

In Vitro* Shoot Organogenesis and Plant Regeneration of *Cymbalaria muralis

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Abstract: We developed an efficient protocol for *in vitro* shoot organogenesis and plant regeneration from *Cymbalaria muralis* leaf explant cultures. Leaf explants were cultured in media containing different concentrations of cytokinins. After determining the most effective cytokinin and cytokinin concentration, we tested the effects of various auxins at different concentrations on the regeneration efficiency and growth of shoots from excised leaf segments. Among the cytokinins tested, zeatin supplementation resulted in the highest shoot initiation efficiency, largest number of shoots per explant, and longest shoots after 6 weeks of culture. In this study, zeatin in combination with an auxin, naphthalene acetic acid, was highly effective for inducing shoot regeneration. Among the cytokinin/auxin combinations tested, supplementation of the culture medium with 1 mg/L zeatin and 0.1 mg/L naphthalene acetic acid resulted in the greatest number of shoots per explant (8.3), highest regeneration efficiency (93%), and longest shoot length (27 mm). Zeatin with 0.5 mg/L naphthalene acetic acid supplementation resulted in the second-highest values for each of these measures. The combined application of cytokinin and auxin might play a vital role in shoot organogenesis of *C. muralis*.

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1. Introduction

Cymbalaria muralis is an herbaceous perennial plant that belongs to the Figwort family, Scrophulariaceae. It is a small flowering herb that is native to the Mediterranean, widely naturalized in several temperate regions of the world, and typically grows on moist rocks and walls. It can be found in the cracks of old walls and is capable of climbing garden fences, reaching a height of over 3 feet. The flowers of *C. muralis* are hermaphroditic and are self-fertile. There are 2 subspecies, of *C. muralis*: *C. muralis muralis* and *C. muralis pilosa* (Vis.) Degen. *C. muralis* contains the following iridoids: antirrhinoside, linarioside, antirrhidine, linaride, 8-epiloganic acid, macfadienoside and muralioside (Bianco et al., 1997). Iridoids are responsible for pharmaceutical activities such as neuroprotective, anti-inflammatory and immunomodulator, hepatoprotective and cardioprotective effects and occur widespread in nature, mainly in dicotyledonous plant families like Scrophulariaceae (Tundis et al., 2008).

While most plants normally reproduce sexually, some species can propagate vegetatively. Vegetative propagation may require any one of several techniques, depending on the plant species. However, regeneration systems based on tissue culture have been recently developed for economically important plants (Chandra et al., 2010;

Debnath et al., 2006). *In vitro* techniques can improve these systems considerably through the application of nutritional and hormonal components under aseptic conditions. Plant proliferation using *in vitro* techniques is referred to as micropropagation because miniature shoots or plantlets are derived in the initial phase of the technique (Ekiert, 2000; Honda et al., 2001).

Many methods for the regeneration of whole plants from excised plant tissue are available. Organogenesis is a developmental system in which shoots or roots (organs) are induced to differentiate from a single cell (or a group of cells). Plant regeneration by organogenesis typically involves induction and development of a shoot from explant tissue, followed by transfer to a different medium, induction of root formation, and plant development (Boudaoud, 2010; Fleming, 2006). The propagation of *C. muralis* is currently achieved either by stem cutting or by planting of seeds. Propagation from seed is difficult and takes time because the seeds are very small and have a low rate of germination. An efficient method for the regeneration of intact plants from tissue culture is essential for establishing a multiple micropropagation system and for use of such a system as a tool for genetic transformation. We therefore developed a method of plant regeneration and micropropagation from *C. muralis* leaf explant cultures.

2. Material and Methods

Seed Sterilization and Germination

Seeds of *C. muralis* were surface-sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite (Sigma, St. Louis, Mo. USA) solution for 10 min, then rinsed 3 times in sterilized water. Ten seeds were placed on 25 mL of agar-solidified culture medium in petri dishes (100 × 15 mm). The basal medium consisted of MS (Murashige and Skoog, 1962) salt and vitamins (Sigma, St. Louis, Mo. USA) solidified with 0.7% (w/v) agar. The pH of the medium was adjusted to 5.8 before the addition of agar, and then sterilized by autoclaving at 121 °C for 20 min. Seeds germinated after 2 weeks of culture in a growth chamber kept at about 70–80% humidity and at 25 °C under standard cool white fluorescent tubes with a flux rate of 35 $\mu\text{mol s}^{-1}\cdot\text{m}^{-2}$ and a 16-h photoperiod.

