Effect of Plant Growth Regulators on Plant Regeneration and *in vitro* Flowering Through Somatic Embryogenesis of *Gentiana scabra*

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ABSTRACT

An efficient plant regeneration system of *Gentiana scabra* through somatic embryogenesis was established. Leaves and roots of seedlings of *Gentiana scabra* excised after germination were cultured on MS basal medium with 2,4-D, NAA or BA. Embryogenic callus was obtained on MS medium with 0.5 mg/L 2,4-D alone or 0.1 mg/L 2,4-D combimation with 1.0 mg/L BA after 45 days of culture. These embryogenic calli gave rise to somatic embryos, which subsequently developed into plantlets on MS medium without PGRs. Also, shoots were effectively differentiated from embryogenic callus when root segments were cultured on MS medium supplement with 0.1 mg/L 2,4-D and 1.0 mg/L BA. Shoots were effectively rooted on MS medium without PGRs. *In vitro* flowers were formed from plantlets cultured on MS medium with 5% sucrose after 60 days of culture.

Key words : *In vitro* flowering, plant growth regulators, somatic embryogenesis,

INTRODUCTION

Gentiana scabra, a perennial herbacious plant, belongs to Gentianaceae, which contains gentiopicroside in it's root. The dried rhizomes and roots of these plant species were used as a substitute for the true gentian. As having a specific aroma and taste, it has been used as a material of chinese drink, and as a cut flower because the color and shape of Gentiana flower are beautiful. Till now, there are a few reports on tissue culture and plant regeneration through somatic embryogenesis in *Gentiana scabra* (Bang *et al*, 1994). Somatic embryogenesis is similar zygotic embryogenesis and has wide application and is highly amenable to experimentation. So, somatic embryogenesis has earlier been reported from various plants (Ammirato, 1983; Gui *et al*., 1990). Flowering is considered to be a complex process regulated by a combination of environmental and genetic factors. Especially, important factors are carbohydrates, growth regulators, light and pH of the culture medium (Heylen and Vendrig, 1988). Also, the C/N ratio plays a critical

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role in the transition to the flowering state (Tanimoto and Harada, 1981). *In vitro* culture, a high C/N ratio promotes plant reproductive development while a high N/C ratio promotes plant vegetative development (Konar and Nataraja, 1964). This study was carried out to examine the effects of plant growth regulators, sucrose on plant regeneration and *in vitro* flowering through somatic embryogenesis.

MATERIALS AND METHODS

Seeds of Gentiana were surface sterilized in 2% (V/V) NaOCl using commercial bleach for 30 min followed by three washes with sterile distilled water. They were then germinated in the dark for 15 days on the half-strength MS medium (Murashige and Skoog, 1962) with 3% sucrose. The pH was adjusted to 5.8 before adding agar and then autoclaved at 121℃ for 15 min. Three investigations were conducted. Firstly, the effect of plant growth regulators (PGRs) on shoot formation from leaf and root of *Geuiana scabra* was tested using MS medium containing 2,4-D/BA 1.0 to 0.1mg/L and NAA/BA(1.0 to 0.1mg/L) combination or 2,4-D alone0.2 or 0.5mg/L. The cultures were incubated at 25±1℃ and in a 16-h photoperiod. Secondly, the response of explants to embryogenic callus induction was examined using leaf and root segments of *Gentiana* seedlings germinated *in vitro*. Leaf and root segments from 15 day-old seedlings (root, approximately 7-mm-long segments) were placed on the surface of solid MS medium supplemented with various PGRs (NAA, 2, 4-D, BA). After 60 days of culture, calli induced on the above medium were counted and number of shoot produced was recorded. In addition, somatic embryos formed from embryogenic callus were transferred to MS media without PGRs for plantlet regeneration. Thirdly, the effect of sucrose (3, 5, 7, 9%) on *in vitro* flowering from plantlets cultured on MS medium was examined.

