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Effects of calcium gluconate and ascorbic acid on controlling shoot necrosis during micropropagation of primocane-fruiting raspberry (*Rubus idaeus* L.) cultivars

Fridia Amalia[1], Samir C. Debnath[2] and Young Rog Yeoung[1]*

[1]Department of Horticulture, Gangneung-Wonju National University, Gangneung 210-702, Korea.
[2]Atlantic Cool Climate Crop Research Centre, Agriculture and Agri-Food Canada, 308 Brookfield Road, Bldg. 25, St. John's, NL A1E 5Y7, Canada.

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***In vitro* shoot necrosis is a quite widespread disorder affecting raspberry micropropagation. This study was conducted to investigate effects of calcium gluconate and ascorbic acid on shoot necrosis and dieback of raspberry shoots during micropropagation. Nodal segments of primocane-fruiting raspberry cultivars 'Allgold', 'Erika', and 'Polka' when cultured on Murashige and Skoog (MS) medium containing 0.6 mg·L^{-1} 6-benzyladenine (BA) and 1 g·L^{-1} calcium gluconate, showed lower explant browning and shoot necrosis and resulted in higher shoot initiation rate in all three cultivars. Ascorbic acid, at 50 and 100 mg·L^{-1}, increased fresh weight of microshoots of all three cultivars. Although culture medium containing calcium gluconate was found to reduce shoot growth and multiplication of 'Allgold' and 'Erika' compared to control, an addition of 1 g·L^{-1} calcium gluconate into MS medium containing 0.6 mg·L^{-1} BA, at shoot induction stage, is recommended to prevent explants browning and shoot necrosis during raspberry micropropagation.**

Key words: Explant dieback, nodal culture, shoot multiplication, tissue culture.

INTRODUCTION

Raspberries (*Rubus idaeus* L.) are traditionally propagated using adventitious buds that arise laterally on cold-treated root cuttings. In the 1980's, micropropagation was introduced as an option in raspberry propagation (Privé and Sullivan, 1991). The use of tissue culture propagation has increased by years as it can produce disease-free plants with rapid and uniform growth. The micropropagated plants spread faster and produce enhanced vegetative growth promoting early establishment and production in the field (Debnath et al., 2012).

However, the *in vitro* establishment of raspberry cultivars is still problematic (Wu et al., 2009). One of the most important problems in raspberry micropropagation is the rapid browning, necrosis, and dieback of the

*Corresponding author. E-mail: yryeoung@gwnu.ac.kr. Tel: +82-33-640-2356. Fax: 82-33-640-2909.

Table 1. Medium compositions tested in raspberry micropropagation.

Medium	Medium supplement substances	
	Calcium gluconate ($g \cdot L^{-1}$)	Ascorbic acid ($mg \cdot L^{-1}$)
Control	0	0
1	0.5	0
2	1	0
3	0	50
4	0	100

explants. These problems are partly caused by oxidation of polyphenols which are abundant in raspberry (Mederos-Molina and Trujillo, 1999) and/or by calcium deficiency that occurs during shoot initiation of culture (Abousalim and Mantell, 1994). Other reasons of shoot necrosis during micropropagation include drop of pH of the culture medium, prolonged subculture, and low temperature for the shoot growth (De Block, 1990).

Raspberries are rich in phenols and the phenolic content varies among cultivars (Liu et al., 2002). Phenolic substances, especially oxidized phenols, generally have negative effect on plant micropropagation (Arnaldos et al., 2001). These phenolic compounds are responsible for browning of the culture medium followed by subsequent lethal necrosis of explants (Ozyigit, 2008). The darkening or browning of the medium in tissue culture is caused by exudation and oxidation of phenolic compounds which results in the formation of quinones which are highly reactive and toxic to plant tissues (Ko et al., 2009). Ascorbic acid has been known as a phenol exudation inhibitor (Gil et al., 1998) and used as a medium supplement to decrease the symptoms of necrosis or browning leading to dieback of explants in various plant species (Ko et al., 2009). Ascorbic acid acts as an antibrowning agent in tissue culture by inhibiting the formation of strongly-oxidizing quinones and by inhibiting the activity of phenolases (Chikezie, 2012).

Another problem associating with browning and necrosis of shoots during *in vitro* culture is calcium deficiency. Calcium deficiency in plant can result from limited uptake of calcium ion and inadequate transport, because calcium is not remobilized or non-translocatable (Hepler, 2005). Smaller amount of calcium supply during *in vitro* culture is caused by low transpiration rate due to high humidity in the culture vessel and can introduce undesirable anions.

