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Full Length Research Paper

Successful plant regeneration of *Orthosiphon stamineus* from petiole

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We present a successful regeneration of *Orthosiphon stamineus* plant from petiole. All of the explants were cultured on Murashige and Skoog (MS)-based medium supplemented with various concentrations of benzylaminopurine (BAP) and naphthalene acetic acid (NAA) at the growth condition (25 ± 2°C, 16-h photoperiod at light intensity of 22.85 µmol /m^2 /s) for 8 weeks. Results showed that petiole explants cultured at combination of 1.0 mg/L BAP and 0.2 mg/L NAA gave highest number of shoot produced per explants (4.33 ± 0.33) and a maximum callus fresh weight gains (3.2 ± 1.9 g) as compared to other treatments. The first shoot was observed from petiole cultured at all treatments in 4 weeks culture except for 0.1 mg/L BAP + 0.5 mg/L NAA treatment. For root induction, treatment at 0.1 mg/L NAA produced the highest number of roots produced per shoot (9.8 ± 3.1) compared to other treatments. Regeneration comparison between petiole, young leaf and stem segments showed that petiole is the most suitable explant for an efficient plant regeneration system of *O. stamineus*.

Key words: *Orthosiphon stamineus*, plant regeneration, shoot regeneration, benzylaminopurine (BAP), naphthalene acetic acid (NAA).

INTRODUCTION

Orthosiphon stamineus Benth. [syn: *O. aristatus* (Bl.) Miq.] or 'cat whisker' is a medicinal plant that belongs to the family Lamiaceae. Several compounds such as betulinic acid and caffeic acid derivatives (rosmarinic acid) have been isolated and identified from leaves of *O. stamineus* (Hossain et al., 2006). The *O. stamineus* plant has been used as antihypertensive, anti-inflammatory, anti-allergic and anti-cancer (Matsubara and Bohgaki, 1999). Furthermore, it has been reported for treatments of various diseases such as fever, syphilis, gonorrhea, menstrual disorder, kidney stones and influenza (Akowuah et al., 2004).

Currently, commercial plantation is utilizing conventional propagation of *O. stamineus*. However, this is untenable as cutting stem supply is inadequate for large market demand. Therefore, *in vitro* propagation of *O. stamineus* could offer plant improvement such as high bioactive compound accumulation and constant supply of propagules. To date, the successful plant regeneration of *O. stamineus* has been established from stem nodal explants treated with benzyl adenine (BA) and indole butyric acid (IBA) (Elongomathavan et al., 2003; Lee and Chan, 2004). However, there is no report on plant regeneration from petiole and leaf explants of *O. stamineus*. Furthermore, a combination of benzylaminopurine (BAP) and naphthalene acetic acid (NAA) has been reported to produce high shoot frequency or number of shoots produced per explant from leaf explants (Koroch et al., 2002). Therefore, in this study, we reported the effects of different combination

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Abbreviations: BAP, 6-Benzyl adenine; **NAA,** α-naphthalene acetic acid; **MS,** Murashige and Skoog salt medium.

Figure 1. Callus formation from petiole explant after 2 weeks culture. Scale bar represents 1 cm.

concentrations of BAP and NAA on different types of explants and its regeneration were discussed in detail.

MATERIALS AND METHODS

The *O. stamineus* plants were obtained from a local nursery and maintained in the greenhouse. The leaf (5 mm^2), stem (7 - 10 mm) and petiole (8 - 10 mm) were used as explants. Explants were first washed under running tap water for 30 min, followed by immersing in 70% (v/v) ethanol for 30 s, surface-sterilized with 15% NaOCl (v/v) for 30 min (for leaf explant) and 20% NaOCl (v/v) for 45 min (for stem and petiole explants) with addition of few drops of Tween 20 and agitated at 180 rpm in the orbital shaker. Sterilized explants were then rinsed with sterile distilled water to remove any bleach traces. Explants were then cultured on MS plates (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose and 8.0 g/L agar. Finally, the MS medium was adjusted to pH 5.7 - 5.8 before autoclaving at 121°C for 15 min. All cultures were incubated in the culture room (25 ± 2°C, 16-h photoperiod at light intensity of 22.85 µmol/m^2/s) for 8 weeks.

Shoot regeneration

Stem, leaf and petiole explants were cultured on MS-based medium supplemented with BAP (0.1 – 2.0 mg/L) and NAA (0.2 – 0.5 mg/L). All the explants were subcultured into fresh medium every week for the first month and every two weeks for the second month. Each treatment was conducted in five replicates. The percentage of shoot regeneration, number of shoots produced per explant, and callus fresh weight were recorded after 8 weeks culture.

Root regeneration

Multiple shoots (3 - 4 cm long) were excised and transferred onto MS plates containing different combination concentrations of BAP (0 - 0.5 mg/L) and NAA (0 - 0.5 mg/L). The shoots were incubated in the culture room for 4 weeks to initiate root formation. The percentage of root regeneration and number of roots produced per shoot were assessed after 4 weeks culture.

Statistical analysis

Data were analyzed by one-way ANOVA using SPSS Version 12 and followed by Bonferroni multiple comparisons test for mean comparison at P = 0.05.