RESULTS AND DISCUSSION

Effect of plant growth regulators on shoot formation

The explants (leaves and roots) from 15-day-old *Gentiana* seedling were cultured on MS medium with various PGRs to investigate the effect of the PGR on shoot formation. After 20 days of culture white friable callus was visible along the cut edge of the explant in the all media supplemented with 2,4-D, NAA and BA (0.1-1.0 mg/L) except hormone free medium (Data of hormone free medium were not shown). The calli grew slowly and some of them turned brown as culture time proceed. In most media containing 2,4-D alone or 2, 4-D combination with BA, callus induction was high, which showed vigorous growth. But in media supplemented with NAA and BA, callus induction was low, which showed poor growth. On the other hand, embryogenic callus was induced on MS medium with 0.5 mg/L 2,4-D or 0.1 mg/L 2,4-D combination with 1.0 mg/L BA. Compact and yellowish callus more induced on MS medium with 0.1 mg/L 2,4-D combination with 1.0 mg/L BA than NAA/BA combination. Shoot primodia occurred on surface of yellowish and compact embrtogenic callus after 5 weeks of culture and grew vigorously and showed normal shoot growth. Shoot formation rate was examined after 8 weeks of culture and the results obtained are presented in Fig 3. Although some shoot-like structure were seen on the media containing 0.5 mg/L 2,4-D, no shoots were formed in leaf culture. On medium containing 2,4-D and BA, shoots formation was maximum (13.6%). When normal shoots were transferred on MS medium without plant growth regulators, roots were differentiated easily. The effect of various PGRs on somatic embryogenesis has earlier been reported. For instance, 2, 4-D can improve the rate of embryogenic cell mass formation but inhibits their further development in mature embryo in *Acanthopanax* (Gui *et al*., 1990). Compact, friable and embryogenic calli

were initiated from young leaves of *Paspalum scrobiculatum* cultured on MS medium with 2, 4-D (Nayak and Sen, 1989). Also, in case of *Brassica nigra*, supplementation with GA_3 enhanced embryogenic response ten-fold (Gupta *et al*, 1990).

Bang *et al* (1994) reported that leaf segments formed calli when cultured on MS medium supplement with 0.5 mg/L 2,4-D and 2 mg/L BA and callus became embryogenic after transferred to SH medium supplimented with 0.5 mg/L 2,4-D, 2 mg/L P-CPA and 0.5 mg/L kinetin. In *Gentiana axillariflora* (Cho *et al*, 1992), optimal level of growth regulators for callus initiation from leaf explants was 10 μM NAA and 5 μM BA. In the present study, the 2,4-D affected the induction of embryogenic callus. Most embryogenic calli were induced on medium containing 2,4-D alone or 2,4-D combination with BA. We therefore suggested that optimal medium shoot regeneration through somatic embryogenesis in Gentiana was MS medium supplemented with 0.5 mg/L 2,4-D or 0.1 mg/L 2,4-D combination with 1.0 mg/L BA. It has long been observed that appropriate medium can promote embryogenesis (Arnold *et al*, 2002). In general, somatic embryo induction usually occurs on medium with a high auxin concentration, and the medium without auxin or with low auxin concentration allows embryo development (Ammirato, 1983). In *Acanthopanax*, secondary somatic embryos developed when somatic embryos were transferred to medium supplemented with IAA (1-3 mg/L) or zeatin (0.5 mg/L) (Gui *et al*, 1990). In the present study, somatic embryos were formed in the medium with 2,4-D or 2,4-D/BA. These results suggest that the requirement of PGRs was different from plant species or position of explants in tissue culture.