Addition of supplemental calcium into culture medium has been proved to decrease the necrosis and dieback of explants in micropropagation of some plant species. Calcium chloride added into MS (Murashige and Skoog, 1962) medium in range of 50-100 $mg \cdot L^{-1}$ efficiently prevented necrosis and dieback of shoots of banana and plantains (Martin et al., 2007). De Block (1990) suggested the use of calcium gluconate instead of calcium chloride or other calcium salts to increase calcium levels in the medium without changing the concentrations of other ions. Calcium gluconate, an organic form of calcium, effectively reduced frequencies of shoot tip necrosis in pistachio shoot cultures although it did not prevent the occurrence of symptoms (Abousalim and Mantell, 1994).

The objective of this work was to evaluate the effectiveness of ascorbic acid and calcium gluconate in eliminating tissue browning of three primocane-fruiting raspberry cultivars. The emphasis is particularly focused on the use of suitable substance concentration to decrease explant browning and shoot necrosis during raspberry micropropagation.

MATERIALS AND METHODS

Plant materials

Primocane-fruiting raspberry cultivars 'Allgold', 'Erika', and 'Polka' were grown in the greenhouse using drip irrigation under 10-12 h photoperiod (10-12 day h) at approximately 250 $\mu moles \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) at 22-25°C with 65-75% relative humidity. Standard cultural practices including spraying fungicide were applied when needed to keep the plants healthy.

Culture initiation and subculture

Ten-centimeter long actively growing apical shoots with removal of leaves, collected from healthy plants were washed under running tap water for 15 min and then surface-sterilized in a 1% (v/v) NaOCl solution containing few drops of Tween-20 for 15 min followed by rinsing four times in sterile distilled water. Nodal segments (0.5-1 cm-long) were excised aseptically and cultured in 25 × 200 mm glass vials containing 12 mL MS medium supplemented with 30 $g \cdot L^{-1}$ sucrose, 8 $g \cdot L^{-1}$ agar, 0.6 $mg \cdot L^{-1}$ 6-benzyladenine (BA), and two kinds of supplement substances calcium gluconate and ascorbic acid. Five treatments consisting of control (0.6 $mg \cdot L^{-1}$ BA), 0.6 $mg \cdot L^{-1}$ BA + calcium gluconate (0.5 and 1 $g \cdot L^{-1}$), and 0.6 $mg \cdot L^{-1}$ BA + ascorbic acid (50 and 100 $mg \cdot L^{-1}$) were used in a completely randomized design with 10 replications (Table 1). Each replication consisted of 20 tubes and each tube contained one explant. The pH of medium was adjusted to 5.7 prior to autoclaving at 121°C for 15 min. Cultures were maintained at 23 ±2°C, recommended by Isac and Popescu (2009) for raspberry micropropagation, under a constant lighting with a PPFD of = 27 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at culture level provided by fluorescent lamps (36 W) and subcultured every 30 days onto the same medium for shoot initiation and multiplication.

