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Original Article

Micropropagation through adventitious shoot regeneration from leaf culture of *Torenia fournieri* Lind.

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Abstract

A tissue culture system was established from young leaves of *in vitro* grown seedling of *Torenia fournieri* Lind. Leaf segments were cultured on Murashige and Skoog medium (MS) medium supplemented with combinations of NAA and BA. Shoot organogenesis was observed in all growth regulators containing medium. High frequency regeneration was obtained from leaves cultured on MS medium supplemented with 0.05 mg/l NAA plus 3 mg/l BA. When individual isolated shoots were transferred to MS medium devoid of growth regulators, complete plantlets were obtained. Adventitious shoots were also regenerated from callus that was derived from leaf explants and a high frequency of shoot organogenesis was found on MS medium containing 0.1 mg/l NAA plus 3 mg/l BA.

Keywords: adventitious organogenesis, leaf culture, micropropagation, *Torenia fournieri*, Wishbone flower

1. Introduction

Tissue culture techniques are becoming the most popular system to yield large numbers of new and true-to-type plants in a relatively short period of time. A large number of plant species that can be propagated by tissue culture methods are presently known, including *Torenia fournieri* Lind. The genus *Torenia* of the family Scrophulariaceae was originally found in tropical Asia and Africa. This genus contains about 50 species and almost of them are from Cambodia, Laos, Vietnam and Thailand (Yamazaki, 1985). The most cultivated species in the world is Wishbone flower, *Torenia fournieri* Lind. which grown as an ornamental plant and is usually found in home gardens and landscaping situations (Tanimoto and Harada, 1990). *T. fournieri* is also used as plant model to study fertilization and movement of chromosomes (Kikuchi *et al.*, 2005). Seeds normally propagate this plant; therefore there has been no need to propagate by tissue culture. However, tissue from *T. fournieri* such as stem segment can regenerate adventitious buds (Ishioka and Tanimoto, 1992). Based on this result, there are a few reports describing tissue culture methods for propagating *T. fournieri* (Tanimoto and Harada, 1986; Kobayashi *et al.*, 1995). To date there have been several reports on genetic transformation in *T. fournieri* (Aida *et al.*, 2000; Li *et al.*, 2006; Li *et al.*, 2007). Leaves have been used as explants to induce axillary shoots in several ornamental plants such as *Phlox paniculata* (Declerck and Korban, 1995); *Tagetes erecta* (Vanegas *et al.*, 2002); *Dianthus chinensis* (Kantia and Kothari, 2002); *Anthurium andraeanum* (Martin *et al.*, 2003); *Hydrangia quercifolia* (Ledbetter and Preece, 2004); *Caladium* 'Pink Cloud' (Ahmed *et al.*, 2004). In the case of *T. fournieri*, there has been no report of shoot regeneration from leaf. Therefore, the present investigation was undertaken with the objective of developing an efficient protocol for regenerating *T. fournieri* from leaf culture.

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2. Materials and Methods

2.1 Plant material

T. fournieri seeds were germinated under aseptic conditions. Briefly, they were surface sterilized using 95% ethanol for 1-2 min and then treated with 10% (v/v) Clorox™ solution containing 2 drops of Tween-20 emulsifier per 100-ml solution for 20 min. The sterilized seeds were washed with sterile distilled water 3 times and sown on MS (Murashige and Skoog, 1962) basal medium without plant growth regulator. After 3 weeks of culture, seedlings with well developed shoots and roots were obtained.

2.2 Preparation of explants

Young leaves of 3-week-old seedlings from the first and second nodes were excised and cut into 0.5x1 cm segments. *In vitro* grown leaves did not require sterilization. Explants were subcultured to fresh medium with the same component every 4 weeks.

2.3 *In vitro* conditions

Leaf segments were cultured for 4 weeks with their abaxial surface in contact with MS basal medium supplemented with six NAA concentrations, 0.05, 0.1, 0.5, 1, 1.5 or 2 mg/l and four BA concentrations, 0, 1, 3 or 5 mg/l. For *in vitro* rooting, green and normal adventitious shoots (1.5-2 cm long) from shoot multiplication cultures were excised and placed on MS basal medium without growth regulators.