RESULTS AND DISCUSSION

Effect of different BAP and NAA concentrations on shoot induction

After three weeks, all the explants showed greenish callus formation (Figure 1). The maximum mean callus fresh weight was recorded for petiole explants cultured at combination of 1.0 mg/L BAP and 0.2 mg/L NAA (3.19 ± 1.87 g) (Table 1). Among three type of explants tested, petiole showed the highest percentage of shoot regeneration (100%) when cultured at combination of 1.0 mg/L BAP and 0.2 mg/L NAA (4.33 ± 0.33 shoots per explants) (Figure 2). The leaf explants showed a maximum number of shoots produced per explant (2.50 ± 0.29) when the explants were cultured at combination of 2.0 mg/L BAP and 0.2 mg/L. In a previous study, nodal segments of *O. stamineus* were reported to produce 82% of shoot regeneration (32 shoots per explant) when cultured at 0.5 mg/L of BAP (Elongamathavan et al., 2003). In contrast, Lee and Chan (2004) demonstrated that nodal stems cultured at 1.5 mg/L BAP produced highest number of shoots (6.1 shoots per explant). Moreover, in our study, stem explants with the nodes removed failed to regenerate any shoots at 0.5 mg/L of BAP and only produced very low number of shoots at 0.5 mg/L for both BAP and NAA (Table 1). The difference in response to hormone combination could be due to factors such as plant genotype and physiological states of the explant that influence shoot regeneration of a plant

Table 1. Shoot regeneration of *Orthosiphon stamineus* cultured on MS plates containing BAP and NAA.

Explants	Growth regulators		Percentage of shoot regeneration (%)	No. of shoots produced per explant[1]	Mean callus fresh weight gains (g) [1]
	BAP (mg/L)	NAA (mg/L)			
Leaf	0.2	0.5	10	0.53 ± 0.16	0.30 ± 0.08
	0.5	0.5	10	0.67 ± 0.23	0.68 ± 0.13
	0.5	0.2	30	0.87 ± 0.26	0.47 ± 0.16
	1.0	0.2	20	1.20 ± 0.24	0.42 ± 0.07
	2.0	0.2	80	2.50 ± 0.29	1.24 ± 0.37
Petiole	0.1	0.5	0	0	0.15 ± 0.02
	0.2	0.5	60	1,47 ± 0.43[a]	0.76 ± 0.52
	0.5	0.5	80	2.33 ± 0.37[b]	3.02 ± 1.54
	1.0	0.2	100	4.33 ± 0.33[b]	3.19 ± 1.87
	2.0	0.2	60	1.47 ± 0.36	0.77 ± 0.76
Stem	0.5	0	0	0	0.28 ± 0.13
	0.5	0.7	0	0	0.66 ± 0.23
	0.1	0.5	0	0	0.43 ± 0.14
	0.2	0.5	10	1.00 ± 0.29	0.69 ± 0.29
	0.5	0.5	20	1.40 ± 0.32	0.74 ± 0.40
	0.2	0.2	0	0	0.34 ± 0.08
	1.0	0.2	0	0	0.69 ± 0.19
	2.0	0.2	10	0.87 ± 0.36	0.71 ± 0.17

[1]Mean ± Standard Error Mean. The different letters in a column were significantly different by the ANOVA at P<0.05 level.

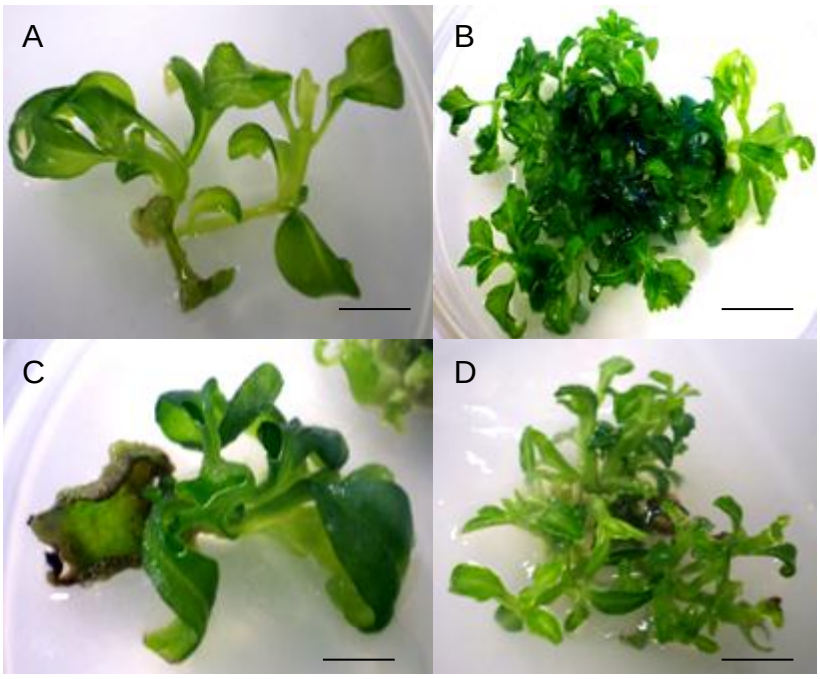

Figure 2. Shoot regeneration of *Orthosiphon stamineus* from different type of explants. A, Petiole explants cultured at 1.0 mg/L BAP and 0.2 mg/L NAA after 4 wks culture. B, Petiole explants cultured on 1.0 mg/L BAP and 0.2 mg/L NAA after 8 wks culture. C, Leaf explants cultured on 2.0 mg/L BAP and 0.2 mg/L NAA after 8 weeks. D, Stem explants cultured on 0.5 mg/L BAP and 0.2 mg/L NAA after 8 wk. Scale bars represent 1 cm.

