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Appropriate choice of antibiotics for plant regeneration and optimization of selective agents to be used in genetic transformation of chrysanthemum

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Abstract

Plant genetic transformation requires appropriate choice of antibiotics for regeneration of transformed tissues and elimination of *Agrobacterium*, and minimal concentration of selective agents for the selection of putative transformants during transformation. This study was conducted to examine the effects of three antibiotics (carbenicillin, cefotaxime, and Clavamox) on *in vitro* plant regeneration from leaf segments of *Chrysanthemum morifolium* (Ramat.) 'Vivid Scarlet', and percentages of shoot induction and number of shoots per explant were recorded after 5 weeks of culture. Carbenicillin and Clavamox had less inhibitory effects on number of shoots per explant than cefotaxime, but superior plant growth (i.e. number of leaves, number of roots, plant height, and fresh weight) was observed in shoots treated with 125 $mg{\cdot}L^{-1}$ Clavamox. Analysis of stomata size indicated that there was no variation in ploidy level between mother plants (controls) grown in the greenhouse and plants regenerated in vitro with 125 $mg{\cdot}L^{-1}$ Clavamox. These findings suggest that Clavamox can effectively replace carbenicillin or cefotaxime in *Agrobacterium*-mediated genetic transformation studies of 'Vivid Scarlet'. In addition, screening of the selective agents revealed optimal concentrations of kanamycin 30 $mg{\cdot}L^{-1}$ and phosphinothricin 0.4 $mg{\cdot}L^{-1}$, respectively, for the selection of putative transgenic 'Vivid Scarlet' plants.

Keywords *Agrobacterium tumefaciens*, Antibiotics, Ornamental plant, Ploidy level, Selective agents.
Abbreviations: BA_6-benzyladenine; Carb_Carbenicillin; Cef_Cefotaxime; Cav_Clavamox; Kan_Kanamycin; MS_ Murashige and Skoog; NAA_Naphthaleneacetic acid; PGRs_ Plant growth regulators; PPT_Phosphinothricin.

Introduction

A limited gene pool and cross incompatibility have constrained the improvement of chrysanthemum by conventional breeding methods (Rout and Das, 1997). *Agrobacterium*-mediated transformation has recently been considered as a promising method for introducing desired genes into higher plants; however, this approach requires efficient procedures for *in vitro* regeneration. A protocol for efficient in vitro regeneration from leaf segments of the chrysanthemum cultivar 'Vivid Scarlet' was established previously (Naing et al. 2014). To achieve genetic transformation, plant tissues are infected by co-cultivation with *Agrobacterium tumefaciens* carrying a gene of interest. After co-cultivation, elimination of the bacterium is normally achieved by transferring plant tissues to a regeneration medium containing *Agrobacterium*-suppressing antibiotics. Currently, carbenicillin and cefotaxime are the most commonly used antibiotics for this purpose because they specifically inhibit the synthesis of prokaryotic cell walls and thereby kill bacteria (Pollock et al. 1983; Asbel and Levison 2000). Studies of shoot regeneration and *Agrobacterium*-mediated transformation in chrysanthemum have also been conducted using carbenicillin or cefotaxime (Fukai et al. 1993; Urban et al. 1994; Seo et al. 2003; Song et al. 2012). However, these studies did not investigate the effects of these antibiotics on plant regeneration potential. Several studies have reported that involvement of these antibiotics in shoot regeneration media inhibited plant cell growth, organogenesis, and embryogenesis (Nauerby et al. 1997; Yu et al. 2001; Ogawa and Mii 2005; Wiebke et al. 2006), especially in herbaceous plants. In addition, these antibiotics are expensive as well. Therefore, it is necessary to find an effective and inexpensive alternative antibiotic that has less inhibitory effect on shoot regeneration and can effectively eliminate *A. tumefaciens*. Clavamox (Pfizer Animal Health) has been widely used in animal hospitals to inhibit the infection of wounds, abscesses, cellulitis, and superficial/juvenile and deep pyoderma by susceptible strains of β-lactamase-producing *Staphylococcus aureus*, non-β-lactamase-producing *Streptococcus* spp., and *Escherichia coli*. Clavamox combines the properties of a broad-spectrum antibiotic (amoxicillin trihydrate) and a β-lactamase inhibitor (clavulanate potassium) and has been used to suppress *Agrobacterium* growth during the genetic transformation of plants (Kim et al. 2004; Ren et al. 2012). However, the effects of Clavamox on plant cell growth, organogenesis, and embryogenesis are unknown. In vitro screening of putative transgenic plants has been achieved using selective agents such as kanamycin or phosphinothricin (PPT), which kill or prevent the growth of surrounding non-transgenic cells. However, the minimum concentration of selective agents required to destroy non-transgenic cells must be determined because higher-than-optimal concentrations can also destroy transgenic cells.

