1983).

Nowadays various biocides are industrially used as bactericidal and fungicidal compounds, but careful checks need to be performed to confirm that they do not inhibit or alter plant growth during in vitro culture procedures. Therefore, in this study we describe contamination inhibition using different biocides incorporated into the culture media and verify their effects on plantlet growth during the in vitro culture of blueberries.

Materials and Methods

In vitro culture of blueberry plantlets

Three blueberry cultivars (‘Spartan’, ‘Northland’ and ‘Woodard’), which were cultured for in vitro shoot proliferation in woody plant medium (WPM) and Murashige and Skoog medium adjusted to pH 5 and supplemented with growth regulators were used in our experiments. These plantlets were individually obtained from meristem cultures and incubated in 450 ml glass vessels containing 90 ml of culture medium (medium composition depended on the cultivar) at 23±1 °C with a 16h photoperiod (40 μmol·m⁻²·s⁻¹ light intensity).

Biocide addition into the culture medium

Four biocides were used: Plant Preservative Mixture (PPM™), vancomycin, nystatin and penicillin G. For culturing, 0, 0.5, 1, 2 and 4 ml/L PPM™ were added into the blueberry culture medium before autoclaving. Stock solutions of other biocides were made up fresh, filter-sterilized and added to the medium after autoclaving. Vancomycin (0, 1, 2.5, 5, and 10 mg/L), nystatin (0, 10, 25, 50, and 100 mg/L) and penicillin G (0, 0.5, 1, 2, and 4 mg/L) were tested at various concentrations. Blueberry plantlets cultured in proliferation medium were transferred into a separate biocide-containing medium, and each treatment was replicated 5 times. After three months, contamination occurrence, as well as growth characteristics, such as shoot number, shoot length and death rate, were calculated.

Statistical analysis

Data were subjected to a two-way analysis of variance.
Addition with higher concentrations of biocides decreased occurrence in blueberry cultivar ‘Northland’

**Effect of biocide addition on plantlet growth and contamination**

Results and Discussion

**Effect of biocide addition on plantlet growth and contamination occurrence in blueberry cultivar ‘Northland’**

Addition with higher concentrations of biocides decreased *in vitro* contamination, increased death rate, and prevented the overall plantlets growth of blueberry cultivar ‘Northland’ (Table 1). In particular, nystatin treatment at a low concentration induced plantlet death, as well as growth retardation.

### Table 1 Effect of biocide type and concentration on plantlet growth and contamination after three-month culture of blueberry cultivar ‘Northland’

| Biocides Type | Conc. | Shoot No. (plantlet) | Shoot length (mm) | Death rate (%) | Contamination rate (%) |
|---------------|-------|----------------------|-------------------|----------------|------------------------|
| Vancomycin   | 0 mg/L | 9.8a                 | 20.4a             | 12.2de         | 19.2a                  |
|              | 0.5 mL/L | 9.6ab               | 19.0ab            | 10.5e          | 13.4bc                |
|              | PPM    | 1 mL/L               | 9.6ab             | 19.2ab         | 14.8cede              | 9.2def                |
|              |        | 2 mL/L               | 9.2ab             | 17.0abc        | 25.2abde              | 8.5def                |
|              |        | 4 mL/L               | 8.4b              | 17.8abce       | 26.2abcede            | 7.9ef                 |
| Penicillin G | 0 mg/L | 9.8a                 | 20.4a             | 12.2de         | 19.2a                  |
|              | 1 mg/L | 9.5ab                | 20.3a             | 15.9cede       | 16.4ab                |
|              | 2.5 mL/L | 9.6ab               | 17.9abce          | 33.1ab         | 14.5b                  |
|              | 5 mL/L | 9.7a                 | 16.8abc           | 31.7abc        | 11.5bced              |
|              | 10 mL/L | 9.3ab                | 17.3abce          | 25.3abde       | 7.7ef                 |
| Nystatin     | 0 mg/L | 9.8a                 | 20.4a             | 12.2de         | 19.2a                  |
|              | 10 mL/L | 9.7a                | 16.0abc           | 20.2abde       | 10.7de                |
| Penicillin G | 25 mL/L | 9.7a               | 15.0bcd           | 19.7abede      | 9.4def                |
|              | 50 mL/L | 9.7a                | 12.9cd            | 30.5abc        | 8.2def                |
|              | 100 mL/L | 9.5ab            | 11.1d             | 35.9a          | 6.8f                  |
|              | 0 mg/L | 9.8a                 | 20.4a             | 12.2de         | 19.2a                  |
|              | 0.5 mL/L | 9.5ab              | 18.9ab            | 21.2abede      | 14.8b                 |
|              | PPM    | 1 mL/L               | 9.6a              | 18.7ab         | 18.8bcde              | 13.1bcde              |
|              |        | 2 mL/L               | 9.5ab             | 16.6abc        | 28.2abcd              | 10.1cdef              |
|              |        | 4 mL/L               | 9.3ab             | 16.7abc        | 30.9abc               | 7.1f                  |

