BIOLOGIA PLANTARUM 54 (2): 361-365, 2010

BRIEF COMMUNICATION

Direct somatic embryogenesis and shoot organogenesis from leaf explants of *Primulina tabacum*

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Abstracts

An efficient propagation system *via* somatic embryogenesis and shoot organogenesis and plant regeneration system for endangered species *Primulina tabacum* Hance was established. Thidiazuron (TDZ) was the key plant growth regulator for inducing somatic embryogenesis and kinetin (KIN) and 6-benzylaminopurine (BAP) were the key cytokinins for inducing shoot organogenesis from leaf explants. TDZ combined with BAP or KIN in the induction Murashige and Skoog medium induced both somatic embryos and adventitious shoots. Leaf explants with abaxial site in contact with the medium induced less somatic embryos or adventitious shoots compared to inversely placed leaf explants and the optimum pH was 6.5 - 7.0. Secondary somatic embryos or adventitious shoot could be induced from primary somatic embryos using TDZ and BAP. Shoots developed adventitious roots on rooting medium containing 0.5 μM indole-3-butyric acid and 0.2 % activated carbon. Over 90 % of plantlets survived following acclimatization and transfer to potting mixture (sand:*Vermiculite*:limestone; 1:2:1).

Additional key words: adventitious shoot, 6-benzylaminopurine, 3-indolebutyric acid, kinetin, micropropagation, thidiazuron.

Primulina tabacum Hance was first discovered by Hance on the limestone Lianjiang drainage areas in Lianzhou (North Guangdong, China) in 1881, followed shortly thereafter by its disappearance for more than 120 years. The rediscovery of *P. tabacum* has important scientific value in the study of both ancient and recent climate, soil and co-evolutionary biology of animals and plants in South China in the Five Ridges Region. It has been listed as a 'first grade' critically endangered species in China (Xin 2005). *P. tabacum* grows only in limestone cave environments with high relative humidity, stable temperatures, high CO_2 concentration and feeble light (Ren *et al*. 2003). In order to preserve and utilize the rare and endangered plant species, it is very necessary to establish an efficient propagation and plant regeneration system in the event of sudden deterioration or loss of the natural environment. In the family *Gesneriaceae*, adventitious shoot regeneration from leaf discs and *in vitro* propagation in *Sinningia* spp. have been reported (Scaramuzzi *et al*. 1999, Nhut *et al*. 2005). Somatic embryogenesis and rapid multiplication *in vitro* has been reported in *Chirita longgangensis* (Tang *et al*. 2007). However, we are not aware of any reports on tissue culture or biotechnology with *P. tabacum*. Hence our current study is focused on somatic embryogenesis and adventitious shoot formation (from leaf explants) for establishment of efficient propagation and regeneration methods. As the species grows in limestone caves where soil pH value is high, we tested the effects of various *in vitro* pH values on inducing somatic embryo or adventitious shoot formation.

Primulina tabacum Hance plants at vegetative stage (about 10 cm high) were harvested in December 2006 in Lianzhou Underground River in Northern Guangdong.