All media were supplemented with 3% sucrose and 8.2 g/l of Mermaid™ agar. The pH of all media was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg/cm^2, 121^{o}C for 20 min. Cultures were maintained at 25±1^{o}C air temperature in a culture room with a 16-h photoperiod under an illumination of 20 mmolm^{-2}s^{-1} photosynthetic photon flux density provided by cool-white fluorescent light. Plant materials were stored in glass-capped culture jars (115 ml capacity) each containing 20 ml of medium.

2.4 Statistical analysis

One leaf segment was implanted per plate and all experiments were conducted on two different days with 15 replicates per treatment. Data on regeneration of shoot number per leaf segment, the presence or absence of roots and callus on leaf segment after 4 weeks of culture were recorded. Shoot numbers were submitted to analyses of variance and mean numbers were compared using Tukey's test at $p \leq 0.05$.

3. Results

The cultured leaf segments enlarged and began to respond in terms of shoot bud formation (Figure 1). Adventitious shoot initiation was evidenced, arising in clump from the entire leaf surface within 12-14 days (Figure 2). There was no difference in regeneration frequency between the first and the second leaf. However, there was significant difference in shoot number per leaf segment. Shoots were formed at a high frequency of 50 shoots on 0.05 mg/l NAA and 3 mg/l BA (Table 1). There was a considerable increase in shoot number on lower NAA (0.05, 0.1 or 0.5 mg/l) and higher BA (3 or 5 mg/l). Higher levels of NAA (1.5 or 2 mg/l) with BA at all concentrations were less effective in shoot regeneration. No adventitious shoot was formed when 1 or 2 mg/l NAA was added solely. Clumps of shootlets from leaf segments were subcultured to MS medium devoid of growth regulators for 4 weeks and the full, expanded leaves were obtained (Figure 3).

Root and callus formation occurred on almost every combination of NAA and BA tried (Table 1). The roots (5 to 6) usually arising from the basal end of shoots were large and vigorous and sufficient for acclimatization. The plants propagated by leaf culture did not show any morphological abnormality when compared with the original plants. Callus was pale yellow to green in color and started from the cut ends or from the incision on the leaf. In order to optimize the shoot regeneration from callus, a second experiment was conducted by transferred these calluses to the previous shoot induction medium (Table 1). No shoot bud induction was

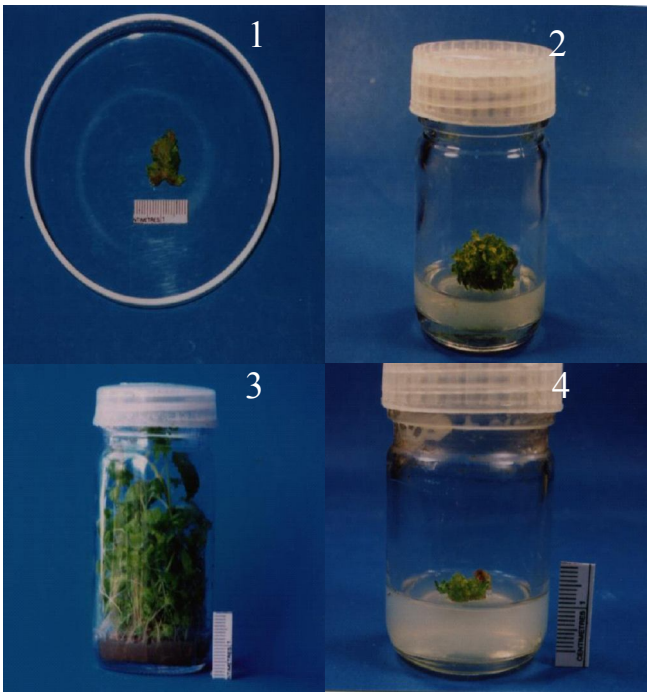

Figure 1-4 Micropropagation of *Torenia fournieri*. (1) Adventitious shoot buds formed on leaf segment. (2) Clump of adventitious shoots cultured on MS medium containing 0.05 mg/l NAA and 3 mg/l BA. (3) Complete plantlets grown on MS medium without growth regulators. (4) Callus-derived shoots developed on MS medium with 0.1 mg/l NAA and 3 mg/l BA.