Two-way ANOVA:

| Type(A) | Conc.(B) | A × B |
|---------|----------|-------|
| 1.07<sup>NS</sup> | 1.91<sup>NS</sup> | 0.37<sup>NS</sup> |
| 6.66*** | 6.95*** | 0.62<sup>NS</sup> |
| 1.80<sup>NS</sup> | 10.48** | 0.92<sup>NS</sup> |
| 7.18** | 81.18** | 1.58<sup>NS</sup> |

*Mean separation within columns by Duncan's multiple range test at *P* = 0.05.

<sup>NS</sup>, **Nonsignificant or significant at *P* ≤ 0.05 or 0.01, respectively.
Niedz (1998) demonstrated that PPM could be used with citrus culture medium as a powerful biocide without deteriorating the plant material. On the other hand, Crompton and Koch (2001) tested the effects of PPM on adventitious shoot regeneration in petunia, somatic embryogenesis in melon and androgenesis in tobacco, and reported that the effectiveness of PPM was largely dependent on the plant species tested. Most studies demonstrated the positive effect of PPM to control contamination and emphasized the importance of PPM at the proper concentration, which was indicated to be different depending on the plant species. Therefore, PPM should be used at the proper concentration, as comparatively high concentrations could induce negative effects on the growth and development of plant tissue.

Effect of biocide addition on plantlet growth and contamination occurrence in blueberry cultivar ‘Spartan’ and ‘Woodard’

Biocide addition at relatively higher concentrations also decreased in vitro contamination, increased death rate and prevented plantlet elongation of the blueberry cultivar ‘Spartan’ (Table 2). Nystatin was found to be effective in reducing the contamination rate by half, but had a phytotoxic effect on plantlets in vitro at low concentrations. Vancomycin and penicillin G treatments at higher concentrations decreased

### Table 2 Effect of biocide type and concentration on plantlet growth and contamination after three-month culture of blueberry cultivar ‘Spartan’

| Biocides   | Shoot No./plantlet | Shoot length (mm) | Death rate (%) | Contamination rate (%) |
|------------|---------------------|-------------------|----------------|------------------------|
| 0 ml/L     | 10.0a               | 23.7a             | 13.7d          | 20.2a                  |
| 0.5 ml/L   | 9.4a                | 22.4ab            | 12.6d          | 13.0cd                 |
| PPM        | 0.5 ml/L            | 9.7a              | 19.7ab         | 13.2d                  |
|            | 2 ml/L              | 9.6a              | 19.8ab         | 15.8cd                 |
|            | 4 ml/L              | 9.4a              | 17.8bc         | 21.3abc                |
| 1 ml/L     | 10.0a               | 22.8ab            | 12.5d          | 16.8b                  |
| 2.5 mg/L   | 9.5a                | 21.9ab            | 21.2abc        | 14.0c                  |
| 5 mg/L     | 9.3a                | 19.9ab            | 18.2abcd       | 10.5defg               |
| 10 mg/L    | 9.5a                | 19.7ab            | 21.5abc        | 8.5fghij               |
| 0 mg/L     | 10.0a               | 23.7a             | 13.7d          | 20.2a                  |
| 10 mg/L    | 9.9a                | 22.6ab            | 18.1abcd       | 9.7efgh                |
| Nystatin   | 25 mg/L             | 9.8a              | 20.1ab         | 21.0abc                |
|            | 50 mg/L             | 9.9a              | 14.6cd         | 22.8ab                 |
|            | 100 mg/L            | 9.8a              | 13.2d          | 23.9a                  |
| Penicillin | 0 mg/L              | 10.0a             | 23.7a          | 13.7d                  |
| G          | 0.5 mg/L            | 9.5a              | 22.7ab         | 16.1bcd                |
|            | 1 mg/L              | 9.5a              | 21.0ab         | 15.7cd                 |
|            | 2 mg/L              | 9.4a              | 19.1ab         | 20.6abc                |
|            | 4 mg/L              | 9.5a              | 20.9ab         | 24.4a                  |

