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BRIEF COMMUNICATION

Effects of antibiotics and bialaphos on the growth and development of embryogenic callus cultures of *Muscari armeniacum*

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Abstract

Effects of 4 potentially selective agents for transformed cells, 3 antibiotics [kanamycin, geneticin (G418) and hygromycin] and bialaphos, as well as 2 antibiotics for eliminating *Agrobacterium*, carbenicillin and cefotaxime on growth and somatic embryogenesis of embryogenic calli of *Muscari armeniacum* cv. Blue Pearl were evaluated. Callus growth was completely inhibited by 75 mg dm^{-3} hygromycin or 4 mg dm^{-3} bialaphos, and somatic embryos were never produced on media containing 25 mg dm^{-3} hygromycin or 3 mg dm^{-3} bialaphos. Kanamycin and G418 less inhibited growth and somatic embryogenesis of the calli. On the contrary, carbenicillin and cefotaxime promoted both callus growth and somatic embryogenesis at all concentrations tested.

Additional key words: genetic transformation, grape hyacinth, selective agent, somatic embryogenesis

Muscari armeniacum Leichtl. ex Bak., which belongs to the family *Liliaceae*, is commonly called muscari or grape hyacinth and cultivated for pot and garden uses throughout the temperate regions of the world. Recently, genetic transformation has commenced to be applied to breeding in a number of plant species. This strategy may enable efficient genetic improvement of *M. armeniacum* in relation to floral as well as marketable qualities. In order to develop an efficient transformation system, it is important to select transformed cells or tissues (Dekeyser *et al.* 1989, Wilmink and Dons 1993, Suzuki *et al.* 2002). For *Agrobacterium*-mediated transformation, it is also necessary to eliminate *Agrobacterium* from co-cultivated cultures using appropriate antibiotics (Suzuki *et al.* 1998, 2002). Therefore, we examined here the effects of 4 selective agents, 3 antibiotics (kanamycin, G418 and hygromycin) and bialaphos, as well as 2 antibiotics for eliminating *Agrobacterium*, carbenicillin and cefotaxime, on the growth and somatic embryogenesis of leaf-derived embryogenic calli.

Muscari armeniacum Leichtl. ex Bak. cv. Blue Pearl, which has deep blue flowers, was used. Embryogenic callus cultures of this cultivar were induced from young leaf segments as previously described (Suzuki and Nakano 2001). The cultures consisted of friable cell clusters white to light yellow in colour. They were monthly subcultured on MS medium containing 10 mg dm^{-3} NAA, 30 g dm^{-3} sucrose and 2 g dm^{-3} gellan gum (C medium). Following transfer to half-strength MS medium lacking PGRs but supplemented with 15 g dm^{-3} sucrose and 2 g dm^{-3} gellan gum (E medium), embryogenic calli developed numerous somatic embryos. In the present study, all culture media were adjusted to pH 5.7 before autoclaving at 121 °C for 20 min, and all cultures were maintained at 25 °C under continuous irradiation with white fluorescent tubes (30 μmol $m^{-2}s^{-1}$).

To examine the effect of various agents, 0.5 g fresh mass (FM) of embryogenic calli 2 weeks after subculture were inoculated on C or E media, each of which was further supplemented with various concentrations (Fig. 1) of a selective agent (kanamycin, G418, hygromycin or bialaphos) or an antibiotic for eliminating *Agrobacterium* (carbenicillin or cefotaxime). Kanamycin, G418, hygromycin and carbenicillin were purchased from *Wako Pure Chemical Industries*, Osaka, Japan, and cefotaxime (*Claforan*) was purchased from *Aventis Pharma Ltd.*,

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Abbreviations: FM - fresh mass; G418 - geneticin; MS medium - Murashige and Skoog medium; NAA - α-naphthalene acetic acid; PGR - plant growth regulator.

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Frankfurt, Germany. Purified bialaphos was kindly provided by Dr. H. Anzai, Pharmaceutical Research Center, *Meiji Seika Ltd.*, Yokohama, Japan. Data on the callus growth on C media (% increase in callus FM) and on the production of somatic embryos on E media (number of somatic embryos) were recorded 4 and 8 weeks after culture, respectively. Each experiment was repeated 3 times, and the data obtained were subjected to analysis of variance using statistical analysis system (*SAS/PC package, version 6.11;* SAS Institute, Japan).

Among 4 selective agents, kanamycin and G418 had no significant effect on callus growth at all concentrations tested (Fig. 1). In contrast, hygromycin and bialaphos inhibited callus growth and induced callus browning to various extents. The calli started to brown after 2 weeks of culture initiation on C media containing 50 mg dm^{-3} or more of hygromycin, or 3 mg dm^{-3} or more of bialaphos. On C media containing 75 mg dm^{-3} hygromycin or 4 mg dm^{-3} bialaphos, callus growth was completely inhibited, and all of the calli showed necrosis within 4 weeks.