After 30 days of culture, 2-5 cm-long shoots were separated, the

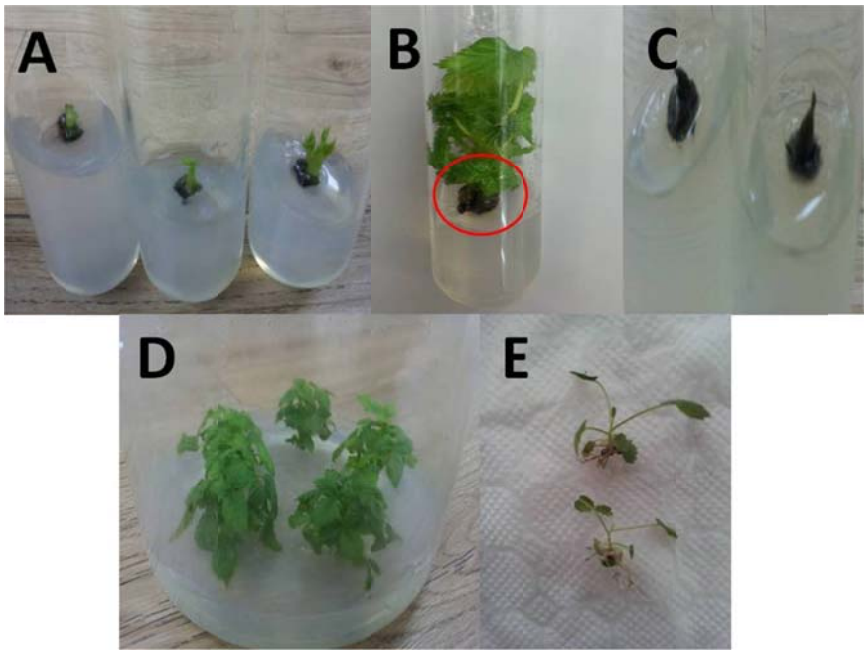

Figure 1. Primocane-fruiting raspberry micropropagation. **A,** Bud formation of 'Allgold' 'Erika' and 'Polka' respectively (from left to right), 10 days after initation of culture on MS medium containing 0.6 mg·L^{-1} BA and 1 g·L^{-1} calcium gluconate (scale =1 : 1.25). **B.** Shoot cluster and basal callus formation within 39 days of culture initiation on MS medium containing 0.6 mg·L^{-1} BA and 1 g·L^{-1} calcium gluconate in 'Erika' (scale =1: 1.25). C. explant browning of 'Allgold' during shoot induction stage on MS medium containing 0.6 mg·L^{-1} BA (panel; scale = 1 : 1). **D.** shoot clusters of 'Erika' at shoot multiplication stage on MS medium containing 0.6 mg·L^{-1} BA and 1 g·L^{-1} calcium gluconate after 30 days of culture (scale =1 : 1.8). **E.** Rooted shoots of 'Allgold' after 30-45 days of culture on MS medium containing 1.0 mg·L^{-1} IBA (scale = 1 : 7).

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leaves were removed, and the stem segments (3-5 cm-long) were transferred into 330-ml light transparent polycarbonate jars containing 100 ml of similar medium used in initiation culture. The treatments then were arranged in incomplete randomized block design, block as replication, where the experimental unit was obtained from survived shoots in previous culture. The replicates were made by considering the amount of the survived shoots from previous culture in each treatment. Transferred explants developed new shoots within 30 days of culture. In subsequent subcultures during the next 30 days, the number of shoots increased substantially.

Rooting and acclimatization

Well-grown shoot clusters were seperated and cultured in 330 ml light transparent polycarbonate jars containing 100 mL of half-strength MS medium supplemented with 1 mg•L^{-1} Indole-3-butyric acid (IBA) for rooting. Each vial contained five explants and there were ten vials per treatment. Rooted shoots were removed from tissue culture after 30-45 days, rinsed free of tissue culture medium, and planted in plug trays containing peat moss + vermiculite (1:1 v/v) (Nongwoo Bio Co., Ltd., Korea). Trays were kept in a growth chamber at 23 ±2°C, covered with transparent plastic covers and gradually acclimatized to ambient humidity. After three weeks, the plants were moved into pots (14 cm-diamater, and 14 cm-deep, equivalent to 1.5 L) and placed in a greenhouse exposed to 10-12 h photoperiod (PPFD = 250 µmoles·m^{-2}·s^{-1}) at 22 - 25°C with 50-65% relative humidity. The survival rate of potted plants was recorded at one month after transfer in greenhouse.

Data collection and statistical analysis

Data were recorded for each treatment on percentage of explant browning, shoot initiation, and shoot necrosis after 30 days of culture. Observation was also done on shoot necrosis percentage, shoot number, and fresh shoot weight (g) per explant after 60 days of culture initiation. Data were subjected to analysis of variance with the SAS statistical software package (Release 9.0, SAS Institute, Inc., Cary, N.C.). Duncan's multiple range test was used to compare treatment means at $P \leq 0.05$.

RESULTS

Nodal segment of three cultivars formed buds within 10-14 days of culture. This response might be attributed to the pre-existing meristems available in the nodal segments. Shoot initiation on 'Erika' explants was observed within 10 days of culture while 'Allgold' and 'Polka' took 12-14 days for shoot initiation (Figure 1A). Analysis of variance indicated that browning explants (30 days), shoot initiation rate (30 days), and survived shoots (60 days) were different among treatments (Tables 2a and b).

Table 2a. Effects of cultivar and medium composition in raspberry micropropagation on explant browning (30 days).

Medium	Browning explant (%)		
	'Allgold'	'Erika'	'Polka'
Control	42.5 ± 0.04^{c}	27.5 ±0.08^{b}	57.5 ± 0.04^{c}
1	30.0 ± 0.02^{b}	25.0 ± 0.08^{b}	32.5 ± 0.13^{b}
2	17.5 ± 0.05^{a}	4.5 ± 0.11^{a}	17.5 ± 0.05^{a}
3	27.5 ± 0.12^{b}	37.5 ± 0.02^{bc}	35.0 ± 0.11^{b}
4	45.0 ± 0.05^{c}	62.5 ± 0.02^{d}	40.0 ± 0.15^{b}

Mean separation within columns and factors by Duncan's multiple range test; $P \leq 0.05$. Means associated with different lower-case letters signify significant differences.