Two-way ANOVA

| Type(A) F value | 1.52NS  | 2.49NS  | 0.65NS  | 14.87** |
| Conc.(B) F value | 2.05NS  | 7.89**  | 10.21** | 89.86** |
| A × B F value  | 0.54NS  | 0.84NS  | 1.11NS  | 1.70**  |

*Mean separation within columns by Duncan’s multiple range test at \( P = 0.05 \).

### Table 3 Effect of biocide type and concentration on plantlet growth and contamination after three-month culture of blueberry cultivar ‘Woodard’

| Biocides   | Shoot No./plantlet | Shoot length (mm) | Death rate (%) | Contamination rate (%) |
|------------|---------------------|-------------------|----------------|------------------------|
| 0 ml/L     | 9.0a                | 23.0a             | 24.3d          | 21.5a                  |
| 0.5 ml/L   | 9.0a                | 22.1a             | 23.3d          | 14.0bcd                |
| PPM        | 1 ml/L              | 8.8ab             | 20.4a          | 24.9d                  |
|            | 2 ml/L              | 7.8abc            | 21.7a          | 24.7d                  |
|            | 4 ml/L              | 7.8abc            | 20.2a          | 29.8d                  |
| 10 mg/L    | 9.0a                | 23.0a             | 24.3d          | 21.5a                  |
| 1 mg/L     | 8.0abc              | 23.7a             | 25.9d          | 16.0b                  |
| Nystatin   | 2.5 mg/L            | 8.1abc            | 20.4a          | 23.4d                  |
|            | 5 mg/L              | 8.1abc            | 20.5a          | 26.4d                  |
|            | 10 mg/L             | 6.7bcd            | 18.4ab         | 35.6cd                 |
| Penicillin | 0 mg/L              | 9.0a              | 23.0a          | 24.3d                  |
| G          | 0.5 mg/L            | 8.2abc            | 20.1a          | 19.1d                  |
|            | 1 mg/L              | 8.0abc            | 23.7a          | 25.9d                  |
|            | 2 mg/L              | 7.3abcd           | 20.1a          | 23.0d                  |
|            | 4 mg/L              | 7.4abcd           | 19.3a          | 25.1d                  |

Two-way ANOVA

| Type(A) F value | 30.45**  | 9.51**  | 20.66** | 9.34** |
| Conc.(B) F value | 16.63**  | 10.06** | 6.48**  | 181.22** |
| A × B F value  | 3.83**  | 2.56**  | 3.04**  | 1.22NS  |

*Mean separation within columns by Duncan’s multiple range test at \( P = 0.05 \).

NS**, **Nonsignificant or significant at \( P \leq 0.05 \) or 0.01, respectively.
contamination efficaciously, but phytotoxicity, as indicated by plantlet death, increased severely. When PPM\textsuperscript{TM} was incorporated into medium at 1 ml/L, the negative effect impairing shoot growth was rarely observed, as contamination considerably declined by 58%. Table 3 shows that 1 ml/L PPM\textsuperscript{TM} was consistently very effective in controlling contamination while being gentle to the shoot growth and proliferation of the blueberry cultivar ‘Woodard’.

Our results from these experiments demonstrated that the industrial biocide PPM\textsuperscript{TM} can be used effectively to control the growth of bacteria and fungi in blueberry tissue cultures. The isothiazolones present in PPM\textsuperscript{TM} exhibited little phytotoxicity at the recommended levels. PPM\textsuperscript{TM} appears to be most effective in inhibiting the growth of air- and waterborne bacteria and fungi. Since it is heat stable and can be autoclaved with the plant growth medium, it may be best suited for use as a preservative agent in culture medium to control contamination.

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