In vitro Plant Regeneration

Leaf explants of *C. muralis* grown *in vitro* were aseptically cut into pieces that were approximately 0.7 × 0.7 cm. The basal medium consisted of MS medium solidified with 0.7% (w/v) plant agar. The medium was sterilized by autoclaving before use. To determine the optimal cytokinin concentration for shoot regeneration from leaf explants, the MS medium was supplemented with 0, 0.1, 0.5, 1, and 2 mg/L 6-benzylaminopurine (BAP), kinetin (N6-furfuryladenine), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), and zeatin. To improve shoot regeneration, the culture medium was optimized by testing the effect of different concentrations (0, 0.1, 0.5 and 1.0 mg/L) of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthalene acetic acid (NAA) on shoot formation and growth. Cultures were maintained at 25 ± 1 °C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 $\mu\text{mol s}^{-1}\cdot\text{m}^{-2}$) for 6 weeks.

Rooting of Regenerated Shoots

Regenerated shoots (~30 mm in length) were transferred to 1/2 MS medium in a Magenta box. The medium was solidified with 8 g/L plant agar and 50 mL was placed in each culture vessel. Four shoots were cultured in each culture vessel. Regenerated shoots were incubated at 25 ± 1 °C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 $\mu\text{mol s}^{-1}\cdot\text{m}^{-2}$) for 4 weeks. After 4 weeks, the rooted plants were washed with water to remove agar, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for 1 week to maintain high humidity. The plants were then transferred to soil and maintained in a growth chamber with a 16-h photoperiod and a day/night temperature of 18/20 °C

for 2 weeks. These hardened plants were then transferred to the greenhouse for further use.

3. Results and Discussion

Effect of Cytokinins on shoot organogenesis

A simple and effective protocol was developed for the *in vitro* plant regeneration and micropropagation of *C. muralis*. Cytokinins play an important role in plant organ initiation (Shani et al., 2006; Perilli et al., 2010). In this study, we investigated the effect of different cytokinins on the efficiency of shoot organogenesis. Shoot development from leaf explants was successful only in media containing zeatin, TDZ, and the medium containing 1 mg/L BAP (Table 1).

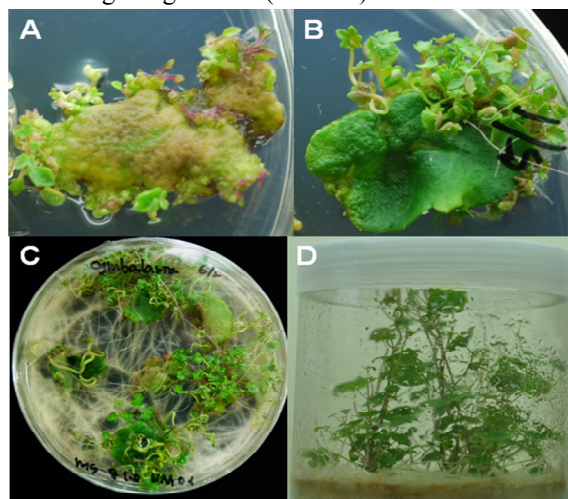


Figure 1. *In vitro* shoot organogenesis and plant regeneration of *Cymbalaria muralis*. (A) Shoot primordia emerging from a leaf explant after 3 weeks of cultivation on MS solid media supplemented with 1.0 mg/L zeatin. (B) After 5 weeks of culture, fully developed shoots were produced from the leaf explant culture. (C) After 6 weeks of culture, shoot regeneration from the leaf explant culture (D) Plantlets were regenerated on 1/2 MS solid medium after 4 weeks in culture. Magnification A-B X5.

The number of shoots and regeneration rate increased with increasing concentrations of TDZ (0.5–2.0 mg/L) and zeatin (0.1–1.0 mg/L). Among the media tested, the medium that contained 1.0 mg/L of the cytokinin zeatin produced the largest number of shoots from each explant (5.4), resulted in the maximum shoot length (13 mm) and had the highest regeneration efficiency (89%). As concentrations of zeatin increased above 1.0 mg/L, the number of shoots, shoot length, and regeneration efficiency decreased. No shoot regeneration was observed in media containing kinetin (N6-furfuryladenine) at any concentration. The regeneration efficiency was only 20% in the medium containing 1.0 mg/L BAP (6-benzylaminopurine), and there were very few shoots

per explant. After 3 weeks of culture on the medium supplemented with zeatin, formation of shoot primordia and small elongated shoots adjacent to the cut surface were observed (**Figure 1A**). Cells of the epidermis proliferated to produce shoots directly (direct shoot organogenesis) without an intervening

callus phase. Regenerated shoots developed from shoot primordia within 5–6 weeks. After 6 weeks of culture, fully developed shoots were produced from leaf explants (**Figure 1B**). The addition of 1 mg/L zeatin resulted in the most robust shoot initiation.

