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Research Article

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^{TM}), 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^{TM} 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

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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^{TM}) is a combination of two broad-spectrum industrial isothiazolone biocides, chloromethylisothiazolone and methylisothiazolone. The active ingredients in PPM^{TM} 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^{TM} 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^{TM} 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^{TM} 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 (Brezis et al. 1984). Its main action is against *Candida species*. It is also effective against *Aspergillus, Coccidioides immitis, Cruptococcus 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㎖ glass vessels containing 90ml 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^{TM}), vancomycin, nystatin and penicillin G. For culturing, 0, 0.5, 1, 2 and 4 ml/L PPM^{TM} 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

(ANOVA) and the differences among means were compared using Duncan's multiple range test (Duncan 1955).

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		Shoot No. (/plantlet)	Shoot length (mm)	Death rate (%)	Contamination rate (%)
Type	Conc.				
PPM	0 ml/L	9.8a[z]	20.4a	12.2de	19.2a
	0.5 ml/L	9.6ab	19.0ab	10.5e	13.4bc
	1 ml/L	9.6ab	19.2ab	14.8cde	9.2def
	2 ml/L	9.2ab	17.0abc	25.2abcde	8.5def
	4 ml/L	8.4b	17.8abc	26.2abcde	7.9ef
Vancomycin	0 mg/L	9.8a	20.4a	12.2de	19.2a
	1 mg/L	9.5ab	20.3a	15.9cde	16.4ab
	2.5 mg/L	9.6ab	17.9abc	33.1ab	14.5b
	5 mg/L	9.7a	16.8abc	31.7abc	11.5bcd
	10 mg/L	9.3ab	17.3abc	25.3abcde	7.7ef
Nystatin	0 mg/L	9.8a	20.4a	12.2de	19.2a
	10 mg/L	9.7a	16.0abc	20.2abcde	10.7cde
	25 mg/L	9.7a	15.0bcd	19.7abcde	9.4def
	50 mg/L	9.7a	12.9cd	30.5abc	8.2def
	100 mg/L	9.5ab	11.1d	35.9a	6.8f
Penicillin G	0 mg/L	9.8a	20.4a	12.2de	19.2a
	0.5 mg/L	9.5ab	18.9ab	21.2abcde	14.8b
	1 mg/L	9.6a	18.7ab	18.8bcde	13.1bcd
	2 mg/L	9.5ab	16.6abc	28.2abcd	10.1cdef
	4 mg/L	9.3ab	16.7abc	30.9abc	7.1f
		F value			
Two-way ANOVA	Type(A)	1.07^{NS}	6.66**	1.80^{NS}	7.18**
	Conc.(B)	1.91^{NS}	6.95**	10.48**	81.18**
	A × B	0.37^{NS}	0.62^{NS}	0.92^{NS}	1.58^{NS}

[z]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.

Nystatin, a fungicide known to be mainly active against *Candida species* and yeast, increases cell membrane permeability of sensitive fungi by binding to ergosterol and hinders cell wall synthesis. Therefore, it was suggested that this inhibitory action results in blueberry plantlet growth inhibition and death. When vancomycin and penicillin G were added at relatively higher concentrations, contamination rates decreased by 40~48%, but phytotoxicity, as indicated by plantlet death, was observed. Although both vancomycin and penicillin G have been used as popular antibiotics to prevent the growth of various bacteria, these agents showing phytotoxicity to plants could not be recommended in our experiment.

Other studies also reported different effects of these antibacterial agents. Martina (1999) evaluated the effect of vancomycin on the growth of *in vitro Primula vulgaris*; a 40~80 mg/L vancomycin application had no effect on plant growth in comparison to control groups grown on the same medium without vancomycin. Gholamhoseinpour et al. (2012) reported that 200 mg/L vancomycin resulted in the least amount of bacterial infection without having any deleterious effect on the growth and proliferation of peach x almond hybrids. Penicillin G was effective in eliminating bacterial contamination on ginseng, but caused abnormal and depressed callus formation and an inhibition of somatic embryogenesis (Teng and Nicholson 1997). In another study, a penicillin agent had a positive effect on the growth of *Oryza sativa* seedlings (Mukherji and Biswas 1985). Such stimulatory effects have not yet been fully explained, but these antibiotics are not likely to act as growth regulator substitutes.