Received 28 July 2008, *accepted* 2 March 2009.
Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indole-3-butyric acid; KIN - kinetin; NAA - α-naphthalene acetic acid; TDZ - thidiazuron;.
Acknowledgement: Supports from the Corporation Program of National Basic Research Program of China (973 Program) (2007CB411600), the Program for Tropical & Subtropical Plant Germplasm Construction of Guangdong Province (2005B60301001, 2006B60101034) and Guangdong Scientific Program (2007A060306011) are gratefully acknowledged.
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Leaves were surface sterilized in 70 % (v/v) alcohol for 10 s and 0.1 % (m/v) mercuric chloride for 8 min, rinsed with sterile distilled water 3 times, then leaves were cut into 0.6 cm^2 explants and inoculated inversely on MS basal media (Murashige and Skoog 1962) supplemented with various combinations of plant growth regulators (PGR) for inducing somatic embryogenesis or adventitious shoot formation. The induction media contained 5.0 μM 2, 4-D, 5.0 μM NAA, 5.0 μM TDZ, 5.0 μM KIN, 5.0 μM BAP, 5.0 μM BAP + 2.5 μM NAA, 1.0 μM TDZ + 5.0 μM BAP, 1.0 μM TDZ + 5.0 μM KIN and 5.0 μM TDZ + 1.0 μM BAP, respectively (Table 1). All the media contained 30 g dm^{-3} sucrose, were adjusted to pH 6.5 and solidified with 0.7 % (m/v) agar before autoclaved. Culture jars were incubated in a culture room with temperature set at 26 ± 2 °C and with 14-h photoperiod (low irradiance < 10 μmol m^{-2} s^{-1}). After culturing for 8 - 12 weeks, induction of somatic embryogenesis or adventitious shoot formation was investigated.

For induction of secondary somatic embryogenesis or adventitious shoot formation, different explants (*in vitro* grown leaves, shoots and primary somatic embryos; 50 per treatment) were inoculated on the different induction media and grown 20 d at dark and then under low irradiance (< 10 μmol m^{-2} s^{-1}). After culturing for 6 to 8 weeks, induction of somatic embryogenesis or adventitious shoot formation was investigated and the number of somatic embryos or shoots per explant recorded.

Leaf explants were placed with adaxial or abaxial site in contact with the induction medium and incubated for 6, 8, 10 weeks under low irradiance and somatic embryogenesis or adventitious shoot formation was investigated. The induction medium contained either 5.0 μM TDZ or 1.0 μM BAP. There were 50 explants per treatment, the experiment was repeated and data statistically analyzed as above.

Primary somatic embryos were isolated and then transferred to media with different pH containing 5.0 μM BAP and 1.0 μM TDZ in darkness for 20 d then transferred to low irradiance. After 8 weeks somatic embryogenesis or adventitious shoot formation was investigated.

For multiple shoot formation, the induced adventitious shoots were transferred to propagation media containing different combinations of plant growth regulators, subcultured every month and grown under standard irradiance (50 μmol m^{-2} s^{-1}). After five subcultures, the average propagation coefficient was calculated.

To induce rooting, single shoots were isolated from multiple shoot clusters and transferred to rooting media containing 0.5 μM indole-3-butyric acid (IBA) and 0.2 % activated carbon. After one month of incubation under irradiance of 50 μmol m^{-2} s^{-1} plantlets were removed from the jars, roots were washed of agar residue and root percentage and growth were recorded. Rooted shoots were transferred to sand:*Vermiculite*:limestone (1:2:1) mixture and acclimatized in a glasshouse under shade, temperature 26 ± 4 °C and relative humidity 90 ± 5 % and then survival rate and growth were monitored.

There were usually 50 - 70 explants per treatment and experiments were done twice. All data were statistically analyzed using one way *ANOVA* with LSD applied to separate treatment means ($P \leq 0.05$).

Leaf explants remained largely uncontaminated even over long periods and responses differed between media treatments. Leaf explants cultured on induction medium supplemented with 5.0 μM 2,4-D produced very little callus tissue at cut surfaces even after 8 - 9 weeks and the callus was granular and did not exhibit somatic embryogenesis or adventitious shoot formation. However, if these callused explants were transferred to media containing 5.0 μM TDZ or 5.0 μM BAP (or combinations of these) somatic embryos or adventitious shoot formation could be induced. On the medium containing 5.0 μM NAA, a small amount of callus was induced (with root formation visible on some callus tissues) after 8 - 9 weeks culture. However, no somatic embryos or adventitious shoot were observed. Leaf explants cultured on medium supplemented with 5.0 μM BAP or 5.0 μM KIN for 9 weeks, many bud-like protuberances were induced on the leaf surface or leaf edge (Fig. 1*A*). As the culture incubation period increased more and more adventitious shoot buds were induced and on some explants multiple shoot formation was observed on (Fig. 1*B*). The maximum number of adventitious shoots exceeded 100 per one leaf explant.