Table 2b. Effects of cultivar and medium composition in raspberry micropropagation on shoot initiation rate (30 days) and survived shoots (60 days).

Medium	Shoot initiation rate (%)			Survived shoots (%)		
	'Allgold'	'Erika'	'Polka'	'Allgold'	'Erika'	'Polka'
Control	87.5 ± 0.84^{bc}	90.0 ± 0.01^{b}	70.0 ± 1.68^{b}	85.0 ± 1.19^{bc}	88.5 ± 0.65^{bc}	65.0 ± 1.19^{d}
1	90.0 ± 1.18^{b}	100.0 ± 0.04^{a}	80.0 ± 0.04^{b}	85.0 ± 1.19^{bc}	90.0 ± 1.68^{b}	65.0 ± 2.06^{d}
2	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}	100.0 ± 0.04^{a}
3	87.5 ± 0.84^{bc}	82.5 ± 0.84^{c}	90.0 ± 0.04^{b}	77.5 ± 0.84^{cd}	62.5 ± 1.88^{d}	72.5 ± 1.88^{cd}
4	82.5 ± 0.84^{c}	70.0 ± 1.19^{d}	80.0 ± 1.68^{b}	62.5 ± 0.84^{d}	72.5 ± 1.46^{d}	77.5 ± 1.19^{cd}

Mean separation within columns and factors by Duncan's multiple range test; $P \leq 0.05$. Means associated with different lower-case letters signify significant differences.

An addition of 1 mg·L^{-1} calcium gluconate into MS medium containing 0.6 mg·L^{-1} BA at shoot induction stage resulted in highest shoot development rate for three cultivars. Cultivar 'Polka' showed the lowest survival rate (observed at 60 days after initiation). Clusters of 2-3 shoots per explant were developed within 30 days in all three cultivars. Nodal segments also developed calli at the base of each explant (Figure 1B). Browning symptoms appeared at first seven days of culture initiation. Severe browning symptoms on the entire parts of explants caused the death of explants that makes them unable to generate shoot (Figure 1C).

After 30 days of culture initiation, the initiated shoots were sub-cultured into the same medium for shoot multiplication. The shoot necrosis symptoms appeared at this stage. Addition of 1 g·L^{-1} calcium gluconate was observed to be effective in reducing frequency of shoot necrosis (Figure 2). Despite its beneficial effect to reduce shoot necrosis symptoms on *in vitro* culture, addition of calcium gluconate resulted in slower growth of shoot and reduced multiplication rate for cultivars, 'Allgold' and 'Erika', compared to control medium (BA 0.6 mg·L^{-1}). However, in 'Polka', addition of 1 g·L^{-1} calcium gluconate into culture medium reduced shoot necrosis symptoms, but did not reduce the rate of shoot growth. Presence of ascorbic acid at both concentration of 50 and 100 mg·L^{-1} in culture medium slightly prevented necrosis in 'Polka' and 'Allgold, but not in 'Erika'.

Browning symptoms and shoot necrosis showed negative correlation with shoot initiation rate and survived shoots. The lower explants browning and shoot necrosis resulted in higher shoot initiation rate and survived shoots and vice versa (Figure 3). There were significant differences on shoot number and shoot weight among cultivars and medium compositions after 60 days of culture (Figure 4A). Slow shoot multiplication of 'Allgold' and 'Erika' due to addition of calcium gluconate was indicated by their lower number of shoots per explant than the control (Figure 4A). Calcium gluconate supplementation also led to lower weight of shoot on cultivar 'Erika' (Figure 4B). On the other hand, addition of ascorbic acid into culture medium significantly increased shoot weight of all cultivars. The shoot weight of 'Polka' was increased with increasing the concentrantion of ascorbic acid. Shoot weight of 'Allgold' and 'Erika' increased at 50 mg·L^{-1} ascorbic acid but decreased at a concentration of 100 mg·L^{-1} (Figure 4B).

After 30-45 days of culture in shoot multiplication medium, raspberry shoot clusters were ready to transfer onto the rooting medium. Culture shoots rooted within 30-45 days (Figure 1E) and they were planted and maintained in the greenhouse. The survival rate of acclimated planlets reached 90-95% and abnormalities in plant development were not observed.