Moreover, the optimal concentration of selective agents differs among cultivars (Urban et al. 1994; Boase et al. 1998; Seo et al. 2003; Kumara et al. 2012; Song et al. 2012).. Optimization of selective agents thus plays a critical role in successful transformation of plants. The objective of this study was to evaluate the effects of Clavamox on shoot regeneration relative to the effects of widely used antibiotics (carbenicillin and cefotaxime), and to determine the minimal concentrations of kanamycin or PPT required to screen putative transgenic chrysanthemum 'Vivid Scarlet' plants.

Results

Effects of antibiotics on shoot regeneration

To evaluate the effects of the antibiotics on shoot regeneration, leaf explants were cultured on shoot regeneration medium containing PGRs alone (control) or in combination with different concentrations of carbenicillin, cefotaxime, and Clavamox. Antibiotic concentration did not affect shoot regeneration percentage (i.e. 100 % in all treatments), but the number of usable shoots per explant was inhibited by the antibiotics. The number of usable shoots formed was almost identical in treatments containing carbenicillin and Clavamox and was lower in treatments containing cefotaxime. Shoot inhibition was highly dose-dependent; the higher the concentration of antibiotics, the greater the inhibition (Fig. 1). Shoots treated with Clavamox appeared more vigorous and healthy than the controls or those treated with carbenicillin or cefotaxime; control and carbenicillin-treated shoots were of similar quality and better than those grown in media containing cefotaxime. These findings indicate that the antibiotics are likely to have affected the physiological activity of regenerated shoots and that Clavamox had a more positive effect than the other antibiotics. We next attempted to clarify whether the antibiotics affected the physiology of regenerants by examining rooting efficiency and plant growth parameters using in vitro experiments.

Determination of plant growth parameters and acclimatization

Shoots obtained from the control and antibiotic treatments were cultured on PGR-free MS medium; growth parameters were evaluated and were found to be dependent on the treatment (Fig. 2A–D). As expected, growth of shoots in Clavamox-treated media (125 $mg{\cdot}L^{-1}$) was superior to growth in other media. However, growth of shoots obtained from treatments with 200 $mg{\cdot}L^{-1}$ carbenicillin or cefotaxime was superior to that of shoots from treatments with 100 $mg{\cdot}L^{-1}$ of these antibiotics, but 200 mgl^{-1} exerted a more negative effect on shoot regeneration than 100 $mg{\cdot}L^{-1}$ (Fig. 1A, B). The highest mean number of roots was obtained in shoots derived from the media with the lowest concentration of antibiotics. Shoots from treatments containing 125 $mg{\cdot}L^{-1}$ Clavamox performed better than shoots produced in the other treatments for all parameters except regeneration efficiency, for which plants treated with Clavamox and carbenicillin performed equally. These results suggest that the choice and concentration of antibiotic used for genetic transformation is important for the successful production of good-quality transgenic plants. Plantlets that reached approximately 4–5 cm in height were transferred into acclimatization pots, and >80% of these plants survived in the greenhouse.

Effects of Clavamox on the elimination of Agrobacterium growth

The Clavamox concentration (125 $mg{\cdot}L^{-1}$) that showed a positive effect on the growth of 'Vivid Scarlet' was tested to determine whether it was sufficient to eliminate *Agrobacterium* growth. No growth of *Agrobacterium* strains LBA 4404, C58C1, GV3101, or AGL1 was observed in YEP medium containing 125 mgl^{-1} Clavamox (Fig. 3A–D).

Leaf epidermal impressions

Frequency and size of stomata in plants used as control and plants regenerated from 125 $mg{\cdot}L^{-1}$ Clavamox containing medium was analyzed to investigate possible relationships with ploidy level; the frequency and size of stomata did not differ between the control and the antibiotic-treated plants (Fig. 4A, B). Specifically, the average length and width of stomata in plants cultured on shoot-regeneration media containing 125 $mg{\cdot}L^{-1}$ Clavamox were almost identical to those of the control (Table 1), revealing no variation in ploidy level among the plants.

Effects of selective agents (kanamycin and phosphinothricin) on shoot regeneration

To achieve efficient screening of transgenic plants, we investigated the minimal concentrations of selective agents that could destroy non-transgenic cells by culturing leaf segments on shoot regeneration medium containing 125 $mg{\cdot}L^{-1}$ Clavamox and different concentrations of kanamycin and PPT. Shoot regeneration from leaf explants was not inhibited significantly by 10 mgl^{-1} kanamycin; however, some of the regenerated shoots developed poorly or the shoot tips became white and gradually died. Increasing the concentration of kanamycin to 20 $mg{\cdot}L^{-1}$ caused a distinct reduction in the mean number of shoots per explant, and shoot regeneration was decreased relative to the control (Fig. 5A, B). Moreover, anthocyanin expression was observed in the regenerated shoots. Further increasing the concentration of kanamycin to 30 $mg{\cdot}L^{-1}$ resulted in the induction of a callus that did not form shoots after 4 weeks of culturing explants. Necrosis was observed in explants when the medium was supplemented with higher concentrations of kanamycin (40–50 $mg{\cdot}L^{-1}$). As for PPT, lower concentrations of PPT (0.1–0.2 $mg{\cdot}L^{-1}$) did not significantly inhibit shoot regeneration. A concentration at 0.3 $mg{\cdot}L^{-1}$ PPT led to strong, but not complete, inhibition of shoot regeneration (Fig. 6A, B). In contrast to kanamycin, no damage was observed in the regenerated shoots. However, when the concentration of PPT was increased to 0.4 $mg{\cdot}L^{-1}$, complete necrosis of all explants was observed after 12 days of culture. From these results, we determined that 30 mgl^{-1} kanamycin and 0.4 $mg{\cdot}L^{-1}$ PPT would be useful concentrations for the efficient screening of putative transgenic 'Vivid Scarlet' plants.

