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Full Length Research Paper

An efficient system for *in vitro* multiplication of *Ocimum basilicum* through node culture

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An efficient *in vitro* micropropagation system was developed for direct shoot growth of *Ocimum basilicum*, an important medicinal plant, using nodal explants. The excised nodes were cultured on Murashige and Skoog (MS) medium containing two plant growth regulators (6-benzyladenine and 2-isopentanyl adenine) with various combinations and concentrations for the study of shoot induction. Addition of L-glutamine was essential to induce sprouting of axillary buds. The best condition for shoot growth was with 6-benzyladenine (BA) 10.0 µM + L-glutamine 30 mg/L in MS medium. The optimum shoot formation frequency was 100% and about 13.4 ± 1.80 shoots were obtained from each explant after 8 weeks of culture. Shoots (more than 4 cm) were rooted most effectively in 5.0 µM indole-3-butyric acid (IBA) supplemented with half-strength MS medium. The plantlets thus obtained hardened off and were transferred to natural soil, where they grew well and attained sexual maturity.

Key words: Axillary shoot sprouting, L-glutamine, medicinal plant, micropropagation, *Ocimum basilicum*.

INTRODUCTION

Ocimum basilicum L. commonly known as 'sweet basil' is a small perennial, culinary herb native to Asia and is widely grown as ornamental or field crops in the Mediterranean countries. Basil is not only valued for its pharmaceutical properties but also for the aromatic oil it yields. The oil of basil is used as flavoring agent for confectionary, sausage and beverages (Anonymous, 2003). It is also used for scenting dental and oral preparation and in certain perfume compounds, notably jasmine blends, to impart strength and smoothness. It also possesses insecticidal and insect repellent properties and effective against houseflies and mosquitoes. Besides its economic importance, it also has medicinal properties; it is used in the treatment of headaches, coughs, diarrhea, constipation, warts, worms and kidney malfunction and shows bactericidal property against *Salmonella typhosa* (Chopra et al., 1994; Simon et al., 1999).

Little practical experience has been acquired in *in vitro* propagation of *Ocimum* species, such as *Ocimum americanum* (Pattnaik and Chand, 1996), *Ocimum sanctum* (Singh and Sehgal, 1999; Shahzad and Siddiqui, 2000). To the best of our knowledge, there are few reports on *in vitro* multiple shoot regeneration of *O. basilicum* L. (Sahoo et al., 1997; Phippen and Simon, 2000; Saha et al., 2010). Micropropagation could provide a means to clone particular plant species rapidly from conventional breeding programme, or provide the basis for *in vitro* genetic manipulation or selection. Previous knowledge on tissue culture of *Ocimum* is limited, although plantlets have been obtained from leaf explant, but nodal explant or axillary shoot buds provide good source for obtaining large number of plants from single explant (Pattnaik and Chand, 1996).

Therefore, the present experiment was carried out and the objectives of this research were to establish clonal

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Abbreviations: BA, 6-Benzyladenine; **2-ip,** 2-isopentanyl adenine; **IBA,** indol-3-butyric acid; **MS,** Murashige and Skoog's medium (1962); **PGRs,** plant growth regulator.

material of *O. basilicum* and determine the suitable plant growth regulator type and concentration singly or in combination with L-glutamine for *in vitro* shoot proliferation from nodal explant of *O. basilicum*, rooting *in vitro* microshoots and finally successful field establishment.

MATERIALS AND METHODS

Plant material and explant preparation

Cultures were initiated with young shoots of established potted plant of *O. basilicum* L. grown under green house conditions. These shoots were carefully washed under running tap water for 30 min. Then, they were surface decontaminated sequentially with 70% ethanol (30 s) and 0.1% (w/v) $HgCl_2$ (4 min) and thoroughly rinsed with sterilized deionized water to remove any trace of sterilant. Nodal segments of 10 to 15 mm long were cut from sterilized shoots and placed on culture medium.

Culture medium and conditions

The culture medium used for the shoot bud induction was Murashige and Skoog (1962) basal medium with 3.0% (w/v) sucrose, 0.8% (w/v) agar (Qualigens, Mumbai) with the supplementation of various level of 6-bezyladenine (BA) and 2-isopentanyl adenine (2iP) and organic adjuvant (L-glutamine) in different combinations and concentrations in 25 × 145 mm glass culture tubes (Borosil India) containing 15 ml of the medium. All cultures were placed in growth room under 16 h cool white fluorescent light (50 µmol $m^{-2}s^{-1}$) provided by fluorescent lamps (Crompton, India) at temperature of 25 ± 2°C with 55 to 60% relative humidity. The pH of all the media was adjusted to 5.8 with 1 N NaOH or HCl before agar was added. The media were then sterilized by autoclaving at 121°C and 1.04 kg cm^{-2} for 15 min. All the cultures were transferred to fresh medium after 2 to 3 weeks of incubation.

