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Short Communication

Regenerating plants from *in vitro* culture of *Erigeron breviscapus* leaves

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A protocol to efficiently achieve plant regeneration from leaf explants of *Erigeron breviscapus* (Compositae) has been developed. Leaf explants produced calli on Murashige and Skoog's (1962) basal medium (MS) supplemented with 6-benzylaminopurine (BAP) (0.4 μM) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.4-22.6 μM) under a 16-h photoperiod. Direct adventitious shoots were induced from leaf explants on MS medium containing BAP (22.2 μM) and IAA (5.7 μM) under the light treatment. The effect of the dark incubation on shoot regeneration from leaves indicated that 15 days of darkness permitted a higher regeneration frequency (82.6%) on the medium supplemented with BAP (4.4 μM) and indole-3-acetic acid (IAA) (0.6 μM). Regenerated shoots were rooted on MS medium with or without naphthaleneacetic acid (NAA). Plantlets were cultivatable in the greenhouse after acclimatization.

Key words: *Erigeron breviscapus,* callus induction, leaf explants, dark culture, plant regeneration.

INTRODUCTION

The whole herb of *Erigeron breviscapus* (Vant.) Hand. -Mazz. (Compositae), a well known medicinal herb in China, has been used to treat a variety of paralyses and the sequelae of apoplexy and cerebral atherothrombosis (Jiangsu College of New Medicine ,1986). Presently, phytochemicals from *E. breviscapus* are of growing interest in pharmacology due to their potential utility as stimulant to fibrinolysis and anti-coagulation of endothelial cells (Yue et al., 1994; Chen and He, 1998; Zhang et al., 1999; Zhu et al., 1999). Moreover, the total flavonoids of the plant have been developed as drugs in China (including troche and injection) (Zuo et al., 1991; Zhang et al., 1998). As a result, an overexploitation has been an inevitable reality concerning this species that is endemic exclusively to the very limited temperate mountainous region of southwestern China. Accordingly, there is an urgent need to develop micropropagation for *E. breviscapus* to meet, alternatively, the growing demand for the plant. However, little is reported about the *in vitro* callus culture and regeneration of the plant up to date.

Here we wish to present the establishment of the efficient *E. breviscapus* regeneration system and the evaluation of the *in vitro* regeneration method using the concept of etiolated leaf explants.

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Abbreviations: MS, Murashige and Skoog's (1962) basal medium; **BAP,** 6-benzylaminopurine**; IAA,** indole-3-acetic acid; **NAA,** naphthaleneacetic acid; **2,4-D,** 2,4-dichlorophenoxyacetic acid.

MATERIALS AND METHODS

Actively growing leaves from basal rosettes of *E. breviscapus* were collected in April 2008 in the north suburb of Kunming City, Yunnan Province, China. A voucher specimen was kept in the Herbarium of Soochow University under the No. PT2008421. Leaves, cleaned with running water for about 30 s, were disinfested successively using detergent (1 min), 70% ethanol (1 min) and 0.525% sodium hypochloride (10 min) containing Tween 20 (3 drops per 100 ml) followed by being rinsed twice in sterile water for 5 min. Leaf segments (*ca.* 5 mm long) were excised avoiding the midrib vein and cultured in 100 × 15 mm sterile petri dishes containing 25 ml of medium supplemented with growth regulators. Four explants with abaxial surface down were cultured per plate and six plates were used for each treatment. The modified MS (Murashighe and Skoog, 1962) medium was the basal medium. All media were fortified with