At 1 ml/L PPM^{TM}, shoot proliferation and death rate were not shown to be appreciably different from non-treatment (control), as the contamination rate significantly declined by 52%. On the other hand, death rates doubled at 2 and 4 ml/L PPM^{TM}, as these levels were seen to induce phytotoxicity. PPM^{TM} is a relatively new, broad-spectrum agent developed by Plant Cell Technology, Inc. (USA) and has been increasingly used in the tissue culture of many species, including salad burnet, melon, petunia, tobacco, chrysanthemum, European birch and rhododendron at concentrations ranging from 0.5 to 10 ml/L (Babaoglu and Yorgancilar 2000; Crompton and Koch 2001; George and Tripepi 2001). PPM^{TM} is designed to kill cells of bacteria and fungi and inhibits spore germination (Plant Cell Technology 2006). It is either used for surface sterilization or included in the culture medium to remove the internal contaminants that may be present in explants. Babaoglu and Yorgancilar (2000) reported that the use of PPM^{TM} was very effective in controlling *in vitro* contamination without impairing shoot regeneration from petiole and hypocotyl explants of salad burnet (*Poterium*

songuisorba L.). 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 (%)
Type	Conc.				
PPM	0 ml/L	10.0a	23.7a	13.7d	20.2a
	0.5 ml/L	9.4a	22.4ab	12.6d	13.0cd
	1 ml/L	9.7a	19.7ab	13.2d	8.4fghij
	2 ml/L	9.6a	19.8ab	15.8cd	8.0ghij
	4 ml/L	9.4a	17.8bc	21.3abc	6.1ij
Vancomycin	0 mg/L	10.0a	23.7a	13.7d	20.2a
	1 mg/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
Nystatin	0 mg/L	10.0a	23.7a	13.7d	20.2a
	10 mg/L	9.9a	22.6ab	18.1abcd	9.7efgh
	25 mg/L	9.8a	20.1ab	21.0abc	8.9fghi
	50 mg/L	9.9a	14.6cd	22.8ab	7.2hij
	100 mg/L	9.8a	13.2d	23.9a	5.9j
Penicillin G	0 mg/L	10.0a	23.7a	13.7d	20.2a
	0.5 mg/L	9.5a	22.7ab	16.1bcd	13.1cd
	1 mg/L	9.5a	21.0ab	15.7cd	12.2cde
	2 mg/L	9.4a	19.1abc	20.6abc	11.3cdef
	4 mg/L	9.5a	20.9ab	24.4a	9.1fgh
		F value			
Two-way ANOVA	Type(A)	1.52^{NS}	2.49^{NS}	0.65^{NS}	14.87**
	Conc.(B)	2.05^{NS}	7.89**	10.21**	89.86**
	A × B	0.54^{NS}	0.84^{NS}	1.11^{NS}	1.70^{NS}

[z]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.

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 (%)
Type	Conc.				
PPM	0 ml/L	9.0a	23.0a	24.3d	21.5a
	0.5 ml/L	9.0a	22.1a	23.3d	14.0bcd
	1 ml/L	8.8ab	20.4a	24.9d	8.9fg
	2 ml/L	7.8abc	21.7a	24.7d	8.1gh
	4 ml/L	7.8abc	20.2a	29.8d	7.2gh
Vancomycin	0 mg/L	9.0a	23.0a	24.3d	21.5a
	1 mg/L	8.0abc	23.7a	25.9d	16.0b
	2.5 mg/L	8.1abc	20.4a	23.4d	12.0cde
	5 mg/L	8.1abc	20.5a	26.4d	11.2def
	10 mg/L	6.7bcd	18.4ab	35.6cd	7.3gh
Nystatin	0 mg/L	9.0a	23.0a	24.3d	21.5a
	10 mg/L	6.6cd	21.6a	29.8d	12.2cde
	25 mg/L	5.8d	18.1ab	52.6bc	8.9fg
	50 mg/L	3.8e	13.5b	57.8b	7.7gh
	100 mg/L	1.6f	8.0c	79.7a	5.9h
Penicillin G	0 mg/L	9.0a	23.0a	24.3d	21.5a
	0.5 mg/L	8.2abc	20.1a	19.1d	14.5bc
	1 mg/L	8.0abc	21.3a	23.8d	12.2cde
	2 mg/L	7.3abcd	20.1a	23.0d	9.8dfg
	4 mg/L	7.4abcd	19.3a	25.1d	8.6fg
		F value			
Two-way ANOVA	Type(A)	30.45**	9.51**	20.66**	9.34**
	Conc.(B)	16.63**	10.06**	6.48**	181.22**
	A × B	3.83**	2.56**	3.04**	1.22^{NS}

