[Short Report]

Callus Induction and Adventitious Shoot Regeneration from Petiole of Erigeron breviscapus

Lei Zhang¹, Chenghong Liu¹, Ling Lin¹ and Wansheng Chen², ³

¹Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai 200433, China;
²Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China;
³Modern Research Center for Traditional Chinese Medicine, Second Military Medical University, Shanghai 200433, China)

Key words: Adventitious shoot, Erigeron breviscapus (Vant.) Hand-Mazz, Micropropagation, Morphogenetic callus, Petiole.

Erigeron breviscapus (Vant.) Hand-Mazz, in the Compositae, is a perennial wild plant distributed in southwestern China. It has been a major resource for making various pharmaceutical products ever since researchers found that its extracts could be used to treat diseases like the sequela of apoplexy, and coronary disease. With the recent development of clinical application and animal experimental studies, the therapy application of this traditional Chinese medicine has been extended to treat many diseases such as cardiovascular disease, senile dementia, dizziness, retinal vein emphyaxis, gastropathy and nephrotic syndrome etc (Liu et al., 2002). The demand for raw materials of E. breviscapus in the domestic market has increased. Nowadays, the wild resources of E. breviscapus are in danger of being exhausted due to overcollection and its poor natural reproduction ability. The seed is small (about 2 mm in diameter) and the total rate of well-germinated seeds in one mature infructescence is about 60%-65%, which lead to the inadequate propagation in natural conditions (Yu and Chen, 2002). Artificial cultivation of E. breviscapus has been attempted in Yunnan province of China. However, difficulty in seedling reproduction and germplasm degrading are major problems in large-scale cultivation (Lin et al., 2003a). The development of a rapid mass propagation system through tissue culture would not only be a powerful tool for commercial propagation but also be an important step for further breeding research (Gamborg, 2002). To date, however, there are few reports on the efficient plant regeneration in vitro of E. breviscapus (Yang et al., 2002; Lin et al., 2003b; Liu et al., 2005). Here, we report an efficient micropropagation method for E. breviscapus via morphogenetic calli derived from petiole, which were excised from the aseptic plant germinated from seed.

Materials and Methods

1. Effect of plant growth regulators on callus induction

Aseptic plants developed from seeds were cultured under a 12-h light/12-h dark photoperiod (60 µmol m⁻² s⁻¹ light provided by white fluorescent tubes). Leaf and petiole segments (50 mm × 50 mm and 0.8 cm respectively), excised from aseptic plants after a 1-month culture, were used as explants to induce callus. The two kinds of explants were cultured on MS basal medium containing NAA and BA at different concentrations (Table 1). All the culture media contained 3% sucrose (w/v) and 2.6 g L⁻¹ phytagel (Sigma) and adjusted to pH 5.8 prior to autoclaving for 15 min. at 121°C. All the culture materials were cultured at 25 ± 2°C under a 12-h light/12-h dark photoperiod (60 µmol m⁻² s⁻¹ light provided by white fluorescent tubes). Subculture was carried out at 2-week intervals and percentages of callus formation were scored after a 6-week culture.

2. Shoot regeneration

After 4 to 6 weeks of subculture, the segregated calli (4-8 mm in diameter) were transferred onto MS-based regeneration media containing different concentrations of cytokinin (BA) and auxin (NAA) (Table 2), and on shoot regeneration was examined 1 month later.

3. Rooting, acclimation and transplant of plantlets

Shoots (about 1 cm in length) were excised and transferred to MS medium. They were cultured at 25°C under a 16-h light/8-h dark photoperiod (60 µmol m⁻² s⁻¹ light intensity provided by white fluorescent tubes) for rooting. When plantlets developed at least three roots, the lids of culture vessels were removed for acclimation in a greenhouse. Two days later, the
plantlets were washed gently under running tap water and transplanted into plastic pots containing a mixture of vermiculite, perlite, and peat (4:1:3; v/v/v) in the greenhouse. The plantlets were watered with a diluted solution of MS macronutrients (1:50; v/v) once every week. After seed germination, the third euphylla would protrude soon after cotyledon and hypocotyls formed. Cotyledons or hypocotyls, at the stage of an ideal plantlet regeneration. (Figure 1A, B).