Response of leaf and root segments on organogenesis

In order to compare the response of explants to the organogenesis, leaves and roots of seedlings were cultured on the same media containing various PGRs. When leaves were cultured on MS medium with various PGRs, the explants showed swelling at the surface within 20 days of culture and callus first appeared as tiny protrusion. Such calli were concentrated at the cut ends of explant. After 45 days of culture, callus induction was good on MS medium with 2,4-D/BA in leaf culture. But, on medium containing NAA/BA, callus induction was low and shoot formation was maximum 3.4 %. Callus induction frequency of 2,4-D in combination with BA was maximum 89.8 % but NAA combination with BA was less than 30%. Between 45 - 60 days of culture, some shoots were formed on the surface of compact callus through organogenesis. Also, root explants of *Gentiana* were cultured on same media as that leaf segement was cultured. The frequency of shoot formation was lower than in the case of leaf culture except medium with 0.5 mg/L 2,4-D. But when roots were cultured on media with 0.5 mg/L 2,4-D alone, shoot formation rate was more higher than media with NAA and BA and embryogenic callus was induced on medium with 0.1 mg/L 2,4-D and 1.0 mg/L BA. In root culture, results were similar to those of leaf culture. Especially, embryogenic calli were effectively induced on medium with 0.5 mg/L 2,4-D in root culture. Embryos induced from embryogenic calli were converted to plantlet on MS medium without plant growth regulators (Fig. 2). These results suggest that 2,4-D alone (0.2 or 0.5 mg/L) is more effective than NAA combination with BA combinations for inducing embryogenic callus. On the other hand, no embryogenic callus was formed when explants were cultured on MS medium supplemented with NAA and BA. But when leaf segments were cultured on medium with 0.5 mg/L 2,4-D, a little of embryogenic callus was induced. Therefore root explants were better than leaf explants for embryogenic callus induction. There are many reports on plant regeneration through somatic embryogenesis using

various organs of plant. Immature inflorescence explants of *Setaria italica* showed a higher tendency for regenerating into plantlets through somatic embryogenesis (Xu *et al.*, 1984). Plant regeneration from immature embryos of peanut (Peggy, 1989), oak (Chalupa, 1990) and strawberry (Wang *et al.*, 1984) has been accomplished through somatic embryogenesis. Also, the high frequency of direct somatic embryogenesis was observed using cotyledon explant of *Brpleurum fakatum* (Lee *et al.*, 1988). Somatic embryogenesis of cotton (John, 1988) and cassava (James and Graham, 1987) was reported using cotyledon explants. Somatic embryogenesis from leaf explants (Wit *et al.*, 1990) and adventitious roots (Theo *et al.*, 1996) of rose has been reported. Also, plant regeneration from cultured leaf tissue of M. *truncatula by* somatic embryogenesis has been reported. The frequency of somatic embryogenesis was increased when regenerated plants *in vitro* were used as an explant source (Nolan *et al.*, 1989).

In this study, observations were made 60 days of culture by counting the number of shoot/explants under microscope. All the culture media, embryogenic callus induction was comparing higher from root than leaf explants.

These results together with the somatic embryogenesis suggest that root explants are more useful for somatic embryogenesis than leaf explants in *Gentiana scabra*.

Effect of sucrose concentration on *in vitro* flowering

Various concentration of sucrose was used to compare their effectiveness for *in vitro* flowering from plantlets transferred to MS medium without plant growth regulatros. Within 60 days of culture in the MS media containing up to 3% sucrose, flowers were induced from plantlets. When plantlets were transferred to MS basal medium with 3% suscrose, only one flowering plant was induced. However, 6 normal plants were produced on MS medium containing 5% sucrose, which was maximal number of plants in the sucrose treatment, and each plant had one flwer in that treatment (Fig.4B). The ability of explants to form flowers *in vitro* depends on numerous factors. The C/N ratio can be manipulated *in vitro* by increasing the sucrose content or decreasing the level of NH_4NO_3 (Wang *et al.*, 2001). Flowering can regularly occur from several explants of some woody flowering plant species (Scorza, 1982). Shoots of orange jessamine (*Murraya paniculata*) flowered *in vitro* on half-strength MT basal medium containing 5% sucrose (Jumin and Ahmad, 1999) and flowers were formed from shoot tips of bitter melon cultured on MS medium with 90 mM sucrose (Wang *et al.*, 2001). In this study, maximum 6 plants were produced on MS medium supplemented with 5% sucrose and high concentration of sucrose(up to 7%) decreased the number of flower. This result suggest that 5% sucrose promoted *in vitro* flowering in *Gentiana scabra*.