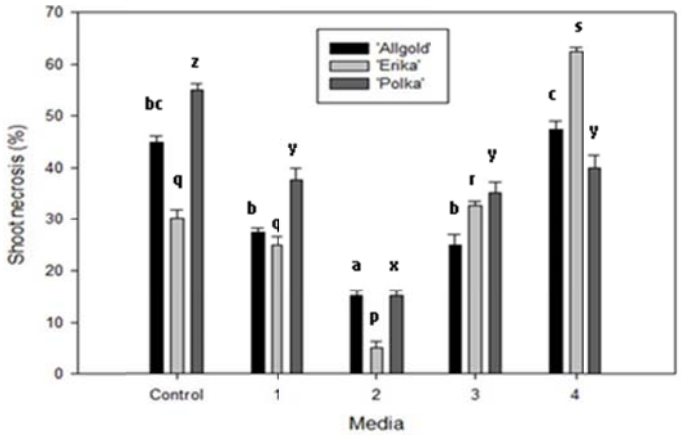

Figure 2. Effects of calcium gluconate and ascorbic acid on shoot necrosis after 60 days of culture on different culture medium compositions. Bars associated with different low case letter show significant difference within cultivar.

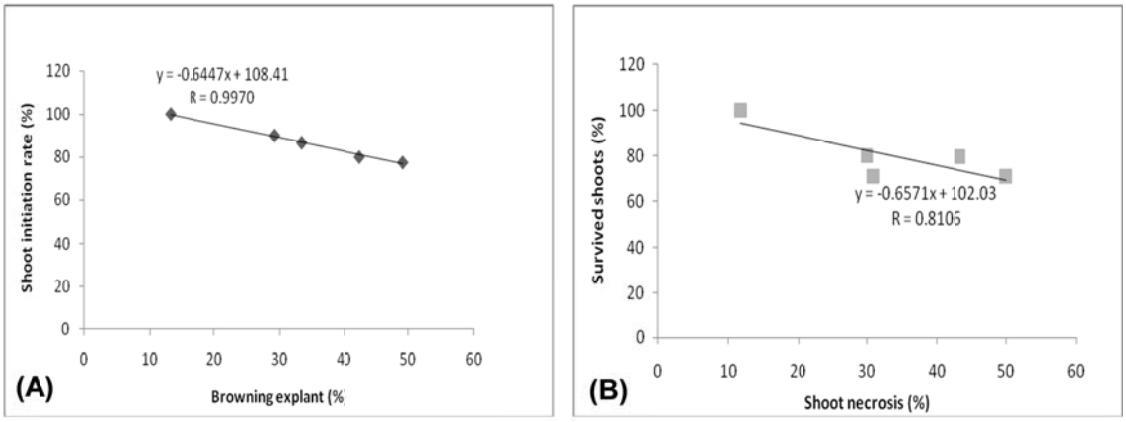

Figure 3. Negative correlation of browning explants on shoot initiation rate (A) and shoot necrosis on survived shoots (B).

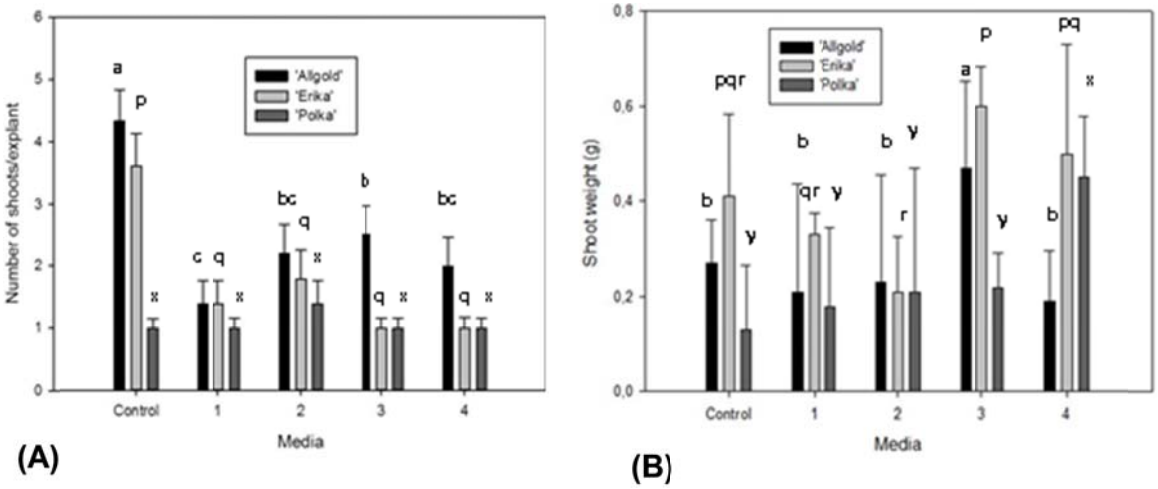

Figure 4. Effects of calcium gluconate and ascorbic acid on number of shoots/explant (A) and shoot weight (B) after 60 days of culture on different culture medium compositions. Bars associated with different low case letter show significant difference within cultivar.