Results and Discussion

Aseptic plants could be generated from sterilized seed, and by this way, it was easy to obtain a large amount of aseptic materials for callus induction and plantlet regeneration in vitro. (Figure 1A, B).

After seed germination, the third euphylla would protrude soon after cotyledon and hypocotyls formed. Cotyledons or hypocotyls, at the stage of an ideal explant type, were too small to use for culture in vitro. Then, two kinds of explants, leaf and petiole segments excised from aseptic plants generated from seeds, were used to induce calli. However, only petiole segments were found to induce calli while leaf segments produced just a few micro-calli. Petiole was reported as an alternative explant for organogenesis with or without an intervening callus phase (Hosokawa et al., 2001; Arockiasamy et al., 2002; Kim et al., 2003), and in our study, no direct organogenesis was observed on the petiole of *E. breviscapus*.

In plant tissue culture, 2, 4-D was suggested as an effective hormone for callus induction (Vasil and Vasil, 1984). In our preliminary experiment, callus were induced on the MS basal medium containing high or low levels 2,4-D alone or in combination with low levels of BA under dark or light condition, but in the subculture, many calli turned to brown, necrosis, or had no differentiation ability (data not shown). In the present study, calli were induced successfully on the basal medium containing different concentration of BA together with NAA (Table 1). Moreover, some of which were compact, in green color and proliferated well in subculture (Figure 1C). A low ratio of cytokinin to auxin promoted callus induction (callus induction rate was up to 100% in the presence of 4.44 µM BA and 5.37 µM NAA), while a high ratio promoted morphogenetic callus proliferation (compact callus with green color grew rapidly in the presence of 8.88 µM BA and 5.37 µM NAA). A few explants turned brown or became necrotized when the concentration of BA was increased to 8.88 µM from 4.44 µM, which often appeared at the initial culture. The results of this work suggested that callus induction and callus subculture of *E. breviscapus* were favored by different media.

When the calli derived from the petiole were used, the highest shoot regeneration frequency (90%) was achieved by applying high levels of cytokinin (17.76 µM BA) together with low levels of auxin (1.07 µM NAA) (Table 2). Multiple adventitious shoots were found to regenerate from callus mass and grew well without vitrification (Figure 1D). The success of shoot induction from callus greatly depended on the success of morphogenic callus induction independent of the kind and concentration of growth regulators. In previous reports, BA was considered to be a useful

| BA (µM) | NAA (µM) | Callus induction frequency (%) | CF* |
|---------|----------|-------------------------------|-----|
| 4.44    | 2.69     | 92.7±1.06d                    | 13.4±1.8f |
| 4.44    | 5.37     | 100a                          | 28.2±2.7e |
| 4.44    | 10.74    | 97.7±0.6ab                    | 55.8±2.2c |
| 4.44    | 16.11    | 95.0±2.06bc                   | 38.7±4.5d |
| 8.88    | 2.69     | 90.3±1.5d                     | 72.1±6.9b |
| 8.88    | 5.37     | 90.0±2.7d                     | 81.2±5.5a |
| 8.88    | 10.74    | 95.3±1.7bc                    | 53.1±4.3c |
| 8.88    | 16.11    | 55.3±3.2e                     | 37.4±3.8d |

Percentages of callus formation were scored for each explant after 6 weeks of culture. Means±SD, n=30, three replicates. Data were analyzed by Duncan’s multiple range test and means followed by identical letters were not statistically different (P<0.05). CF*: frequency of explants that produced compact callus green in color on more than half of explant surface.