Development of somatic embryos was inhibited to various extents by G418, hygromycin and bialaphos (Fig. 1). On E media containing 25 mg dm^{-3} or more of hygromycin, or 3 mg dm^{-3} or more of bialaphos, the calli started to brown after 2 weeks of culture initiation and all of them showed necrosis within another 6 weeks. Somatic embryos were never produced on these media. Although callus browning as well as significant decrease in the number of somatic embryos were observed, embryo production was not completely inhibited on E media containing 75 mg dm^{-3} G418, or 1 or 2 mg dm^{-3} bialaphos. These results indicate that both hygromycin and bialaphos may be suitable agents for selecting both transformed calli and somatic embryos of *M. armeniacum* cv. Blue Pearl.

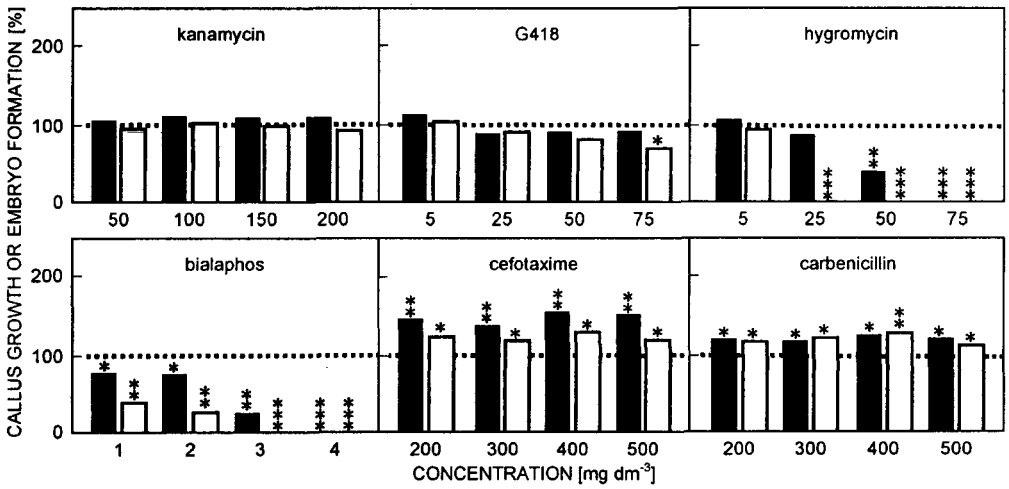

Fig. 1. Growth and somatic embryo formation of embryogenic calli of *Muscari armeniacum* cv. Blue Pearl as affected by various agents. The % callus growth and somatic embryo formation represent the % increase in callus fresh mass (*full columns*) and the number of somatic embryos (*empty columns*), respectively, as a percentage of the value of the control treatment. Data on callus growth and embryo formation were recorded after 4 and 8 weeks of culture, respectively. Significant differences from the control at $P < 0.05$, 0.01 and 0.001 with *t*-test are indicated by *, ** and ***, respectively.

The antibiotic kanamycin, which is most widely used as a selective agent for transformation of dicotyledonous species, cannot easily be applied to monocotyledonous species, since monocots generally has high natural tolerance to kanamycin (Potrykus *et al.* 1985, Wilmink and Dons 1993). Therefore, G418, hygromycin and bialaphos have been alternatively used as a selective agent in *Oryza sativa* (Dekeyser *et al.* 1989), *Zea mays* (Dennehey *et al.* 1994) and *Allium cepa* (Eady and Lister 1998). In the present study, embryogenic calli of *M. armeniacum* was insensitive to relatively high contents of not only kanamycin but also G418. Similarly, *Lilium formosanum* (Suzuki *et al.* 1998) and *Agapanthus praecox* ssp. *orientalis* (Suzuki *et al.* 2002), were also insensitive to relatively high concentrations of G418.

With respect to *Agrobacterium*-eliminating antibiotics, both cefotaxime and carbenicillin did not inhibit the growth nor development of embryogenic calli. They also did not induce callus browning. Callus growth and somatic embryo formation were rather promoted by these antibiotics at all concentrations tested. No differences in the morphology and subsequent growth and development were observed between somatic embryos produced on E media containing antibiotics and those produced on E medium without these antibiotics. These results indicate that these antibiotics might be appropriate for eliminating *Agrobacterium* from embryogenic callus cultures of *M. armeniacum* cv. Blue Pearl.

Generally, betalactam antibiotics are considered to have no effects on plant cells due to their specific action

on bacterial cell walls (Pollock *et al.* 1983). However, promotive effects of these antibiotics on the growth and/or development of cultured tissues have previously been reported for several plant species including *Antirrhinum majus* (Holford and Newbury 1992), *Triticum aestivum* (Mathias and Boyd 1986), *Dianthus* spp. (Nakano and Mii 1993), *Pennisetum americanum* (Pius *et al.* 1993), *Lilium formosanum* (Suzuki *et al.* 1998) and *Coryphantha elephantidens* (Bhau and Wakhlu 2001). A similar result was also obtained in the present study for embryogenic callus cultures of *M. armeniacum* cv. Blue Pearl, although the mechanism of the promotive effect of cefotaxime and carbenicillin is not identified at present.

In conclusion, we determined here appropriate selective as well as *Agrobacterium*-eliminating agents as the first step toward the development of an efficient transformation system of *M. armeniacum* cv. Blue Pearl.

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