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BRIEF COMMUNICATION

In vitro plant regeneration from leaf explants of *Ophiorrhiza japonica*

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Abstract

An efficient *in vitro* plant regeneration system from leaves of *Ophiorrhiza japonica* Blume was established for the first time. Callus formation rate was more than 90.4 % from leaf segments on Murashige and Skoog (MS) supplemented with either α-naphthaleneacetic acid (NAA) alone or in combination with 6-benzyladenine (BA). The highest shoot regeneration (78.9 %) was achieved on MS medium containing 2.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA, with an average of 9.4 shoots developed per leaf segment. Shoot regeneration was also improved when the leaf explants were cultured in MS basal medium supplemented with 0.5 % (m/v) polyvinylpyrrolidone (PVP). The leaf explants from seedlings with age of about 18 - 27 d showed the highest shoot regeneration. The regenerated shoots were rooted on half-strength basal MS medium supplemented with 0.5 mg dm^{-3} indole-3-butyric acid (IBA), which averagely produced 24.8 roots per shoot. The plantlets were transferred to soil, where 100 % survived after 1 month of acclimatization.

Additional key words: camptothecin, growth regulators, medicinal plant, shoot regeneration.

Camptothecin (CPT) is a kind of modified monoterpenoid indole alkaloids, originally identified in the extracts of Chinese tree *Camptotheca acuminata* (*Nyssaceae*) (Wall *et al*. 1966). CPT derivatives such as irinothecan and topothecan have been widely used for the treatment of cancer over the world (Lorence *et al*. 2004). Several *Ophiorrhiza* species (of the family *Rubiaceae*) such as *O. mungos*, *O. filistipula*, *O. pumila*, *O. liukiuensis*, *O. prostrata* have been reported as the new sources of CPT production (Tafur *et al*. 1976, Arbain *et al*. 1993, Saito *et al.* 2001, Sudo *et al*. 2002, Kitajima *et al*. 2005, Beegum *et al*. 2006), respectively. As *O. japonica*, one indigenous Chinese medicinal plant commonly used for ulcers, poisonous wounds and leprosy in China, belongs to *Ophiorrhiza* genus, it is speculated that CPT analogues may also exist in its roots. Because of low germination rate of seeds, *O. japonica* is very scarce naturally, which limited its potential medicinal application. In this paper, we have established an efficient *in vitro* plant regeneration protocol from leaf explants of *O. japonica* for the first time.

Ophiorrhiza japonica (Blume) was collected from Fujian province in China. The leaves of *O. japonica* were surface-sterilized by immersion in 70 % (v/v) ethanol for 30 s and then in 0.1 % (m/v) mercuric chloride ($HgCl_2$) solution for 12 min, followed by rinsing three times with sterile double-distilled water. For callus induction and shoot regeneration, young leaves (about 30-d-old) were cut into 0.5 cm^2 segments and placed on MS (Murashige and Skoog 1962) medium containing different 6-benzyladenine (BA) concentrations (0, 0.2, 0.5, 1.0, 2.0 mg dm^{-3}) either alone or in combination with different α-naphthaleneacetic acid (NAA) concentrations (0, 0.2, 0.5, 1.0 mg dm^{-3}) (Table 1). The effect of basal medium type such as MS, B_5 (Gamborg 1970), N_6 (Chu *et al*. 1975), WPM (Lloyd and McCown 1980) and White (White