| BA (µM) | NAA (µM) | Shoot regeneration frequency (%) | No. of shoots per callus + |
|---------|----------|---------------------------------|----------------------------|
| 4.44    | 1.07     | 20.0±1.3e                       | 1.7±0.3d                  |
| 8.88    | 1.07     | 36.7±1.3d                       | 3.0±0.5ab                 |
| 13.32   | 1.07     | 80.0±2.06                       | 2.3±0.4bcd                |
| 17.76   | 1.07     | 90.0±1.5a                       | 2.5±0.6bc                 |
| 22.20   | 1.07     | 56.7±1.0c                       | 2.2±0.2cd                 |
| 26.64   | 1.07     | 33.3±1.8d                       | 3.4±0.3a                  |

Percentages of shoot regeneration were scored on each callus mass derived from petiole explants after 1 month of culture. Means±SD, n=30, three replicates. Data were analyzed by Duncan’s multiple range test and means followed by identical letters were not statistically different within the column (P<0.05). +: Calli without shoot regeneration were not included.
cytokinin in callus formation and shoot induction (Cuenca and Amo-Marco, 2000; Echeverrigaray et al., 2000; Nguyen Thi et al., 2003). In another medicinal plant *Piper longum*, green morphogenetic callus formed from leaf explants cultured on basal MS medium supplemented with different concentration BA (4.44 -17.76 µM) plus IAA (4.6-11.42 µM) or NAA (Soniya and Das, 2002). In this study, as for *E. breviscapus*, BA accompanied with NAA were found to be effective growth regulators combination for callus induction, callus subculture and shoot induction from callus.

When shoots regenerated from callus were excised and cultured on the MS medium without any hormones, roots were formed in 2 weeks (Figure 1E). Plantlets with multiple roots 3~5 cm in length after acclimation for 2 d were successfully transferred to the greenhouse conditions with 100% survival. All transplants grew well in the greenhouse without morphological variation in appearance (Figure 1F).

In conclusion, an efficient protocol has been developed for plant regeneration from the callus derived from petiole of *E. breviscapus*. This offers a potential system for mass propagation, conserving, and quality improvement of this traditional Chinese medicinal plant.

Acknowledgements

This research was supported by National Natural Science Foundation of China (30371746, 20572130, 30600807), Shanghai Natural Science Foundation (04ZR14051), Shanghai Science and Technology Committee, and Shanghai Rising-Star Program (06QB14010).

References

Arockiasamy, S. et al. 2002. Biol. Plant. 45 : 129-132.
Cuenca, S. and Amo-Marco, J.B. 2000. Plant Growth Regul. 30 : 99-103.
Echeverrigaray, S. et al. 2000. Plant Cell Tiss. Org. Cult. 60 : 1-4.
Gambaro, O.L. 2002. *In vitro* Cell. Dev. Biol. Plant. 38 : 84-92.
Hosokawa, K. et al. 2001. Sci. Hortic. 90 : 143-150.
Kim, S.W. et al. 2003. Plant Cell Tiss. Org. Cult. 74 : 163-167.
Lin, C. et al. 2003a. Chin. Wild Plant Resour. 22 : 8-11.
Lin, C. et al. 2003b. J Yunnan Agirc Univ.18 : 323-326.
Liu, H. et al. 2002. Chin. Trad. Herb Drugs. 33 : 566-568.
Liu, C.H. et al. 2005. Chin. Trad. Herb Drugs. 36 :597-599.
Nguyen Thi, P.T. et al. 2003. Plant Cell Tiss. Org. Cult. 70 : 325-327.
Soniya, E.V. and Das, M.R. 2002. Plant Cell Tiss. Org. Cult. 70 : 325-327.
Vasil, V. and Vasil, L.K. 1984. Orlando. Academic Press. 36-42.
Yang, Y.W. et al. 2002. J. Yunnan Coll. Tradit. Chin. Med. 25 : 122-131.
Yu, H.Y. and Chen, Z.L. 2002. Acta Bot. yunnanica. 24 : 115-120.