On medium supplemented with 5.0 μM TDZ leaf explants seemed not to respond at first. As the culture time progressed to 9 weeks globular-like protuberances protruded from the leaf surface in some of the cultured explants and generally formed somatic embryos (Fig. 1*C*). As the time in culture progressed, more and more somatic embryos were induced on the leaf surface or leaf edge of explants. The somatic embryos appeared to be globular when first observed then progressed to more mature stages over time and finally shoot buds were able to develop from the somatic embryo, but did not develop into whole seedlings on the same induction medium (Fig. 1*D*).

Combining TDZ and BAP or KIN in the same induction medium could induce both somatic

Table 1. Production of adventitious shoots and somatic embryos from the leaf explants of *Primulina tabacum* cultured on different induction media for 12 weeks. The same letter in a column denotes no significant difference according to LSD test ($P \leq 0.05$). PGR - plant growth regulators.

PGR [μM]	Number of adventitious shoots	Number of somatic embryos
TDZ 5.0	0.0 a	65.6 c
KIN 5.0	45.8 c	0.0 a
BAP 5.0	59.5 c	0.0 a
BAP 5.0 + NAA 2.5	90.3 d	0.0 a
TDZ 1.0 + BAP 5.0	55.9 c	25.7 b
TDZ 1.0 + KIN 5.0	33.7 b	24.9 b
TDZ 5.0 + BAP 1.0	18.4 b	67.1 c

embryos and adventitious shoots (Fig. 1*E*). If TDZ concentration exceeded that of BAP, the number of somatic embryos predominated over adventitious shoots. However if BAP or KIN concentration exceeded that of TDZ, more adventitious shoots were induced than somatic embryos (Table 1).

Indole-3-acetic acid (IAA) was the key substance for somatic embryogenesis in many plant species including cassava, peanut and neem. Use of TDZ could also induce somatic embryogenesis in the some species (Murthy and Saxena 1998, Murch and Saxena 1999, Kumari *et al*. 2008). In this study, it is evident that TDZ was the key factor in somatic embryo induction, whether singly or in combination with other plant growth regulators. The results with *P. tabacum* suggested that TDZ plays a different role compared with BAP or KIN. However, TDZ has also been reported to induce shoot organogenesis from leaf or cotyledon explants in many species including *Hydrangea quercifolia*, *Ochna integerrima*, *Fragaria, Melastoma affine* and *Gossypium hirsutum* (Ledbetter and Preece 2004, Faisal and Anis 2006, Landi and Mezzetti 2006, Ma and Wu 2006, Ma *et al*. 2007, Divya *et al*. 2008). The other auxins such as 2,4-D and NAA did not induce somatic embryogenesis or adventitious shoot formation in *P. tabacum*. However leaf explants of *Chirita longgangensis* (also from the family *Gesneriaceae*) could be induced to directly form somatic embryos on induction medium supplemented with 0.5 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA (Tang *et al*. 2007).

When primary somatic embryos used as explants and cultured on various induction media, TDZ aldo induced production of secondary somatic embryos, while BAP induced adventitious shoot formation from primary somatic embryo explants and also from leaf and petiole explants derived from adventitious shoots (or adventitious shoots regenerated from primary somatic embryos. When TDZ and BAP were combined in the same medium, both somatic embryogenesis and adventitious shoot formation were induced, however, the number of somatic embryos or adventitious shoots produced was less that from primary explants. This result may be related to the size of explants because explants produced *in vitro* were much smaller than primary leaf explants. Interestingly, the induction of somatic embryogenesis or adventitious shoot