DISCUSSION

Effects of genotype on shoot initiation rate

Raspberry cultivars used in this study required different period to initiate shoot. Node culture of 'Erika' showed shoot initiation earlier than those of 'Polka' and 'Allgold'. Plant genotype often profoundly affects explant response to *in vitro* culture as proposed by Burbulis et al. (2012). Similar observation on other raspberry cultivars was reported by Georgieva et al. (2004) where raspberry cultivar 'Bulgarski Rubin' started shoot initiation three weeks after culture initiation while 'Shopska Alena' started one week later. The different responses of raspberry cultivars in *in vitro* culture might be caused by the endogenous hormonal balance in plant tissue as mentioned by Tanaka et al. (2012) that the difference in adventitious bud regeneration between cultivars might be caused by differences in endogeneous hormones levels between cultivars.

Effects of calcium gluconate on shoot necrosis and plant growth

To eliminate shoot necrosis during micropropagation, calcium gluconate was used in a range recommended by previous reports on same cases. The use of 0.5 and 1 $g \cdot L^{-1}$ calcium gluconate in this experiment was in consideration with the finding of Singha et al. (1990) which mentioned that 0.03-3.0 $g \cdot L^{-1}$ calcium gluconate could effectively reduce shoot tip necrosis in shoot cultures of quince. Visible symptoms of shoot necrosis in primocane-fruiting raspberry culture showed a pale brown necrosis developed at the tips and margins of young leaves that spread to whole microshoots, and subsequently became darkened and eventually, the shoots died. These symptoms also occurred in *Pistachia* meristem culture where calcium deficiency was a problem (Abousalim and Mantell, 1994). Calcium is a major anion in plant cell and plays a key role in many physiological processes in plants. Thus, calcium deficiency could result in disturbance of metabolic activities of growing tissues, which in turn result in growth abnormalities such as shoot necrosis (Bairu et al., 2008).

Addition of 1 $g \cdot L^{-1}$ calcium gluconate was found to be effective to reduce frequency of explants browning and shoot necrosis on all raspberry cultivars. Alleviation of shoot necrosis by using calcium gluconate to increase calcium concentration in culture medium, as in this study, has been demonstrated in Pistachio (*Pistacia vera*) (Abousalim and Mantell, 1994), where calcium supplementation in MS medium using 1.5 and 3.0 $g \cdot L^{-1}$ calcium gluconate significantly decreased shoot tip necrosis and increased shoot length and bud number. Despite its benefit to reduce shoot necrosis, presence of calcium gluconate in medium reduced shoot multiplication rate of cultivars 'Allgold' and 'Erika'. An increase of calcium salts in medium of quince culture was reported to cause reduction of shoot proliferation and growth (Singha et al*.,* 1990). Giel and Bojarczuk (2011) mentioned that small amount of calcium salts in the growth substrate stimulates seedling growth of rhododendron cutting, while an excessively high calcium content of the substrate, inhibited their growth and development.

The reduction of raspberry explant growth in cultivar 'Allgold' and 'Erika' is suggested as an indirect influence of calcium on plant cell as proposed by Singha et al*.* (1990). One of the important roles of calcium within plant cells is to determine the structural rigidity of the cell wall. High concentration of calcium will rigidify the cell wall and make it less plastic resulting in slow cell division and tissue elongation (Hepler, 2005). Thus, from this mechanism, it can be assumed that elevating the Ca^{2+} concentration could reduce shoot multiplication. However, the effects of calcium on growth and development of plants is a complicated mechanism as Ca^{2+} influences plant growth through its role in cell wall structure, cellular membrane system, cell division, and its interaction with plant growth regulators (Poovaiah and Leopold, 1973) .

In this study, differential expression of shoot multiplication rate among genotypes of primocane-fruiting raspberry cultivars was observed in the presence of calcium gluconate in culture medium. These results could be attributed to the complicated interactions between calcium and plant hormones, which is varied among cultivars. Santner et al. (2009) suggested that many growth regulators (plant hormones) could potentially affect the plant susceptibility to Ca^{2+}. Specific growth regulator produced at different plant tissues, cultivars and developmental stages could potentially regulate plant Ca^{2+} uptake and translocation.