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Abbreviations: BA - 6-benzyladenine; CPT - camptothecin; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - naphthalene acetic acid; PVP - polyvinylpyrrolidone; WPM - woody plant medium.
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1943) on callus formation and shoot regeneration was determined. All the above media contained 2.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA. To test the effect of seedlings age on callus induction and shoot regeneration, leaf explants from seedlings with various age ranging from 12 - 15 d to 38 - 41 d were put on MS basal medium supplemented with 2.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA. Polyvinylpyrrolidone (PVP) at concentrations ranging from 0 to 3 % (m/v) was added in MS basal medium containing 2.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA, was used to study the effect of PVP concentration on callus initiation and shoot regeneration. The frequency of shoot induction and average number of shoots per culture were determined and recorded after 35 d of culture. For root induction, regenerated shoots (about 2 cm height) were excised and transferred to half-strength MS basal medium supplemented with different indole-3-butyric acid (IBA) concentrations (0, 0.2, 0.5, 1.0 mg dm^{-3}) or NAA (0, 0.2, 0.5, 1.0 mg dm^{-3}). The frequency of rooting and average number of roots per shoot were investigated and recorded after 28d of culture. All the media contained 7 g dm^{-3} agar and 30 g dm^{-3} sucrose. The pH of all the media was adjusted to 5.8 prior to autoclaving (at 121 °C, for 20 min). All cultures were maintained in a growth room at 25 ± 2 °C under a 12-h photoperiod with an irradiance of 50 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Rooted plantlets were removed from rooting medium followed by rinsing out media and then transferred to small pots containing soil and sand (1:1), which were covered with glass beakers and kept under high humidity to prevent wilting for acclimatization in 14 d before being exposed to greenhouse conditions by removing the cover. Each treatment consisted of 30 cultures and all the experiments were repeated three times. Means and standard deviations were used in statistical analysis. Treatment means differing significantly were further compared using Duncan's multiple range test (DMRT) (Duncan 1955).

Initially, the leaf explants gradually enlarged on MS medium supplemented with different concentrations of NAA (0.2, 0.5, 1.0 mg dm^{-3}) alone or in combination with BA (0, 0.2, 0.5, 1.0, 2.0 mg dm^{-3}) after 7 d of culture and more than 90.4 % of leaf segments began to produce callus at the cutting surface after 14 d of culture, while no callus can be obtained on MS basal medium without NAA, irrespective of the presence or absence of BA. In the media containing NAA, callus induction frequencies were 90.4 to 100 %, which showed that some concentration of NAA seemed to be very effective for promoting callus initiation in *O. japonica*. Subsequently, callus began to produce shoot buds on the same media after 21 d of culture. Plant growth regulators and their combinations significantly ($P < 0.05$) influenced shoot regeneration from leaf segments of *O. japonica* (Table 1). We noticed that the calli induced on the media supplemented with NAA without BA could not produce shoot, while the calli induced on the media containing BA and NAA simultaneously could produce shoot. When the concentrations of BA in the media were higher than those of NAA, most calli developed shoots. On the contrary, when the concentrations of BA were lower than those of NAA, most calli developed roots. The best hormone combination for promoting shoot regeneration was 2.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA, at which shoot regeneration frequency was 78.9 % and average 9.4 shoots developed per callus after 35 d of culture (Table 1). Our results revealed that a combination of BA and NAA was found to be suitable for callus formation and shoot regeneration in *O. japonica*. The cytokinin-auxin combination has been widely used for organogenic differentiation in various protocols developed for other species (Lisowska *et al.* 2000, Pereira *et al.* 2000, Pretto *et al.* 2000, Koroch *et al.* 2002, Zhang *et al*. 2004, Agrawal and Sardar 2006, Chen and Chang 2006, Haliloglu 2006).

Table 1. The effect of plant growth regulators [mg dm^{-3}] on callus induction and shoot regeneration from leaf explants of *O. japonica.* The number of shoot per callus was recorded after 35 d of culture. Means ± SE based on three replicates, 30 explants per replicate. Means with the same letter were not significantly different at $P \leq 0.05$ (Duncan 1955) (b - brown, c - compact, g - green, l - loose, y - yellow; shoots with length less than 5 mm were excluded).

BA	NAA	Callus formation [%]	Callus state	Shoot regeneration [%]	Shoot number [$callus^{-1}$]
0.2	0.2	91.1	y,l	5.6 ± 1.5g	1.5 ± 0.4f
0.2	0.5	95.6	g,l	3.3 ± 2.7h	1.2 ± 0.7f
0.2	1.0	97.8	b,l	4.4 ± 1.7h	1.3 ± 0.5f
0.5	0.2	93.3	g,c	47.8 ± 4.3c	3.3 ± 0.1d
0.5	0.5	96.7	y,l	7.8 ± 1.6g	2.2 ± 0.3e
0.5	1.0	98.9	b,c	4.4 ± 1.8h	1.3 ± 0.2f
1.0	0.2	94.4	g,c	71.1 ± 3.1b	8.2 ± 0.5b
1.0	0.5	95.6	g,c	38.9 ± 4.2d	2.4 ± 0.2e
1.0	1.0	100.0	b,l	11.1 ± 4.1g	2.5 ± 0.2e
2.0	0.2	93.3	g,c	78.9 ± 1.9a	9.4 ± 0.6a
2.0	0.5	94.8	y,l	26.7 ± 2.8e	3.9 ± 0.2d
2.0	1.0	95.3	b,l	20.0 ± 2.7f	4.7 ± 0.3c