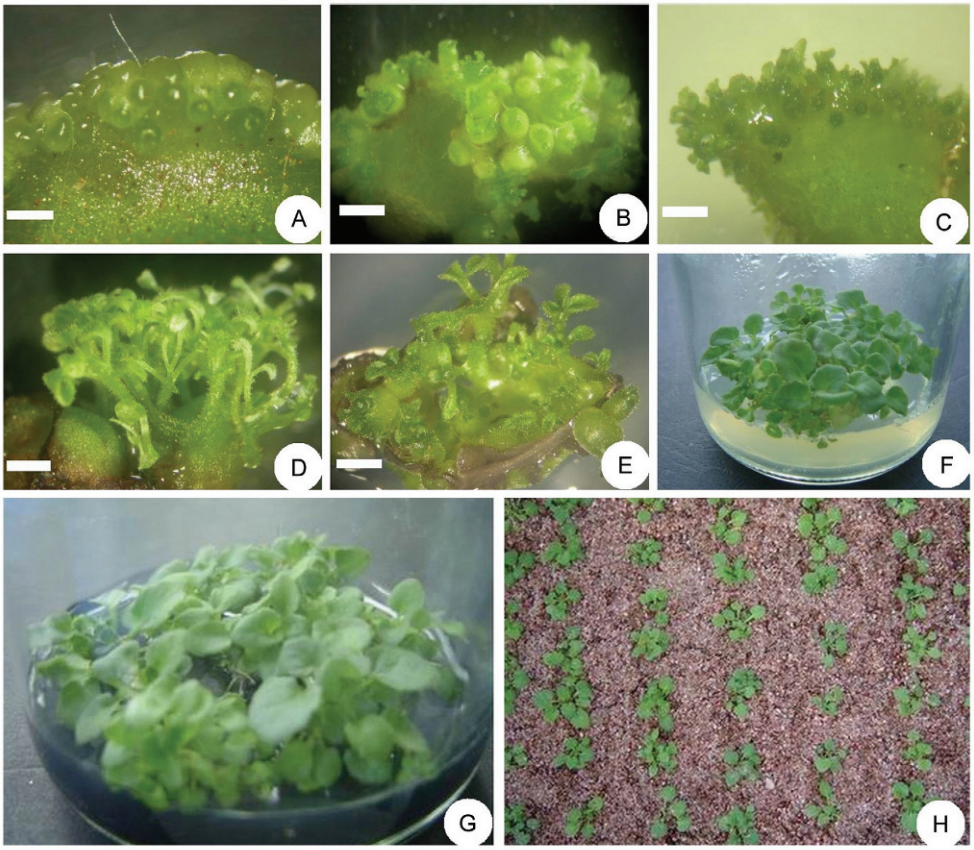

Fig. 1. Somatic embryogenesis and shoot organogenesis in *Primulina tabacum* (*bar* = 2 mm). *A* - Primary somatic embryogenesis induced from leaf explant on the induction medium containing 5.0 μM TDZ; *B* - Growth of somatic embryos; *C* - Adventitious shoots induced from leaf explant on the induction medium containing 5.0 μM BAP; *D* - Growth of adventitious shoots; *E* - Both somatic embryogenesis and adventitious shoots formation induced from leaf explant on the induction medium containing 5.0 μM BAP and 5.0 μM TDZ; *F* - Adventitious shoot propagation on the medium containing 5.0 μM BAP and 0.5 μM NAA; *G* - Root formation on the medium containing 0.5 μM IBA and 0.2 % activated carbon; *H* - Plantlets in a plastic tray with sand:*Vermiculite*:limestone (1:2:1) mixture 30 d after transplanting from *in vitro* conditions.