Table 1. Effect of cytokinins on shoot regeneration and growth from *Cymbalaria muralis* leaf explants after 6 weeks of culture on MS medium

Cytokinin (mg/L)	Regeneration %*	No. of shoots per explant*	Shoot length* (mm)
Control	0	--	--
BAP	0.1	--	--
	0.5	--	--
	1	20	2.0 ± 0.2
	2	--	--
Kinetin	0.1	--	--
	0.5	--	--
	1	--	--
	2	--	--
TDZ	0.1	--	--
	0.5	33	3.2 ± 0.3
	1	40	3.8 ± 0.2
	2	65	4.0 ± 0.2
Zeatin	0.1	67	3.5 ± 0.4
	0.5	74	4.2 ± 0.3
	1	89	5.4 ± 0.1
	2	83	4.8 ± 0.2

Regeneration frequency (%) = (No. of explants with root differentiation/all explants) × 100

-- No response

* Values represent the mean ± standard deviation of 50 shoots

Combined Effects of Cytokinins and Different Concentrations of Auxins

To enhance shoot organogenesis, young leaf explants from *in vitro*-grown *C. muralis* plants were cultured for 6 weeks in media containing 1 mg/L of zeatin and various concentrations of one of the following auxins: IAA, IBA, or NAA. The combination of zeatin and 0.1 mg/L NAA resulted in a higher regeneration rate (93%), more shoots from explants (8.3), and longer shoot length (27 mm) than zeatin alone (**Table 2**). These results agree with those of Lainé and David (1994) who observed shoot formation from *Eucalyptus grandis* leaf explants cultured on medium containing a combination of zeatin and NAA. In the present study, zeatin combined with NAA also resulted in the development of numerous adventitious roots. *C. muralis* appears to have a very prompt response to auxin that may be associated with its creeping habit. This results in profuse adventitious root development together with shoot organogenesis (**Figure 1C**). Shoot regeneration capacity decreased with increasing NAA concentration (0.1 to 2.0 mg/L).

At high concentrations of NAA, numerous root proliferations developed from the excised leaf segments, but no shoots were developed (data not shown). *C. muralis* cultured in media containing with the range tested of IAA and the medium containing 0.1 mg/L IBA produced fewer and shorter shoots than explants cultured in zeatin alone. However, there was no regeneration response in media with IBA concentrations of 0.5 mg/L and 1 mg/L. Overall, the optimum combination of cytokinin and auxin for shoot regeneration in *C. muralis* was 1.0 mg/L of zeatin supplemented with 0.1 mg/L of NAA.

Plant Micropropagation

In vitro organogenesis is one of the key techniques associated with genetic transformation, as it permits the successful regeneration of transgenic plants after co-cultivation with bacteria. Therefore, the development of efficient regeneration protocols is the most critical step in genetic transformation experiments (Degenhardt-Goldbach et al., 2011). The regenerated plantlets were obtained after 4 weeks of culture on hormone free ½ MS solid medium in Magenta box (**Figure 1D**).

Table 2. Effect of MS medium supplementation with 1 mg/L zeatin and different concentrations of auxins on shoot regeneration and growth from *Cymbalaria muralis* leaf explants after 6 weeks of culture

Auxin	(mg/L)	Regeneration %	No. shoots per explant *	Shoot length* (mm)
Control	0	89	5.4 ± 0.1	13.0 ± 0.07
IAA	0.1	67	2.7 ± 0.4	20.0 ± 0.08
	0.5	50	3.5 ± 0.1	18.0 ± 0.05
	1	33	2.3 ± 0.3	12.0 ± 0.08
IBA	0.1	50	1.5 ± 0.2	19.0 ± 0.11
	0.5	--	--	--
	1	--	--	--
NAA	0.1	93	8.3 ± 0.2	27.0 ± 0.12
	0.5	75	7.7 ± 0.1	24.0 ± 0.09
	1	45	2.0 ± 0.1	14.0 ± 0.08

Regeneration frequency (%) = (No. of explants with root differentiation/all explants) × 100

-- No response

* Values represent the mean ± standard deviation of 50 shoots

In the present study, we optimized a rapid protocol for efficient shoot organogenesis and normal plant regeneration from leaf explant cultures of *C. muralis*. The plants regenerated by this method can be multiplied by vegetative multiplication (*in vivo*) for further use in landscaping, ground cover, or as medicinal plants.

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