Effects of ascorbic acid on shoot necrosis and plant growth

To eliminate shoot necrosis during micropropagation, ascorbic acid in a dosage of 50 and 100 $mg \bullet L^{-1}$ was also used in this experiment by considering recommendation by Cassels and Minas (1983). They suggested that the high concentration of ascorbic acid (above 50 $mg \bullet L^{-1}$) is effective to control the shoot necrosis on *Pelargonium* micropropagation.

Ko et al. (2009) also mentioned that the use of ascorbic acid was found to be effective to prevent the development of lethal browning on tissue culture of cavendish banana. In this study, addition of ascorbic acid in culture medium significantly reduced explant browning and shoot necrosis on cultivar 'Polka' although less effectively than the addition of calcium gluconate. On the other hand, addition of 100 $mg \cdot L^{-1}$ ascorbic acid resulted in higher explant browning and shoot necrosis on cultivar 'Erika' compared to control medium. Different response of

raspberry cultivars to application of ascorbic acid can be resulted from the interaction between ascorbic acid and phenolic compounds. Ascorbic acid in high doses causes the production of large amount of hydroxyl radicals by reducing oxygen in the presence of transition iron and copper (John and Borut, 2007) available in culture medium. The large amount of hydroxyl radicals was absorbed and quenched with the excess of phenols (Jin and Russel, 2010) produced by cultivar 'Polka' while in cultivar 'Erika', the production of phenols might be not sufficient to extinguish those hydroxyl radicals. As the level of hydroxyl radicals overcome endogenous phenolic content in 'Erika', oxidative reactions might intensively occur and lead to high level of explant browning and necrosis (Jin and Russel, 2010).

Presence of ascorbic acid in culture medium of three cultivars of primocane-fruiting raspberry significantly affected the growth of cultures. The response of each cultivar on the addition of ascorbic acid was different. In cultivar 'Polka', shoot weight increased with the increase of ascorbic acid concentration to 100 $mg \cdot L^{-1}$, while in 'Allgold' and 'Erika', it increased at 50 $mg \cdot L^{-1}$ ascorbic acid and then decreased when the concentration was 100 $mg \cdot L^{-1}$. The decreased weight could be due to high level of explants browning and necrosis in 'Allgold' and 'Erika' at 100 $mg \cdot L^{-1}$ ascorbic acid. The effects of ascorbic acid on the plant growth were previously reported in some crops. Bathia and Ashwath (2008) found that addition of ascorbic acid in the range of 30-480 µM into medium could significantly promote *in vitro* shoot growth of tomato. They stated that quality of regenerated shoots could be improved by using ascorbic acid due to its function to oxidize growth inhibitory substances produced in tomato *in vitro* culture. Antioxidants including ascorbic acid may affect morphogenic processes by providing protection against oxidative stress (Leshem, 1988). Addition of antioxidants into tissue culture medium also enhances the development of isolated cells and tissues (Khan et al., 2011).

In conclusion, the present study clearly documented the effects of calcium gluconate and ascorbic acid on the shoot necrosis and growth of explants *in vitro* from nodal segments. Diverse responses were observed for shoot initiation rate among primocane-fruiting raspberry cultivars related to calcium deficiency that induced necrosis and dieback of explants. Generally, addition of calcium gluconate decreased explants browning and shoot necrosis during micropropagation of all raspberry cultivars used in this experiment. The decreasing explants browning and shoot necrosis then resulted in increasing shoot initiation rate and survived shoots. Therefore, an addition of 1 $g \cdot L^{-1}$ calcium gluconate into MS medium containing 0.6 $mg \cdot L^{-1}$ BA at shoot induction stage is recommended to promote shoot initiation and to get more viable shoots. We suggest this method is sui-table for shoot multiplication without explant browning and shoot necrosis.

Conflict of Interests

The author(s) have not declared any conflict of interest.

REFERENCES

Abousalim A, Mantell SH (1994). A practical method for alleviating shoot-tip necrosis symptoms in in vitro shoot cultures of *Pistacia vera* cv. Mateur. J. Hortic. Sci. 69(2):357-365.

Arnaldos TL, Munoz R, Ferrer MA, Calderon AA (2001). Changes in phenol content during strawberry (*Fragraria x ananassa*, cv. Chandler) callus culture. Physiol. Plant. 113:315-322.

Bairu MW, Jain N, Stirk WA, Doležal K, Staden JV (2008). Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. South Afr. J. Bot. 75:122-127.

Bathia P, Ashwath N (2008). Improving the quality of in vitro cultured shoots of tomato (*Lycopersicon esculentum* Mill. cv. Red Coat). Biotechnology 7:188-193.