Nutritional requirements for optimal *in vitro* growth may change with the species concerned. Similarly, tissues from different plant organs may have different demands for satisfactory growth (Murashige and Skoog 1962). Because of this reason, one single medium cannot be suggested for all types of plant tissues and organs. So when establishing a new system, it is essential to investigate a medium that can fulfill the specific requirements of that tissue. Among the five different basal media assayed (MS, B_5, N_6, WPM and White's media), MS was the most effective for *in vitro* shoot differentiation of leaf explants of *O. japonica,* which was similar in *Isatis indigotica* (Zhang *et al.* 2004). The highest shoot regeneration frequency of 78.9 % was achieved on MS medium with an average shoot number per explant of 9.4 ± 0.6 within 35 d of culture (Table 2). This result indicates that higher content of inorganic salt from MS is

Table 2. Influence of medium type, seedlings age and PVP concentration on shoot regeneration from leaf explants of *O. japonica.* The number of shoot was recorded after 35 d of culture. Means ± SE based on three replicates, 30 explants per replicate. Means with the same letter were not significantly different at $P \leq 0.05$ (shoots with length less than 5 mm were excluded).

Factors		Shoot regeneration [%]	Shoot number [callus^{-1}]
Medium	MS	78.9 ± 1.9a	9.4 ± 0.6a
	B_5	66.7 ± 2.7b	7.8 ± 0.2c
	N_6	60.1 ± 2.6b	8.9 ± 0.3b
	White	35.5 ± 3.2c	1.8 ± 0.1e
	WPM	42.2 ± 1.6c	3.3 ± 0.2d
Seedling age [d]	12 - 15	79.7 ± 2.7b	8.5 ± 0.1c
	18 - 21	83.3 ± 2.8a	10.5 ± 0.4a
	24 - 27	82.1 ± 1.9a	10.7 ± 0.5a
	31 - 34	72.4 ± 1.6b	9.6 ± 0.3b
	38 - 41	63.2 ± 2.5c	8.4 ± 0.2c
PVP conc. [%]	0	40.1 ± 2.3e	7.4 ± 0.1d
	0.2	62.3 ± 4.6c	9.6 ± 0.2b
	0.5	82.4 ± 1.6a	11.1 ± 0.3a
	1.0	70.2 ± 2.4b	8.5 ± 0.3c
	3.0	50.5 ± 3.2d	7.8 ± 0.2d

beneficial for shoot regeneration from leaf-derived calli of *O. japonica*.

Physiological status of explants is also an important factor on callus induction and shoots regeneration (Liu *et al*. 2006). The ability of shoot differentiation was significantly different ($P<0.05$) in response to the seedlings age (Table 2). The highest frequency of shoot regeneration from leaf-derived callus was 83.3 % with the average number of 11.9 shoots per culture from seedlings at the age of 18 - 27 d. However, the regeneration rate began to decrease sharply using leaf explants from seedlings at the age of 38 - 41 d, by which the frequency of shoots regeneration and the average number of shoots per culture went down to 63.3 % and 8.5, respectively. The effect of explants age on callus induction and shoot regeneration potential is in agreement with the responses of sunflower (Peterson *et al*. 1985), bean, pea (Angelini *et al*. 1989), and safflower (Nikam *et al.* 1999). It has already been established that younger explants exhibit greater morphogenic potential than older explants (Welander 1988, Fasolo *et al.* 1989, Yepes *et al.* 1994), as they might have more metabolically active cells with hormonal and nutritional conditions that are responsible for increased organogenesis (Famiani *et al.* 1994).

Our research results showed (Table 2) that the frequency of shoot regeneration from leaf-derived callus of *O. japonica* would be much lower without PVP due to callus browning, while addition of PVP can effectively overcome this problem and PVP of 0.5 % was the optimal treatment concentration for shoot regeneration. Polyphenols have been reported to associate with mechanical injury and environmental stress responses (Lagrimini 1992). These stress response may involve release of polyphenol from the vacuole, as well as phenol synthesis in the cytoplasm (Vaugh *et al*. 1984). It is known that cell death is correlated with elevated levels of polyphenols during tissue browning, which could be solved by adding anti-oxide such as PVP (Tang *et al*. 2004, Rout *et al.* 1999).