Discussion

Reports on the effects of antibiotics on in vitro shoot regeneration in chrysanthemums are still limited. In this study, we compared the effects of three antibiotics (carbenicillin, cefotaxime, and Clavamox) on the regeneration of chrysanthemum shoots and observed clear inhibitory effects. Similar to these results, carbenicillin and cefotaxime were found to have negative effects on chrysanthemum morphogenesis (Teixeira da Silva and Fukai 2001). Chung and

Table 1. Frequency and size of stomata in plants used as control and plants regenerated on 125 mg·L^{-1} Clavamox containing medium.

Ploidy	Origin	Stomatal frequency	Size of Stomata	
			Length (uM)	Width (µM)
Hexaploid	Mother plant (control)	6.0a	44.9±0.7a	40.4±0.6a
Hexaploid	Regenerated plant from Clavamox125 mg·L^{-1}	6.0a	44.7±.3a	41.6±0.6a

Means with the same letter are not significantly different by Duncan's multiple range test (P< 0.05).

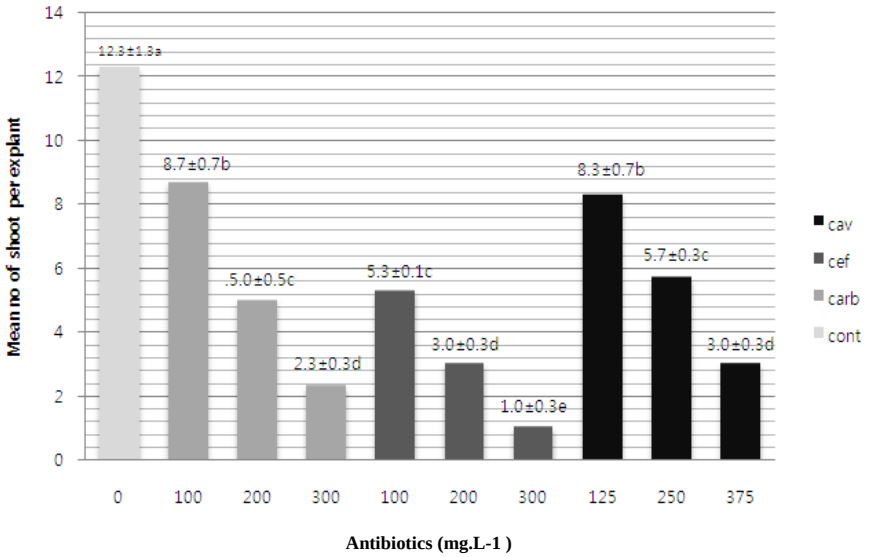

Fig 1. Effects of antibiotics (carbenicillin, cefotaxime, and Clavamox) on the mean number of usable shoots per explant
Control- 1.0 mg·L^{-1} 6-benzyladenine (BA) + 2.0 mg·L^{-1} naphthaleneacetic acid (NAA). Cav, Clavamox; cef, cefotaxime; carb, carbenicillin; cont, control.