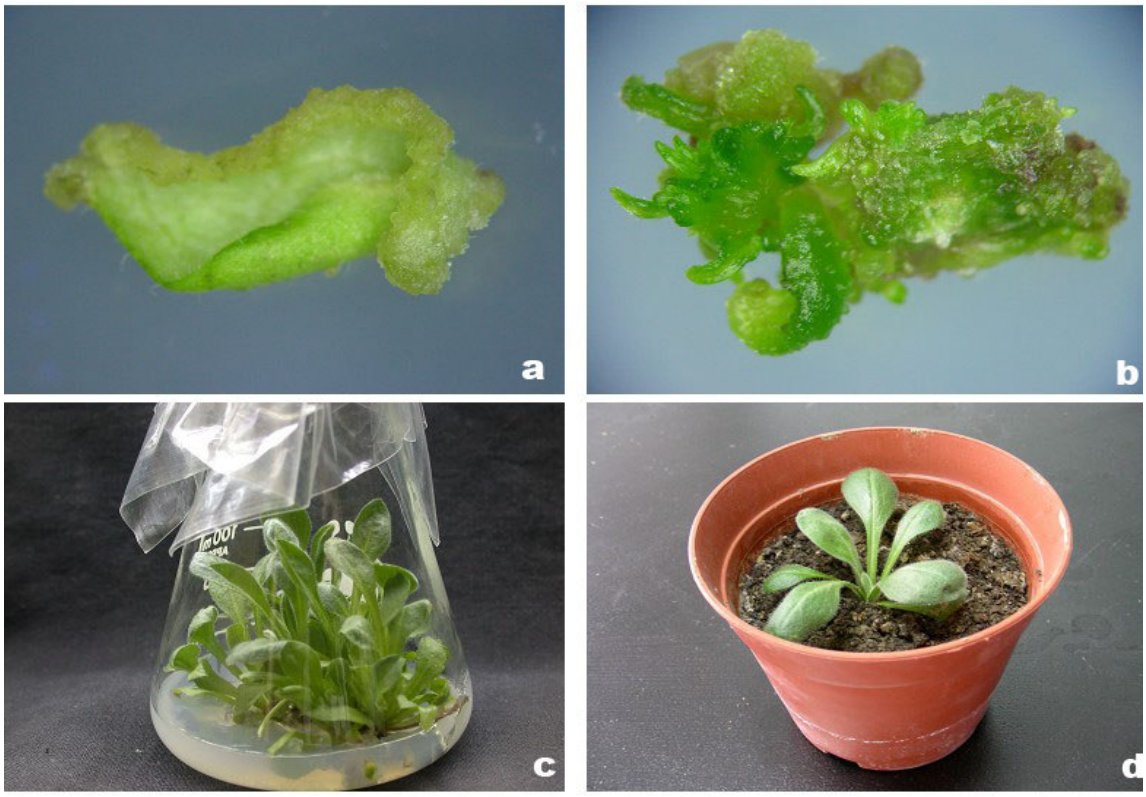

Figure 1. Organogenesis and plantlet regeneration from leaf explant of *E. breviscapus*. (a) Callus generated from the leaf explants for 2 wk; (b) Adventitious buds formed through callus from cultured leaf explants of *E. breviscapus* after 5 wk; (c) Elongated shoots regenerated through leaf-derived callus; and (d) Regenerated plantlets from leaf growing in pots.

with growth regulator at the following concentration combination: BAP (0.4, 4.4 and 22.2 μM) and IAA (0.6, 5.7 and 28.5 μM) or 2,4-D (0.4, 4.5 and 22.6 μM). The cultures were incubated under a 16 h photoperiod (2500 lx) with cool white fluorescent lamps or in darkness at 26 ± 2℃. In the second experiment, the effect of dark incubation was tested. Leaf sections were cultured on MS medium supplemented with BAP (4.4 μM) and IAA (0.6 μM). Five dark pretreatments were performed, during which culture plates were incubated in darkness for 0, 5, 15 or 30 days before being transferred to a 16 h photoperiod. For the control, leaves were maintained in darkness throughout the experiment. In both experiments, explants were transferred to MS without growth regulators after 30 days of culture, and morphogenic responses were recorded after 60 days. To induce rooting, leaf-derived shoots (>1 cm long) were excised and cultured under a 16 h photoperiod in tubes containing 20 ml hormone free MS basal medium. The effect of NAA (2.7 μM) on rooting was also tested on the shoots. After 40 days, rooting shoots were then transferred into sterile peat pots maintained in a controlled-environment room to ambient relative humidity for 1 month acclimatization and then moved to the greenhouse.

RESULTS AND DISCUSSION

When cultured under light, the leaf explants of *E. breviscapus* suffered severe necrosis and callus generally formed on the cut surfaces, especially near the main veins after 14 to 20 day cultures. Callus formation was favored by the high concentration of 2,4-D. The best regulator combinations for callus growth were found to be BAP (0.4 μM) plus 2,4-D (4.5 μM). The callus formed was nodular and yellowish with some zones exhibiting the formation of anthocyanins (Figure 1a). Calli have been used to initiate cell suspension cultures. The morphogenic responses required the presence of growth regulators in the culture medium. BAP and IAA were effective for shoot initiation at relative high concentrations (22.2 and 5.7 μM, respectively) under light. However, the rate of shoot induction and the mean number of shoots per explant were very low (11.4% and 0.8, respectively). In most cases, shoot formation occurred directly from cut areas of the explants. Adventitious shoots were macroscopically visible after 20 days of culture (Figure 1b). No indirect shoot organogenesis through callus was observed. Dark culture significantly enhanced caulogenesis (percentage of explant forming shoots and mean number of shoots per explant) on leaves. For adventitious shoot, initiation in dark culture on MS+BAP (4.4 μM) + IAA (0.6 μM) or 2,4-D (0.4 μM) was found to be more suitable than the other combinations tried. This promotive effect on caulogenic response was not altered significantly by higher IAA concentrations. In contrast, the highest 2,4-D concentration

(22.6 μM) drastically reduced the percentage of shoot forming (12.4% vs. 54.2%) when leaf explants were cultured with BAP (4.4 μM). The best shoot regeneration (66.4%) was obtained with the presence of BAP at 4.4 μM in combination with IAA (0.6 μM) for 60-day culture in darkness. This BAP- and IAA-supplemented medium also increased the mean number of shoots (5.4). The length of etiolated shoots was variable ranging from 0.5 to 6 cm (Figure 1c).

The effect of continuous dark culture on adventitious shoot formations indicated that 15 days of darkness resulted in a high regeneration frequency (82.6%). Dark periods longer than 15 days inhibited this caulogenic response. In contrast, the mean number of shoots per explant did not increase significantly in dark culture. Etiolation as a horticultural method has been reported to improve the rooting of cuttings (Read, 1987), but has scarcely been tried as a pretreatment for establishment of initial cultures *in vitro* as observed with the root formation of apple shoots *in vitro* (Druart et al., 1982), and the caulogenesis in *Malus* spp. leaf cultures (Korban and O'Connor, 1992).

In leaf-derived shoots, roots occasionally formed on the base and elongated stems of individual shoots after 20 day culture on medium without growth regulators, and the rooting rate reached to 71.8% after additional 20 days. Adding NAA (2.7 μM) to the medium slightly increased the percentage of rooting (82.6%). The mean number of roots per rooted shoots ranged from 1 to 6. Rooted plants were successfully acclimated to *in vivo* conditions, and eventually 72.6% of the plantlets survived (Figure 1d).

The present work achieved direct caulogenesis from leaf explants of *E. breviscapus* allowing its germplasm conservation and industrial-scale cultivation for medicinal purpose.

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