Auxins are involved in the process of adventitious root formation, but these hormones affect *in vitro* rooting of various species differently (Zhang *et al.* 2004, Wang *et al*. 2006). Well developed shoots from the culture were excised and transferred to rooting medium (half strength MS) augmented without or with IBA or NAA. The results showed that the addition of auxin IBA or NAA can effectively promote induction of roots. The optimum rooting response was observed on the rooting medium supplemented with 0.5 mg dm^{-3} IBA, at which root frequency reached 100 % with the higher number of 24.8 roots per shoot after 28 d of culture. The results are in agreement with the finding on *Cassia angustifolia* where IBA was found better than NAA to induce the formation of maximum number of roots (Agrawal and Sardar 2006). This may be attributed to the slow movement and slow degradation of IBA, which might facilitate its localization near the site of application and thus its better function in inducing roots. The plantlets with normal shoots and roots were transferred to the soil in the greenhouse and transplantation survival rate was 100 % as recorded after 1 month.

In conclusion, an efficient plant regeneration system from leaf-derived callus of *O. japonica* has been successfully established for the first time, which can offer an effective approach for the rapid propagation of *O. japonica* and also provide the foundation for genetic transformation of *O. japonica* in the future.

References

Agrawal, V., Sardar, P.R.: *In vitro* propagation of *Cassia angustifolia* through leaflet and cotyledon derived calli. - Biol. Plant. **50**: 118-122, 2006.

Angelini, R.R., Allavena, A.: Plant regeneration from immature cotyledon explant cultures of bean (*Phaseolus coccineus*). - Plant Cell Tissue Organ Cult. **19**: 167-174, 1989.

Arbain, D., Putra, D.P., Sargent, M.V.: The alkaloids of *Ophiorrhiza filistipula*. - Aust. J. Chem. **46**: 977-985, 1993.

Beegum, A.S., Martin, K.P., Zhang, C.L., Nishitha, I.K., Ligimol, S.A., Madhusoodanan, P.V.: Organogenesis from leaf and internode explants of *Ophiorrhiza prostrata,* an anticancer drug (camptothecin) producing plant. - Electronic J. Biotechnol. **10**: 1-10, 2006.

Chen, J.T., Chang, W.C.: Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*. - Biol. Plant. **50**: 169-173, 2006.

Chu, C.C., Wang, C.C., Sun, C.S., Hsu, C., Yin, K.C., Chu, C.Y., Bi, F.Y.: Establishment of efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. - Sci. sin. **18**: 659-688, 1975.

Duncan, D.B.: Multiple range and multiple F test. - Biometrics **11**: 1-42, 1955.

Famiani, F., Ferradini, N., Staffolani, P., Standari, A. Effect of leaf excision time and age, BA concentration and dark treatments on *in vitro* shoot regeneration of M.26 apple rootstock. - J. hort. Sci. **69**: 679-685, 1994.

Fasolo, F., Zimmerman, R.H., Fordham, I.: Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple. - Plant Cell Tissue Organ Cult. **16**: 75-87, 1989.

Gamborg, O.L.: The effects of amino acids and ammonium on the growth of plant cells in suspension culture. - Plant Physiol. **45**: 372-375, 1970.

Haliloglu, K.: Efficient regeneration system from wheat leaf base segments. - Biol. Plant. **50**: 326-330, 2006.

Kitajima, M., Fujii, N., Yoshino, F., Sudo, H., Saito, K., Aimi, N., Takayama, H.: Camptothecins and two new monoterpene glucosides from *Ophiorrhiza liukiuensis*. - Chem. Pharmaceut. Bull. **53**: 1355-1358, 2005.

Koroch, A., Juliani, H.R., Kapteyn, J., Simon, J. E.: *In vitro* regeneration of *Echinacea purpurea* from leaf explants. - Plant Cell Tissue Organ Cult. **69**: 79-83, 2002.

Lagrimini, L.M.: Wound-induced deposition of polyphenols in transgenic plants over expressing peroxidase. - Plant Physiol. **96**: 26-31, 1992.