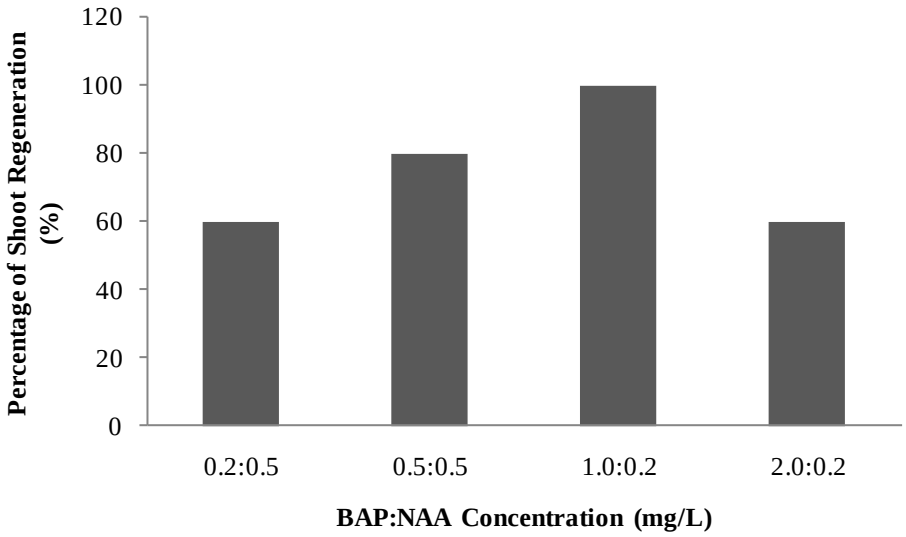

Figure 3. Effect of various concentrations of BAP and NAA on shoot regenerated from petiole of *O. stamineus*.

Table 2. Root regeneration of *Orthosiphon stamineus*.

Growth regulator		% of root regeneration	No. of roots produced per shoot
BAP (mg/L)	NAA (mg/L)		
0	0	100	7.00 ± 1.38
0	0.1	100	9.80 ± 3.10
0.2	0.5	100	5.40 ± 1.69
0.5	0.2	80	1.20 ± 0.49

(Gaba, 2005).

All types of explants showed different responses when cultured at various concentrations of hormones. For example, number of shoots produced per petiole explants decreased as the concentration of BAP became higher than 1.0 mg/L (Table 1). On the contrary, percentage of shoot regenerated from leaf explants increased as the concentration of BAP became higher than 1.0 mg/L. Our results indicate that different types of tissue respond differently to varying hormone combination (Takahashi et al., 2004). In this study, 100% shoot regeneration was achieved when petiole explants were grown at the combination of BAP and NAA (Figure 3). A combination of BAP and NAA has also been shown to induce high shoot regeneration of *Saussurea involucrate* (Guo et al., 2007), *Vitex agnus-ca*stus (Balaraju et al., 2008) and *Centella asiatica* (Mohapatra et al., 2008). These results indicated that petiole has the potential as new source for *in vitro* propagation of *O. stamineus*. The use of petiole has also been reported in other species such as *Heracleum candicans* (Sharma and Wakhlu, 2001), *Saintpaulia ionantha* (Sunpui and Kanchanapoom, 2002) and *Actinidia polygama* (Takahashi et al., 2004).

Root induction

Shoots treated with 0.1 mg/L NAA produced the highest percentage of root regeneration (100%), with an average of 9.8 ± 3.1 roots per shoot after 4 weeks culture. However, shoots were also rooted in the control, MS-free hormone medium (7.0 roots produced per shoot) (Table 2). Other researchers also reported that NAA promoted high number of roots in *Swertia chirayita* (average 6.5 roots per shoot) (Joshi and Dhawan, 2007). Our findings also showed that high BAP: NAA ratio (0.5 mg/L BAP + 0.2 mg/L NAA; 1.2 roots per shoot) produced lesser number of roots than low BAP: NAA ratio (0.2 mg/L BAP + 0.5 mg/L NAA; 5.40 ± 1.69). It is suggested that high BAP: NAA ratio promotes shoot formation but low BAP: NAA ratio induces root formation.

Conclusion

Petiole is the best explant source for plant regeneration of *O. stamineus*. Even though leaf and stem explants were capable of producing shoots, only petiole achieved

100% shoot regeneration with the highest number of shoots produced per explant. The establishment of plant regeneration system of *O. stamineus* will enable future research into production of high secondary product plant lines.

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