Park (2005) also observed an adverse effect of cefotaxime on shoot regeneration in chrysanthemum. In the present study, cefotaxime had stronger inhibitory effects than carbenicillin, in contrast to the findings of Teixeira da Silva and Fukai (2001) who reported that carbenicillin showed greater phytotoxicity than cefotaxime in chrysanthemum. This discrepancy might be a result of the use of different genotypes in the respective studies. Similar to our findings, Tang et al. (2000) showed that carbenicillin led to a less pronounced reduction in somatic embryogenesis than cefotaxime in walnut. Yu et al. (2001) found that media containing carbenicillin induced superior somatic embryogenesis than that containing cefotaxime. Moreover, Tang et al (2003) reported that carbenicillin was associated with a higher frequency of shoot regeneration and mean numbers of shoots per explant than cefotaxime in loblolly pine. Positive effects of carbenicillin on shoot regeneration were also reported for *Dianthus caryophyllus* 'Scania' (Estopa et al. 2001), *Coryphantha elephantidens* (Bhau and Wakhlu 2001), and *Pinus taeda* (Tang et al. 2003). da Silva Mendes et al. (2009) also reported a detrimental effect of cefotaxime on shoot regeneration in *Citrus sinensis.* Unlike the antibiotics mentioned above, Clavamox is not commonly used in *Agrobacterium*-mediated transformation but has been used to suppress *Agrobacterium* growth in some genetic transformation studies (Kim et al. 2004; Ren et al. 2012). However, effects of Clavamox on plant cell growth, organogenesis, and embryogenesis have not been well documented to date. In this study, Clavamox inhibited shoot regeneration to the same extent as carbenicillin, but shoots derived from Clavamox were superior in quality to those derived from other treatments, including the control. Although it had inhibitory effects on shoot regeneration, Clavamox appeared to exert a hormonal effect on plant growth and development. Ren et al. (2012) reported that the presence of timentin resulted in aberrant morphology in 70% of shoots and severely reduced shoot elongation frequency in *Citrus sinensis*, while only 5% of shoots grown on media containing Clavamox were abnormal. These findings suggest that Clavamox is more appropriate for *Agrobacterium*-mediated transformation of the chrysanthemum 'Vivid Scarlet' than carbenicillin, although these antibiotics have the same effect on shoot regenerability. Clavamox tablets are sterile and individually packaged, and the antibiotic is stable and convenient to use in this form since there is no need to prepare a stock solution. Clavamox also has the advantage of being much less expensive than carbenicillin or cefotaxime. The presence of antibiotics in the shoot regeneration medium affected subsequent in vitro growth of shoots on hormone-free medium (Fig. 2A–D), and Clavamox had stronger positive effects than carbenicillin or cefotaxime. These findings suggest the occurrence of different physiological processes among the treatments. Ren et al (2012) recently reported that Clavamox was associated with superior shoot elongation frequency to timentin in *Citrus sinensis*. It is also possible that residual antibiotics from the shoot regeneration media may have subsequently affected in vitro rooting and plant growth. Residual effects of carbenicillin, cefotaxime, or timentin from

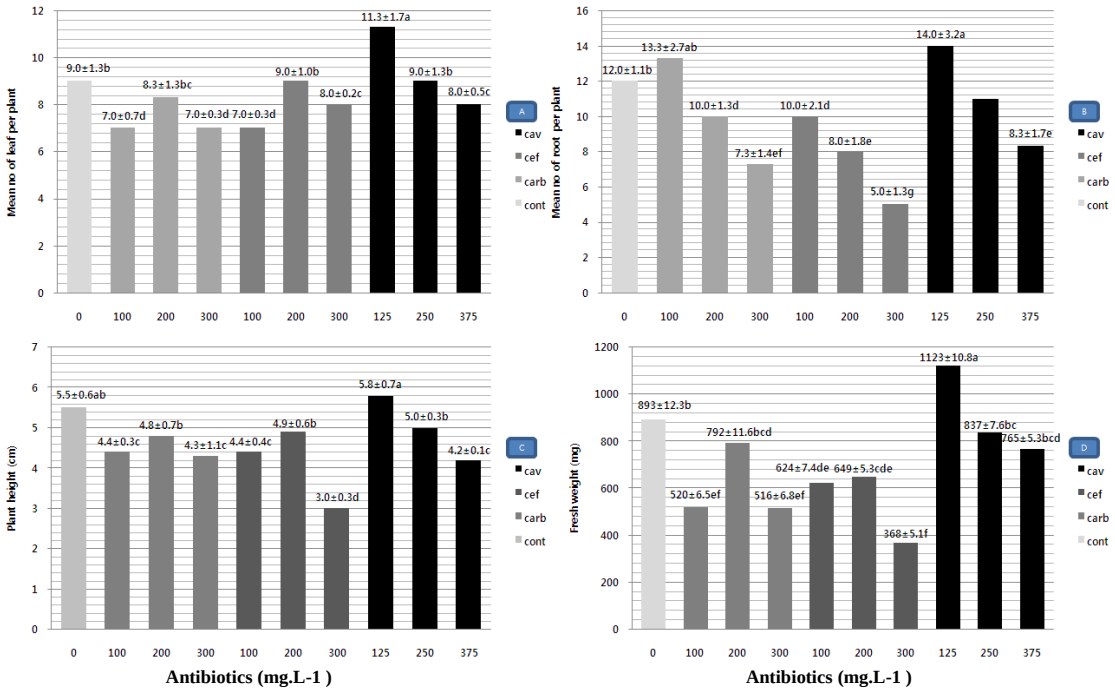

Fig 2. Plant growth parameters in shoots grown on media with antibiotics (carbenicillin, cefotaxime, or Clavamox) and in control treatments. A) mean number of leaves per plant; B) mean number of roots per plant; C) mean plant height; D) fresh weight after 5 weeks of culture. Cav, Clavamox; cef, cefotaxime; carb, carbenicillin; cont, control.