Lisowska, K., Wysokinska, H.: *In vitro* propagation of *Catalpa ovata* G. Don. - Plant Cell Tissue Organ Cult. **60**: 171-176, 2000.

Liu, C.Q., Xia, X.L., Yin, W.L., Huang, L.C., Zhou, J.H.: Shoot regeneration and somatic embryogenesis from needles of redwood (*Sequoia sempervirens* (D.Don.) Endl.). - Plant Cell Rep. **25**: 621-658, 2006.

Lloyd, E., McCown, B.: Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. - Proc. Int. Plant Propag. Soc. **30**: 421-427, 1980.

Lorence, A., Nessler, C.L.: Camptothecin, over four decades of surprising findings. - Phytochemistry. **65**: 2735-2749, 2004.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nikam, T.D., Shitole, M.G.: *In vitro* culture of safflower cv. Bhima: initiation, growth optimization and organogenesis. - Plant Cell Tissue Organ Cult. **55**: 15-22, 1999.

Pereira, A.M., Bertoni, B.W., Appezzato-da-Gloria, B., Araujo, A. R.B., Januario, A.H., Lourenco, M.V., Franca, S.C.: Micropropagation of *Pothomorphe umbellate* via direct organogenesis from leaf explants. - Plant Cell Tissure Organ Cult. **60**: 47-53, 2000.

Peterson, K.E., Everett, N.P.: Regeneration of *Helianthus annuus* inbred plants from callus. - Plant Sci. **42**:125-132, 1985.

Pretto, F.R., Santarem, E.R.: Callus formation and regeneration from *Hypericum perforatum* leaves. - Plant Cell Tissue Organ Cult. **62**: 107-113, 2000.

Rout, G.R., Samantaray, S., Mottley, J., Das, P.: Biotechnology of the rose: a review of recent progress. - Sci. Hort. **81**: 201-228, 1999.

Saito, K., Sudo, H., Yamazaki, M., Nakamura, M.K., Kitajima, M., Takayama, H., Aimi, N.: Feasible production of camptothecin by hairy root culture of *Ophiorrhiza pumila*. - Plant Cell Rep. **20**: 267-271, 2001.

Sudo, H., Yamakawa, T., Yamazaki, M., Aimi, N., Saito, K.: Bioreactor production of camptothecin by hairy root cultures of *Ophiorrhiza pumila*. - Biotechnol. Lett. **24**: 359-363, 2002.

Tafur, S., Nelson, J.D., Delong, D.C., Svoboda, G.H.: Antiviral components of *Ophiorrhiza mungos* isolation of camptothecin and 10-methoxycamptothecin. - Lloydia **39**: 261-262, 1976.

Tang, W., Harris, L.C., Outhavong, V., Newton, R.J.: Antioxidants enhance *in vitro* plant regeneration by inhibiting the accumulation of peroxidase in Virginia pine (*Pinus virginiana* Mill.). - Plant Cell Rep. **22**: 871-877, 2004.

Vaugh, K.C., Duke, S.O.: Function of polyphenol oxidase in higher plants. - Physiol. Plant. **60**: 106-112, 1984.

Wall, M.E., Wani, M.C., Cook, C.E., Palmer, K.H., McPhail, A.T., Sim, G.A.: Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. - J. Amer. Chem. Soc. **88**: 3888-3890, 1966.

Wang, H.-M., Zu, Y.-G., Wang, W.J., Wu, S.-X., Dong, F.-L.: Establishment of *Camptotheca acuminata* regeneration from leaf explants. - Biol. Plant. **50**: 725-728, 2006.

Welander, M.: Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. - J. Plant Physiol. **132**: 738-744, 1988.

White, P.R.: Nutrient deficiency studies and an improved inorganic nutrient for culture of excised tomato roots. - Growth **7**: 53-65, 1943.

Yepes, L.M., Aldwinckle, H.S.: Factors that effect leaf regeneration efficiency in apple, and effect of antibiotics in morphogenesis. - Plant Cell Tissue Organ Cult. **37**: 257-269, 1994.

Zhang, L., Kai, G., Xu, T., Pi, Y., Zhang, H., Sun, X., Tang, K.: Efficient regeneration of tetraploid *Isatis indigotica* plants via adventitious organogenesis from hypocotyl explants. - Biol. Plant. **48**: 121-124, 2004.