formation from leaf and petioles derived from *in vitro* tissues required 5 - 6 weeks for regeneration to occur compared to 8 - 9 weeks typically recorded for leaf explants derived from *in vivo* plants. Similar differences have been observed with other species, *e.g.*, cassava (Raemakers *et al*. 2000) where primary somatic embryogenesis was induced from young leaf explants *in vivo* within 6 weeks compared to induction of primary and secondary somatic embryogenesis in ≤ 2 weeks from young leaf explants from *in vitro* shoots or somatic embryo explants (Mathews *et al*. 1993, Raemakers *et al*. 1993, Ma *et al.* 1998, Ma and Xu 2002). Here we should emphasize that only immature or young leaf explants in cassava induced primary somatic embryogenesis at a low frequency (usually 0 - 30 %) while mature or old leaf explants could not be induced to form somatic embryos (Ma *et al*. 1998, 2002, Raemakers *et al*. 2000). For *P. tabacum*, even mature and older leaves from *in vivo* grown plants or *in vitro* shoots could also be induced to form somatic embryos or adventitious shoots at high frequency (90 - 100 %).

Table 2. Effect of leaf explant orientation with abaxial or adaxial site in contact with the medium and culture time on induction of somatic embryogenesis (on medium with 0.5 μM TDZ) and adventitious shoot formation (on medium with 0.5 μM BAP) in *Primulina tabacum*. The same letter in a row denotes no significant difference at $P \leq 0.05$.

Culture	Number of somatic embryos		Number of adventitious shoots	
	abax	adax	abax	adax
6 weeks	6.5 a	28.3 b	7.1 a	23.1 b
8 weeks	15.3 a	45.5 b	16.8 a	48.4 b
10 weeks	23.7 a	65.6 b	26.4 a	69.5 b

Also leaf explant orientation affected the induction of somatic embryos or adventitious shoot formation. Leaf explants placed with abaxial side in contact induced fewer somatic embryos or adventitious shoot compared to inversely placed leaf explants on the same induction media treatments at all observation times (Table 2).

Induction media treatments with pH settings at 6.5 and 7.0 induced more somatic embryos and adventitious shoots (48.9 and 47.6, respectively) compared to lower pH (5 - 18.7, 5.5 - 22.3 and 6.0 - 26.7) or higher pH (7.5 - 30.4 or 8 - 21.5) treatments. This optimum pH is higher than usual for most other plant species where the optimum pH range is 5.4 - 6.0 (Murashige and Skoog 1962). The reason may be that the species naturally grow on the limestone which has a higher pH.

As the adventitious shoots were transferred to the different propagation media, they developed into multiple shoot clumps. Medium supplemented with 5.0 μM BAP and 0.5 μM NAA induced the higher shoot propagation coefficient (5.6 in one month; data not shown; Fig. 1*F*), compared to medium supplemented with 5.0 μM TDZ and 0.5 μM NAA that induced only somatic embryogenesis.

Single shoots were isolated from multiple shoot clusters and transferred to rooting media. After 20 d, the fastest root formation occurred on medium supplemented with 0.5 μM IBA. If the medium was additionally supplemented with 0.2 % activated carbon, 100 % root formation was induced in 30 d (Fig. 1*G*). Other rooting media (0.5 μM NAA) also induced root formation in 30 d, however, roots were generally shorter with many root hairs. Rooted plantlets tended to be very small (only 1 to 3 cm high) and were prone to desiccation and premature death. To combat this, an early transplant phase was devised. The small rooted shoots were transferred from *in vitro* conditions to plastic trays with mixture of sand *Vermiculite* and limestone placed under black net to keep the environment wet and shade. Survival of plantlets was observed after one month. Plantlets 2 - 3 cm in height recorded high survival (92.8 %) (Fig. 1*H*), while smaller plantlets (1 - 2 cm) exhibited a lower survival rate (51.6 %). We have accumulated a total of 400 plantlets using the protocol described above and some of these plantlets have been reintroduced to original habitats in Lianzhou, Guangdong, China. Over 60 % of the plantlets could survive after two month indicating successful introduction of the species to its original habitat. This is the first report of the reintroduction of an endemic rare and endangered plant species from China as a result of biotechnology research.

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