Rooting and acclimatization

Healthy and normal microshoots, more than 4 cm in length were isolated from cultures for the induction of adventitious roots. These shoots were cultured on MS, ½MS and ¼MS medium supplemented with 5.0 µM indole-3-butyric acid (IBA). Plantlets with well-developed shoot and roots were removed from the culture vessels and thoroughly washed, dipped in 5% w/v bavistin solution and transferred to 8 cm diameter thermocol cups containing soilrite. The plants were completely covered with transparent polyethylene bags to maintain humidity, irrigated regularly with tap water and maintained in growth room at 25 ± 2°C with 16 h photoperiod before they were transferred outside under field conditions.

Statistical analysis

Data obtained on percent shoot regeneration, number of shoots, number of roots/ shoots and root length was analyzed for significance using ANOVA and the differences were contrasted using Tukey's test. All statistical analyses were performed at the 0.05% level, using the statistical software (SPSS inc. Chicago; USA).

RESULTS AND DISCUSSION

Direct multiple shoot induction was observed in nodal explants of *O. basilicum* after 2 weeks of culture on the MS medium containing BA or 2ip either singly or in combination with L-glutamine with varied frequency. With the increase in concentration of BA (10.0 µM), an increase in the percent regeneration frequency (100%) and average number of shoots (5.7 ± 0.35) were observed (Figure 1A) and 10.0 µM (BA) proved to be the optimum concentration for multiple shoot induction (Table 1).

In the second set of experiment, 2-iP was used and the obtained data shows that the concentration of 2-iP must be double of BA (10.0 µM) for obtaining the maximum number of shoots/explant. Of the various concentrations used, 10 µM proved to be the optimum for maximum shoot induction (Table 1). Less effective nature of cytokinin 2-iP in multiple shoot induction and requirement of twice the concentration of BA was also reported in *Bacopa monniera* (Tiwari et al., 2001).

In the other set of experiment, the response of shoot multiplication was investigated on the MS medium containing optimum concentrations of BA (10 µM) and 2ip (20 µM) with the different concentrations of L-glutamine (10, 20, 30, 40 mg/l) (Table 2). Addition of glutamine to the medium containing optimum concentrations of BA showed considerable increase in the number of shoots per explant with better shoot growth. Augmentation of 30 mg/L glutamine with medium containing BA (10 µM) showed the optimum response with 100% regeneration frequency and (13.4 ± 1.80) shoot per explant (Figure 1B). The concentration of glutamine beyond 30 mg/l did not affect the shoot multiplication frequency and resulted in basal callusing and decrease in the number of shoots regeneration per explant was also observed. The beneficial effect of glutamine is also expressed significantly in *B. monniera*. These results are in contrast with the results obtained in *O. sanctum* (Singh and Sehgal, 1999), where BA 1.0 mg/L proved to be the best for maximum shoot induction. This inconsistency may be because of species, explant or auxin: cytokinin combination.

After harvesting the microshoots, the regenerating mother tissues were placed on media containing 1.0 and 5.0 µM BA to check the regenerative capacity of mother tissue and it was found that the mother tissue, initially cultured on BA was more effective in obtaining the maximum number of shoots (12.5 ± 2.17) in comparison with the tissues obtained from the cultures containing 2-iP (Table 3). At optimum concentrations of 2ip or BA with glutamine, the continuous production of shoots was observed when transferred to lower concentrations of BA (5 µM) and the regeneration frequency was maintained upto the 4th subculture, thereafter, the number of shoots/explant decreased considerably (data not shown).

For rooting, individual shoots measuring 4 to 5 cm were transferred to MS, ½MS and ¼MS media supplemented with IBA (5.0 µM). ½MS media showed prolific root growth with 100% rooting frequency and maximum (3.8 ±

Figure 1. *In vitro* shoot regeneration and plant establishment of *O. basilicum*. A: Multiple shoot induction from nodal explant on MS medium containing 10 µM BA; B: Shoot multiplication on MS medium supplemented with 10 µM BA with 30 mg m/L glutamine; C: Rooted microshoot; D; An acclimatized plant.

0.33) roots/shoot with the optimum root length 2.7 ± 0.23 cm (Figure 1C and Table 4). The result thus obtained is in agreement with the results obtained in *B. monniera* (Tiwari et al., 2001).

Table 1. Effects of plant growth regulators on direct shoots regeneration through nodal segments of *O. basilicum* cultured in MS medium.