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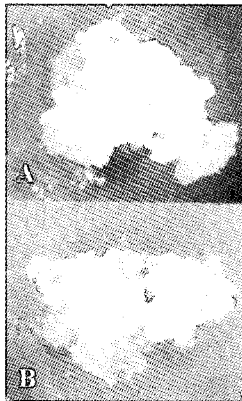

Fig. 1. Calli (A) and shoot (B) formation from leaf culture of *Gentiana scabra*. on MS medium with 0.1 mg/L 2,4-D and 1.0 mg/L BA after 45 days of culture.

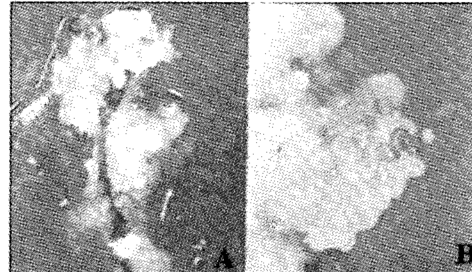

Fig. 2. Somatic embryogenesis from root culture in *Gentiana scabra*. A: Embryogenic callus formed on MS medium with 0.5 mg/L 2,4-D; B: Embryo formation of various shape on MS medium without PGRs.

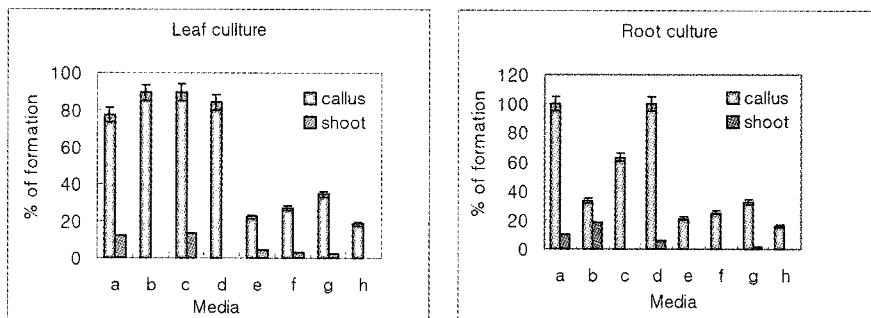

Fig. 3. Effects of plant growth regulators to callus and shoot formation from leaf and root of *Gentiana scabra* after 60 days of culture.

a: 2,4-D 0.2; b: 2,4-D 0.5; c: 2,4-D 0.1 + BA 1.0; d: 2,4-D 0.5 + BA 1.0; e: 2,4-D 1.0 + BA 1.0; f: NAA 0.1 + BA 1.0; g: NAA 0.5 + BA 1.0; h: NAA 1.0 + BA 1.0 mg/L.

Fig. 4. *In vitro* flower on MS medium supplemented with sucrose after 60 days of culture. A: plantlet formed from somatic embryo on MS medium; B: *In vitro* flower on MS medium supplemented with 6% sucrose.

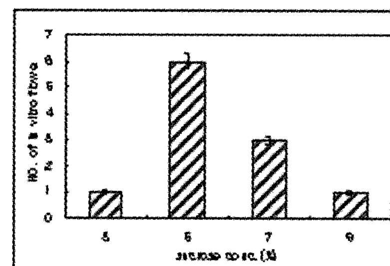

Fig. 5. Effect of various sucrose concentration on *in vitro* flowering from plantlets transferred to MS medium without plant growth regulators.

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