Table 1. Induction of shoots from leaf explants and callus of *Torenia fournieri* cultured on MS medium with combinations of NAA and BA.

MS medium		Number of shoots/leaf (Mean±SE)	Visual Observation	Number of shoots/callus (Mean±SE)
NAA (mg/l)	BA (mg/l)			
0.05	0	5.2±1.8^{efg}	Root, callus	0^{d}
0.05	1	24.9±3.6^{bcd}	Root, callus	1.0±0.3^{cd}
0.05	3	50.9±9.0^{a}	Callus	1.2±0.5^{abcd}
0.05	5	45.9±6.3^{a}	Callus	2.2±0.5^{abc}
0.1	0	9.8±1.7^{defg}	Root, callus	0^{d}
0.1	1	20.4±3.2^{cde}	Root, callus	1.7±0.3^{abcd}
0.1	3	18.3±1.9^{def}	Callus	3.4±0.8^{a}
0.1	5	45.0±5.3^{ab}	Root, callus	3.1±0.7^{ab}
0.5	0	20.9±2.7^{cde}	Root, callus	0^{d}
0.5	1	41.6±4.2^{abc}	Root, callus	0.5±0.2^{cd}
0.5	3	31.8±4.5^{abcd}	Root, callus	0.9±0.3^{cd}
0.5	5	36.0±3.8^{abc}	Callus	1.3±0.4^{bcd}
1.0	0	4.6±2.7^{fg}	Root, callus	0^{d}
1.0	1	7.1±1.6^{defg}	Root, callus	1.5±0.5^{abcd}
1.0	3	19.2±3.8^{cdef}	Callus	1.5±0.4^{abcd}
1.0	5	13.8 ±2.0^{defg}	Root	1.9±0.6^{abc}
1.5	0	0^{g}	Root	0^{d}
1.5	1	21.1±0.4^{fg}	Callus	0^{d}
1.5	3	4.1±0.8^{fg}	-	0^{d}
1.5	5	3.5±1.0^{defg}	Callus	0^{d}
2.0	0	0^{g}	Root	0^{d}
2.0	1	3.9±0.5^{fg}	Root, callus	0^{d}
2.0	3	5.5±1.0^{efg}	Root, callus	0^{d}
2.0	5	5.1±0.8^{efg}	Root, callus	0^{d}

Values within column not having a superscript in common differ significantly ($p<0.05$) by Tukey's test.

formed when NAA was added singly or at high concentrations with BA (1.5 or 2 mg/l). Callus subcultured on MS medium supplemented with 0.1 mg/l NAA and 3 mg/l BA formed green shoot buds within 7-10 days of culture at a frequency of 3 shoots (Figure 4). However, callus-derived shoots did not produce any roots on this medium.

4. Discussion

The aim of the present study was to develop a protocol for the induction of shoot regeneration from *T. fournieri* leaf. For shoot multiplication *T. fournieri* preferred BA at high concentration and NAA at low concentration. This may suggest that bud formation required cytokinin. A conjunction of BA and NAA evoked a better response in shoot multiplication than NAA alone and this is probably due to the difference in endogenous levels of growth regulators in this plant or to a difference in sensitivity (Trewawas and Cleland, 1983). Such a synergistic effect of NAA and BA is in concurrence with the results in other ornamental plants such as *Tagetes* (Kothari 1984; Belarmino, 1992), *Lilium* (Liu, 1986) and *Dianthus* (Jethwani, 1993). In the present study the first and second position of leaves below the shoot tip could produce multiple shoots which is in concurrence with the report of *Dianthus chinensis* (Kantia and Kothari, 2002).

NAA and BA at several combinations resulted in callusing and shoot multiplication on callus indicating that the normal endogenous growth substance levels were conductive to bud formation. A similar result was observed in plant regeneration of *Dianthus barbatus* through organogenesis in callus induced from leaf explants (Pareek and Pareek, 2005). Callus culture with regenerative capacity reported here could be useful for a genetic transformation system in *T. fournieri*. Obviously, the finding that rooting could be obtained readily in the medium implies that the nutrient was benecial hence providing rapid clonal propagation.

In summary, a plant regeneration system was developed for *T. fournieri* utilizing leaf explants. Leaves from *in vitro* grown seedling appeared to be a potentially suitable explant for micropropagation.

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