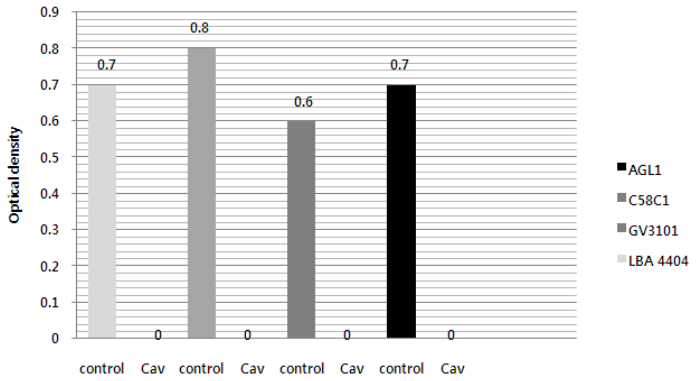

Fig 3. Effects of the antibiotic Clavamox (125 mg·L^{-1}) on growth of different strains of *Agrobacterium tumefaciens* (LBA4404, GV3101, C58C1, AGL1) Data represent the readings of optical density at 600 nm. (OD_{600}) 18 h after inoculation in YEP medium
Control- 50 mg·L^{-1} spectinomycin + 50 mg·L^{-1} rifampicin (LBA 4404, GV3101, and C58C1)
Test-50 mg·L^{-1} spectinomycin + 50 mg·L^{-1} rifampicin + 125 mg·L^{-1} Clavamox (LBA 4404, GV3101, and C58C1)
Control- 50 mg·L^{-1} kanamycin + 50 mg·L^{-1} rifampicin (AGL 1)
Test- 50 mg·L^{-1} kanamycin + 50 mg·L^{-1} rifampicin + 125 mg·L^{-1} Clavamox (AGL1)

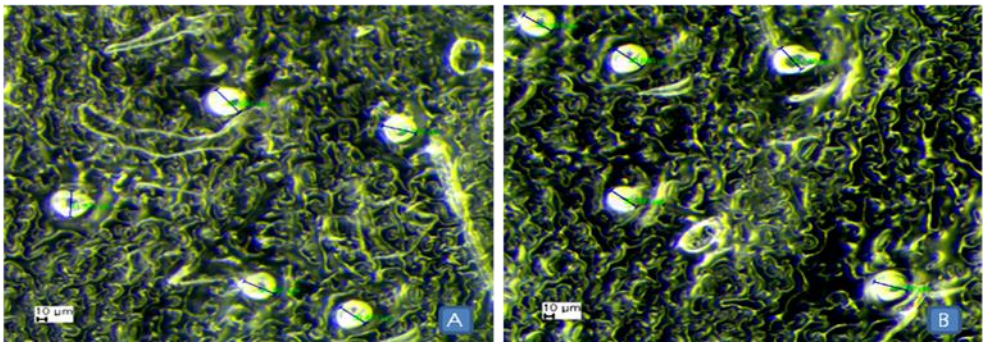

Fig 4. Analysis of ploidy level by using the leaf epidermis of chrysanthemum 'Vivid Scarlet' plants in control plants and plants regenerated from media containing 125 mg·L^{-1} Clavamox, A) Leaf epidermis of mother plants (control, hexaploid) grown in the greenhouse; B) regenerated plants from the medium containing Clavamox.

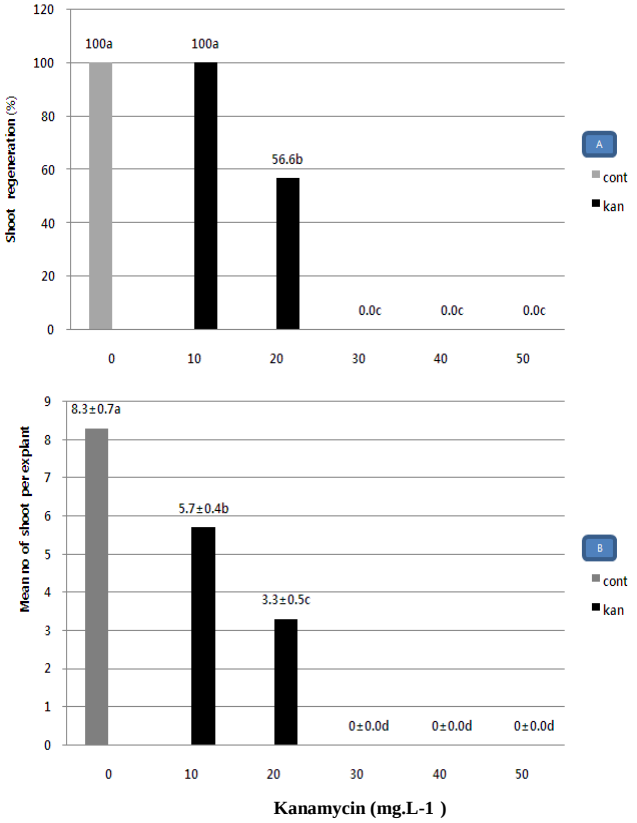

Fig 5. Effects of kanamycin on the percentage of shoot regeneration (A) and mean number of usable shoot per explant (B). *Control*- 1.0 mg·L^{-1} BA+ 2.0 mg·L^{-1} NAA + 125 mg·L^{-1} Clavamox.