PGRs (µM)		Response (%)	Mean number of shoot	Remark
BA	2iP			
1.0	-	60 ± 2.9[b]	3.0 ± 0.30[cd]	Slow growing shoots
5.0	-	70 ± 2.7[c]	4.0 ± 0.36[b]	Moderate growth
10.0	-	100 ± 0.0[a]	5.7 ± 0.35[a]	Moderate growth
20.0	-	100 ± 0.0[a]	4.4 ± 0.46[b]	Slow growth, with slight callusing from cut end
-	1.0	41 ± 1. 7[e]	1.7 ± 0.15[e]	Slow growing shoots
-	5.0	70 ± 2.9[c]	2.5 ± 0.17[d]	Slow growth
-	10.0	70 ± 2.5[c]	2.7 ± 0.18[cd]	Moderate growth
-	20.0	93 ± 3.3[b]	5.3 ± 0.15[a]	Moderate growth with rosaceous appearance

Value represents means ± SE from 20 replicates. Mean followed by the same letter are not significantly different (P = 0.05) using Tukey's test. Data were recorded after 8 weeks of inoculation.

Table 2. Effects of glutamine on direct shoots regeneration through nodal segments of *O. basilicum* cultured in MS medium supplemented with optimum concentration of BA or 2iP.

PGRs (µM)		Glutamine (mg/L)	Response (%)	Mean number of shoot
BA	2iP			
10.0	-	10.0	80 ± 2.50[c]	6.0 ± 0.70[e]
10.0	-	20.0	90 ± 2.71[b]	8.0 ± 1.64[bc]
10.0	-	30.0	100 ± 0.0[a]	13.4 ± 1.80[a]
10.0	-	40.0	100 ± 0.0[a]	8.4 ± 1.43[bc]
-	10.0	10.0	60 ± 1.63[d]	3.7 ± 0.50[f]
-	10.0	20.0	80 ± 2.80[c]	5.5 ± 0.75[e]
-	10.0	30.0	90 ± 3.12[b]	7.7 ± 0.58[c]
-	10.0	40.0	100 ± 0.0[a]	9.3 ± 0.52[b]

Value represents means ± SE from 20 replicates. Mean followed by the same letter are not significantly different (P = 0.05) using Tukey's test. Data were recorded after 8 weeks of inoculation.

Table 3. Effects of 6-benzyladenine (BAP) and 2-isopentanyl adenine (2-iP) on shoots multiplication after well developed shoot harvest, through regenerating tissues of *O. basilicum* cultured on MS + BAP (1 and 5 µM) medium.

MS + BAP (µM)	Regenerating tissues from medium having	Mean number of shoot/explant
1.0	BA	4.6 ± 0.63[c]
5.0	BA	12.5 ± 2.17[a]
1.0	2iP	2.7± 0.35[d]
5.0	2iP	7.6 ± 1.63[b]

Value represents means ± SE from 20 replicates. Mean followed by the same letter are not significantly different (P = 0.05) using Tukey's test. Data were recorded after 8 weeks of inoculation.

Well rooted plantlets were taken out and washed with tap water, dipped in Bavistin (5%) and subsequently transferred individually to plastic cups filled with soilrite and placed in growth room and hardened by following the procedure stated in materials and methods and they were then transferred to botanical garden of the University. These plantlets resumed normal growth and development. Out of the 150 plantlets of *O. basilicum* transferred to soil, 135 survived with the survival rate of 90% under the green house conditions and thereafter, were transferred to open environment where they flowered and had fruits normally (Figure 1D).

Table 4. Effects of Murashige and Skoog (MS) salts concentration with 5.0 µM IBA on root, inducing efficiency in microshoots of *O. basilicum*.

MS Strength	Response (%)	Mean number of roots/shoot	Mean shoot length (cm)
MS full strength	72 ± 4.40[b]	2.7 ± 0.15[b]	2.5 ± 0.15[a]
MS half strength	100 ± 0.00[a]	3.8 ± 0.33[a]	2.7 ± 0.23[a]
MS one fourth	48 ± 3.40[c]	1.5 ± 0.16[c]	1.4 ± 0.14[b]

Value represents means ± SE from 20 replicates. Mean followed by the same letter are not significantly different (P = 0.05) using Tukey's test. Data were recorded after 4 weeks of inoculation.

Shoot multiplication in the present study was obtained by enhanced axillary branching, which is very crucial in employing tissue culture techniques for micropropagation and conservation as it ensures genetic stability. Micropropagation of *O. basilicum* could support domestication of *O. basilicum* and ultimately lead to the commercial cultivation in a short time span with the conservation of mother plant. The other advantage of our experiment was the absence of somaclonal variation which could be as a result of the lack of callus phase in the process of shoot induction and multiplication.

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