Burbulis N, Blinstrubienė A, Masienė R, Jankauskienė Z, Gruzdevienė E (2012). Genotypic and growth regulator effects on organogenesis from hypocotyl explants of fiber flax (*Linum usitatissimum* L.). J. Food Agric. Environ. 10:397-400.

Cassels AC, Minas GJ (1983). Plant in vitro factors influencing the micropropagation of Pelargonium cultivars by bud-tip culture. Sci. Hortic. 21:53-65.

Chikezie UNY (2012). Effect of ascorbic acid on blackening and sprouting of *Musa* spp shoot tips. J. Biotechonol. Bioinform. 2: 11-17.

De Block M (1990). Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediumted transformation of hybrid aspen and poplar clones. Plant Physiol. 93: 1110-1116.

Debnath SC, Vyas P, Goyali JP, Igamberdiev AU (2012). Morphological and molecular analyses in micropropagated berry plants acclimatized under ex vitro condition. Can. J. Plant Sci. 92:1065-1073.

Georgieva M, Djilianov D, Kondakova V, Boicheva R, Kontantinova T, Parvanova D (2004). Regeneration from leaf explants of Bulgarioan raspberry cultivars and elites. Biotechnol. Biotechnol. Equip. 18: 8-14.

Giel P, Bojarczuk K (2011). Effects of high concentrations of calcium salts in the substrate and its pH on the growth of selected rhododendron cultivars. Acta Soc. Bot. Pol. 80:105-114.

Gil MI, Gorny JR, Kader AA (1998). Responses of 'Fuji' apple slices to ascorbic acid treatments and low oxygen atmospheres. HortScience 33:305-309.

Hepler PK (2005). Calcium: a central regulator of plant growth and development. Plant Cell 17: 2142-2155.

Isac V, Popescu AN (2009). Protocols for *in vitro* micropropagation of raspberry. http://www.euroberry.it/documents/wgm08/Book%20of%20abstracts Cacak2009.pdf. Accessed on June, 2012.

Jin D, Russel JM (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15: 7313-7352.

John GI, Borut P (2007). Metal ions mediated pro-oxidative reactions with vitamin C: possible implications for treatment of different malignancies. Int. J. Cancer Prev. 3:1-26.

Khan TA, Mazid M, Mohammad F (2011). Ascorbic acid: an enigmatic molecule to developmental and environmental stress in plant. Int. J. Appl. Biol. Pharm. Technol. 2:468-483.

Ko WH, Su CC, Chen CL, Chao CP (2009). Control of lethal browning of tissue culture plantlets of Cavendish banana cv. Formosana with ascorbic acid. Plant Cell Tissue Organ 96: 137-141.

Leshem Y (1988). Plant senescence processes and free radicals. Free Radic. Biol. Med. 5:39-49.

Liu M, Li XQ, Weber C, Lee CY (2002). Antioxidant and antiproliferative of raspberries. J. Agric. Food Chem. 50: 2926-2930.

Martin KP, Zhang CL, Slater A, Madassery J (2007). Control of shoot necrosis and plant death during micropropagation of banana plantains (*Musa* spp.). Plant Cell Tissue Organ 88:51-59.

Mederos-Molina S, Trujillo MI (1999). Elimination of browning exudate and in vitro development of shoots in *Pistacia vera* L. Cv. Mateur and *Pistacia atlantica* desf. Culture. Acta Soc. Bot. Pol. 68:21-24.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-97.
Ozyigit II (2008). Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips. Afr. J. Biotechnol. 7:1145-1150.
Poovaiah BW, Leopold AC (1973). Deferral of leaf senescence with calcium. Plant Physiol. 52:236-239.
Privé JP, Sullivan JA (1991). Performance of tissue-cultured primocane-fruiting red raspberries following chilling. HortSci. 26:590-592.
Santner A, Calderon-Villalobos LIA, Estelle M (2009). Plant hormones are versatile chemical regulators of plant growth. Nat. Chem. Biol. 5:301-307.
Singha S, Townsend EC, Oberly GH (1990). Relationship between calcium and agar on vitrification and shoot tip necrosis of quince (*Cydonia oblonga* Mill.) shoots *in vitro*. Plant Cell Tissue Organ 23:135-142.
Tanaka H, Johkan M, Mitsukuri K, Tezuka T, Furukawa H, Oda M (2012). Intact roots promote shoot regeneration from hypocotyl independent of exogeneous plant growth regulators in eggplant in vitro. Plant Root 7:5-11.
Wu JH, Miller SA, Hall HK, Mooney PA (2009). Factors affecting the efficiency of micropropagation from lateral buds and shoot tips of *Rubus*. Plant Cell Tissue Organ 99:17-25.