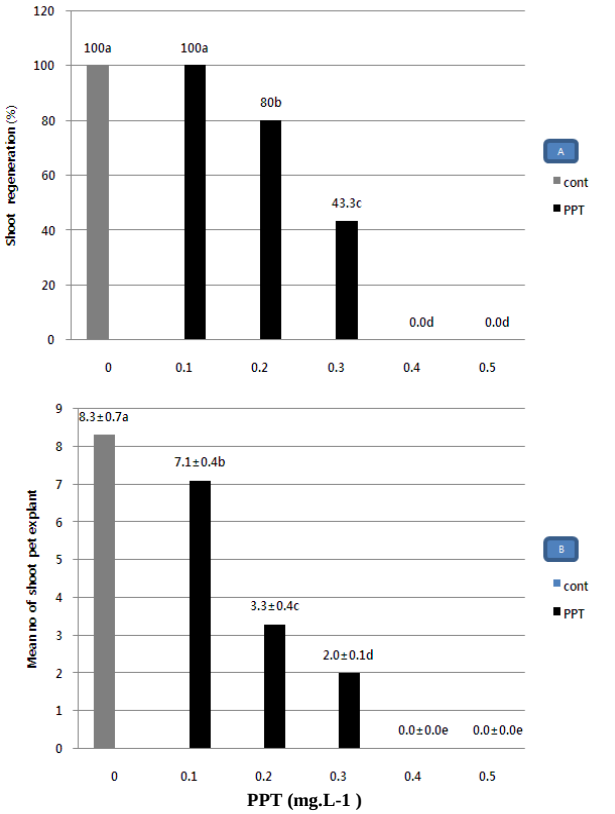

Fig 6. Effects of phosphinothricin (PPT) on percentages of shoot regeneration (A) and mean number of usable shoots per explant (B). *Control*- 1.0 mg·L^{-1} BA+ 2.0 mg·L^{-1} NAA + 125 mg·L^{-1} Clavamox.

shoot regeneration media on subsequent rooting ability were previously reported for tobacco (Nauerby et al. 1997), tomato (Costa et al. 2000), and citrus (da Silva Mendes et al. 2009). However, residual effects of Clavamox on in vitro rooting and plant growth have not been reported previously. Our results indicated a positive effect of Clavamox at a concentration of 125 mg·L^{-1}; Clavamox is thus predicted to provide a good replacement for carbenicillin or cefotaxime in *Agrobacterium*-mediated transformation studies of chrysanthemum.

Kim et al. (2004) and Ren et al. (2012) used 250 mg·L^{-1} Clavamox to suppress *Agrobacterium* overgrowth in explants inoculated with LBA 4404, while we found that treatment with 125 mg·L^{-1} Clavamox was effective in eliminating four *Agrobacterium* strains. We conclude that 125 mg·L^{-1} Clavamox can be used for both elimination of *Agrobacterium* and efficient regeneration of genetically transformed plants. Genetic variation has the potential of producing new cultivars with valuable traits in plant breeding; however, it may be an undesired issue for genetic transformation. A few studies have reported that the involvement of antibiotics in plant regeneration media causes genetic variation (Schmitt et al. 1997; Bardini et al. 2003, and Sun et al. 2013). Stomata frequency and size were successfully used as indicators of ploidy level in *Bixa orellana* (Carvalho et al. 2005), *Chrysanthemum cinerariifolium* (Liu and Gao 2007), and *Passiflora cincinnata* (Pinto et al. 2010). In the present study, stomata frequency and size indicated an absence of polyploidy. Teixeira da Silva et al. (2001) also reported that application of antibiotics did not cause polyploidy in chrysanthemum, but the effects of Clavamox on ploidy level have not been reported until now. Kanamycin is commonly used as a selective agent for screening transgenic chrysanthemum plants (Urban et al. 1994; Boase et al. 1998; Seo et al. 2003; Kumara et al. 2012; Song et al. 2012). However, the optimal concentration of kanamycin differs among cultivars. Urban et al (1994) screened transgenic chrysanthemum 'Hekla and Polaris' by using 50 mgl^{-1} kanamycin, while Boase et al. (1998) and Seo et al. (2003) used 20 mg·L^{-1} for 'Peach Margaret' and 'Puma and Subangryeok.' Similarly, 7.5 mg·L^{-1} was optimal for the selection of transgenic plants in 'Orlando' (Song et al. 2012), while 100 mg·L^{-1} was used for 'Kundan.' Here, 30 mg·L^{-1} kanamycin was observed to be optimal for screening 'Vivid Scarlet.' In addition, the basal leaves of some shoots induced by kanamycin showed anthocyanin expression, and these shoots gradually died. It would be of interest to determine whether kanamycin promoted the accumulation of anthocyanins in leaves or whether shoots were stressed by kanamycin. Unlike kanamycin, PPT has not been used in the genetic transformation of chrysanthemum, although it has been widely used in other crops. In this study, shoots derived from media containing PPT did not show disorder or damage; thus, the susceptibility of explants was more severe to kanamycin than to PPT.

Materials and Methods

Plant materials

Chrysanthemum morifolium (Ramat.) 'Vivid Scarlet,' provided

by the National Institute of Horticultural and Herbal Science (South Korea), were transplanted into a greenhouse.
Explants for shoot regeneration were prepared as described by Naing et al. (2014).