[z]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.

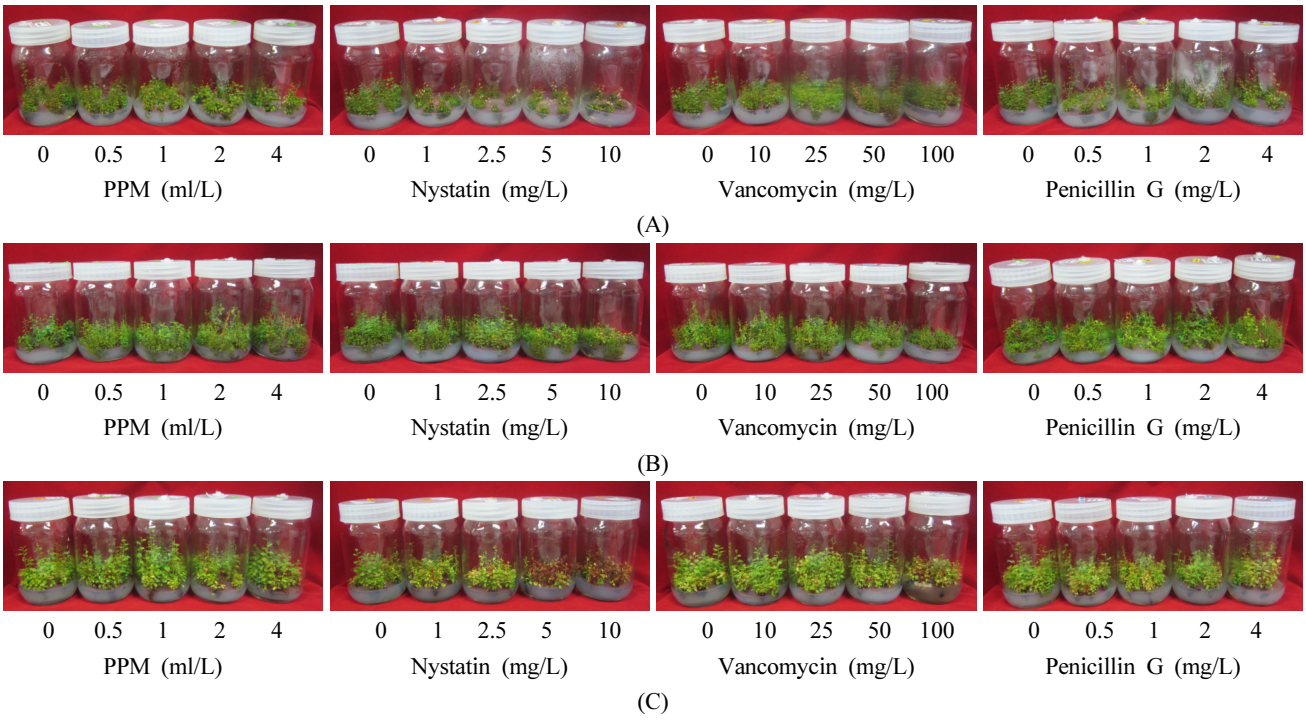

Fig. 1 Comparison of blueberry plantlets growth as affected by biocide type and concentration. (A): Northland, (B): Spartan, (B): Woodard

contamination efficaciously, but phytotoxicity, as indicated by plantlet death, increased severely. When PPM^{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^{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^{TM} can be used effectively to control the growth of bacteria and fungi in blueberry tissue cultures. The isothiazolones present in PPM^{TM} exhibited little phytotoxicity at the recommended levels. PPM^{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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