Effects of antibiotics on shoot regeneration

Combination of 1 $mg{\cdot}L^{-1}$ 6-benzyladenine (BA) and 2 $mg{\cdot}L^{-1}$ naphthaleneacetic acid (NAA) was determined by Naing et al. (2014) to be optimal and was used in the present study. Leaf segments (0.5–1.0 cm) were cultured on Murashige and Skoog (MS) medium containing the optimal combination of PGRs and different concentrations of carbenicillin (Duchefa, The Netherlands), cefotaxime (Duchefa), and Clavamox (Pfizer Animal Health), which were added to the medium after autoclaving.

Determination of plant growth parameters and acclimatization

Shoots derived from media containing PGRs alone or in combination with each antibiotic treatment were placed onto PGR-free MS medium for the determination of plant growth. Five plantlets per treatment were used with three replications. Growth parameters were recorded after 5 weeks of culture, after which plantlets that reached 4–5 cm in height were planted in a cell tray with vermiculite soil and acclimatized in a greenhouse at 25 °C. After 3 weeks, the plantlets were transferred to pots containing peat-based soil and were grown in the greenhouse.

Effects of Clavamox on the elimination of Agrobacterium growth

We used *Agrobacterium tumefaciens* strains LBA4404, GV3101, and C58C1 harboring plasmids pB7WG2D and AGL1 carrying pJAM1980. *Agrobacterium* maintained in glycerol stock at –80 °C were grown at 28 °C overnight in an orbital shaker at 250 rpm on YEP medium containing appropriate antibiotics (control) or in combination with 125 $mg{\cdot}L^{-1}$ Clavamox. Optical density at 600 nm (OD_{600}) was read 18 h after *Agrobacterium* inoculation.

Leaf epidermal impressions

Three fully expanded leaves were sampled from each plant, and epidermal impressions of the leaf abaxial surfaces were made using clear nail polish. The impressions were examined and photographed using a light microscope (Olympus AX-70) with a coupled U-photo camera system and a computer with isolation software. For determination of stomatal length and width, three fields were photographed with a 11.5-mm objective lens, and five stomata in each field were randomly selected for measurement.

Effects of selective agents (kanamycin and phosphinothricin) on shoot regeneration

In order to determine optimal concentration of selective agents [kanamycin (Duchefa, The Netherlands), and phosphinothricin (PPT) (Duchefa)], that can destroy growth of non-transgenic cells, leaf segments were cultured on MS medium containing the optimal combination of PGRs, 125 $mg{\cdot}L^{-1}$ Clavamox, and different concentrations of the selective agents, which were added to the medium after autoclaving.

The percentages of sucrose and Gelrite used for all cultures were 3% and 0.3%, respectively. All cultures were incubated under fluorescent lights at an intensity of 37–40 $\mu mol\ m^{-2}\ s^{-1}$ with a 16-h photoperiod.

Experimental design and statistical analysis

All cultures were placed in a completely randomized design. Concentrations of the antibiotics used in these experiments are shown in each representing figure. Each treatment consisted of three replicates of 30 explants. The total number of usable shoots (about 3 cm in height) per explant was counted after 5 weeks of culture. All data were analyzed using analysis of variance (ANOVA), and means were compared using Duncan's multiple range test ($p < 0.05$).

Conclusion

We evaluated the effects of antibiotics on shoot regeneration from leaf segments of the commercial cultivar 'Vivid Scarlet.' Clavamox was found to be the most appropriate antibiotic for shoot regeneration and subsequent plant regeneration. No variation in ploidy level was observed between mother plants and regenerated plants. To the best of our knowledge, this is the first report of the effects of Clavamox on shoot regeneration in chrysanthemum.

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References

Asbel LE and Levison ME (2000) Cephalosporins, carbapenems, and monobactams. Infect Dis Clin North Am. 14:435–447.

Bardini M, Labra M, Winfield M, Sala F (2003) Antibiotic-induced DNA methylationchanges in calluses of *Arabidopsis thaliana*. Plant Cell Tiss Organ Cult. 72:157–162.

Bhau BS and Wakhlu AK (2001) Effect of some antibiotic on the in vitro morphogenetic response from callus cultures of *Coryphantha elephantidens*. Biol Plantarum. 44:19–24.

Boase MR, Bradley JM, Borst NK (1998) Genetic transformation mediated by *Agrobacterium tumefaciens* of florists' chrysanthemum (*Dendranthema grandiflorum*) cultivar 'Peach Margaret". *In Vitro* Cell Dev Biol—Plant. 34:46-51.

Carvalho JFP, Carvalho CR, Otoni WC (2005) *In vitro* induction of polyploidy in annatto (*Bixa orellana* L.). Plant Cell Tiss Organ Cult. 80:69–75.

Chung KM and Park YD (2005) Development of an *Agrobacterium-mediated* transformation system for regenerating garland chrysanthemum (*Chrysanthemum coronarium* L.). J. Plant. Biol. 48:136-141.

Costa MGC, Nogueira FTS, Figueira ML, Otoni WC, Brommonschenkel SH, Cecon PR (2000) Influence of the antibiotic timentin on plant regeneration of tomato (*Lycopersicon esculentum* Mill.) cultivars. Plant Cell Rep. 19:327–332.

da Silva Mendes AF, Cidade LC, de Oliveira MLP, Otoni WC, Soares-Filho WDS, Costa MGC (2009) Evaluation of novel beta-lactam antibiotics in comparison to cefotaxime on plant regeneration of *Citrus sinensis* L. Osb. Plant Cell Tiss Organ Cult. 97:331–336

Estopa M, Marfa V, Mele E, Messeguer J (2001) Study of different antibiotic combinations for use in the elimination of *Agrobacterium* with kanamycin selection in carnation. Plant Cell Tiss Organ Cult. 65:211–220.

Fukai S, De Jong J, Rademaker W (1993) Efficient genetic transformation of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) using stem segments. Breed Sci. 45:179-184.

Kim CK, Chung JD, Park SH, Burrell AM, Kamo KK, Byrne DH (2004) *Agrobacterium tumefaciens*-mediated transformation of *Rosa hybrid* using the green fluorescent protein (GFP) gene. Plant Cell Tiss Organ Cult. 78: 107–111.

Kumara S, Raja SK, Sharmab AK, Varmac HN (2012) Genetic transformation and development of Cucumber mosaic virus resistant transgenic plants of *Chrysanthemum morifolium* cv. Kundan. Scientia Hortic. 134:40–45.

Liu Z and Gao S (2007) Micropropagation and induction of autotetraploid plants of *Chrysanthemum cinerariifolium* (Trev.) Vis. *In Vitro* Cell Dev Biol-Plant. 43:404–408.

Naing AH, Jeon SM, Han JS, Lim SH, Lim KB, Kim CK (2014) Factors influencing in vitro shoot regeneration from leaf segments of Chrysanthemum, C R Biologies. 337: (6): 383-390.

Nauerby B, Billing K, Wyndaele R (1997) Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. Plant Sci. 123:169–177.

Ogawa Y and Mii M (2005) Evaluation of 12 b-lactam antibiotics for *Agrobacterium*-mediated transformation through in planta antibacterial activities and phytotoxicities. Plant Cell Rep. 23:736–743.

Pollock K, Barfield DG, Shields R (1983) The toxicity of antibiotics to plant cell culture. Plant Cell Rep. 2:36–39.

Pinto DLP, Barros B de A, Viccini LF, Campos JMS de, Silva ML da, Otoni WC (2010) Ploidy stability of somatic embryogenesis-derived *Passiflora cincinnata* Mast. plants as assessed by flow cytometry. Plant Cell Tiss Organ Cult. 103:71–79.

Rout GR and Das P (1997) Recent trends in the biotechnology of Chrysanthemum: a critical review. Scientia Hortic. 69: 239–257.

Ren Y, Bang H, Gould J, Patil BS, Crosby KM (2012) *Agrobacterium*-mediated transformation and shoot regeneration in elite breeding lines of western shipper cantaloupe and honeydew melons (*Cucumis melo* L.). Plant Cell Tiss Organ Cult. 108:147–158.

Schmitt F, Oakeley EJ, Jost JP (1997) Antibiotics induce genome-wide hypermethylation in cultured *Nicotiana tabacum* plants. J Biol Chem. 272:1534–1540.

Seo SY, Choi DC, Kim JM, Lim HC, Kim HJ, Choi JS, Choi YG (2003) Plant regeneration from leaf explant and efficient *Agrobacterium* mediated transformation system of chrysanthemum (*Dendranthema grandiflorum*). Acta Hortic. 620:333–338.

Song JY, Sivanesan I, Jeong BR (2012) Use of petal explants for successful transformation of *Dendranthema* × *grandiflorum* Kitamura 'Orlando' mediated by *Agrobacterium tumefaciens.* African Journal of Biotechnology. 11:9141–9148.

Sun SL, Zhong JQ, Li SH, Wang XJ (2013) Tissue culture-induced somaclonal variation of decreased pollen viability in torenia (*Torenia fournieri* Lind.). Botanical Studies. 54:36.

Tang W, Harris L, Newton RJ (2003) Influences of antibiotics on plantlet regeneration via organogenesis in loblolly pine (*Pinus taeda* L.). J For Res. 14:185–190.

Teixeira da Silva JA and Fukai S (2001) The impact of carbenicillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and *Agrobacterium* growth. J Appl Hort. 3:3–12.

Urban LA, Sherman JM, Moyer JW, Daub ME (1994) High frequency shoot regeneration and *Agrobacterium* mediated transformation of chrysanthemum (*Dendranthema grandiflora*). Plant Sci. 98: 69–79.

Wiebke B, Ferreira F, Pasquali G, Bodanese-Zanettini MH, Droste A (2006) Influence of antibiotics on embryogenic tissue and *Agrobacterium tumefaciens* suppression in soybean genetic transformation. Bragantia. 65:543–551.

Yu TA, Yeh SD, Yang JS (2001) Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. Bot Bull Acad Sin. 42:281–286.