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Effect of explants source and different hormonal combinations on direct regeneration of basil plants (*Ocimum basilicum* L.)

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Abstract

Ocimum basilicum L. a herbaceous species belonging to the lamiaceae family is considered as a valuable plant for its pharmaceutical, aromatic and culinary properties. The major problem with the use of Lamiaceae species for pharmaceutical purposes is the plant to plant variability, mainly due to genetic and biochemical heterogeneity. *In vitro* shoot regeneration and multiplication is an impressive mean for precipitate propagation of species in which it is necessary to obtain a progeny with a high level of uniformity. In this research, two successive experiments were performed: first, the effects of explants source on MS medium supplemented with four different concentrations of BAP were studied in order to investigate the morphogenic responses; and second, the effects of different levels of two growth regulators (BAP and IAA) either individually or in combination on multiple shoot induction from nodal segments were evaluated. Maximum percent of regeneration (96.67±0.33) and average number of shoot (5.6±1.15) were observed on the medium containing 11 µM BAP + 0 µM IAA. Regenerated shoots were separated and rooted on the same half strength MS medium supplemented with 3.42 µM IAA alone for two weeks. Similarly in the second experiment, increasing BAP concentration led to decreased rooting. Moreover, a positive correlation between increasing the BAP level in culture media and vitrification of regenerated shoots was observed. The lowest and the highest vitrification values were achieved in the media containing 0 and 33 µm BAP, respectively.

Keywords: *Ocimum basilicum*., Direct Regeneration, Vitrification, BAP, Nodal Segment.
Abbreviations: BAP: 6-Benzylaminopurine, IAA: Indole-3-Acetic Acid, MS: Murashige and Skoog.

Introduction

Sweet basil (O*cimum basilicum* L.) is an annual and aromatic herb belonging to the Lamiaceae family, native to Iran, Afghanistan and India. It represents an important source of essential oil used in food, pharmaceutical, perfumery and cosmetics industries (Simon et al., 1990). Its aromatic leaves are used in fresh or dried forms as drug in traditional medicine and as a flavoring agent in food and confectionary products as well as beverages (Prakash, 1990; Marotti et al., 1996). *Ocimum* is also used as a stomachic, antihelmintic, antipyretic, diaphoretic, expectorant, carminative, stimulant and pectoral (Siddique and Anis, 2008). There has been a growing interest and support in the conservation and development of medicinal plants globally. This is in part, due, to the growing recognition given to the role of medicinal plants in the provision of culturally relevant and affordable health care in creating sustainable livelihoods and in the vital conservation of biodiversity.

As World Health Organisation (WHO) estimates, almost 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care necessities. The conventional propagation method of *O. basilicum* has been via seed. However, seedling progeny shows a high degree of variability because of cross-pollinated nature of the plant. In the recent years, there has been an increasing interest in *in vitro* culture techniques posing as a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Kumar and Seeni, 1998; Arumugan and Bhojwani, 1990; Babu et al., 2000; Bakos et al., 2000; Chitty et al., 2003, Maleki et al., 2011). *In vitro* micropropagation is an effective tool for rapid multiplication of species in which it is necessary to obtain a high progeny uniformity. In case of *Ocimun* genus, different explants, like nodal segments (Ahuja et al., 1982; Shahrzad and Siddiqui, 2000; Begun et al., 2000), leaf explants (Phippen and Simon, 2000), apical buds (Kanebo, 1992; Banu and Bari, 2007), adventitious buds (Pattnaik et al., 1995), leaves and internodal stem segments (Makri and Kintzios, 1999), young inflorescence (Singh and Sehgal, 1999) and axillary buds (Begum et al., 2000, 2002) have been used for plant propagation. Bicca Dode et al. (2003), reported the highest efficiency of shoot formation using cotyledonary leaf, obtained in MS medium containing 5 $mg.l^{-1}$ BAP and 0,2 $mg.l^{-1}$ NAA. In a different study, Banu and Bari (2007), showed that among the different concentrations and combinations of growth regulators, the highest percentage of shoot formation (90%) and the highest average number of shoots (5.88%) were observed in 0.2$mg.l^{-1}$ BAP from shoot tip explants. This study was aimed at identifying the best type of explants obtained from *in vitro* grown seedlings of sweet basil as well as the most efficient growth regulator concentration and combinations for shoot formation and regeneration.

Results

Seed germination

Seeds of *Ocimum basilicum* were germinated on MS medium without any growth regulators two days after inoculation.

Evaluation of the explants type effect on adventitious bud induction

Adventitious bud induction was observed after three weeks of culture initiation (Figure 1 a–d). Multiple shoots were initiated from all of the explants after 4 weeks of culture. The type of explant and BAP dosage influenced the percentage of shoot formation. Explants planted on MS without BAP (control) did not respond. The highest frequency of shoot bud induction was observed in cotyledon explants followed by the nodal and hypocotyls ones (Table 1). As the BAP concentration in the medium increased, the shoot regeneration and the number of produced shoots increased. The maximum shoot regeneration (93.33%) and shoot number (10.53 shoots per explant) were obtained from cotyledon segments on media supplemented with 10 µM BAP (Table 1). None of the explants responded to hormone free medium.

Adventitious bud induction by different hormonal combinations

There was a large difference in the response of nodal segments to the different media used. These differences were dependent on BAP concentration alone or in combination with IAA. Nodal explants placed on hormone-free media did not respond and did not produce any adventitious bud (Table 2). These results showed that BAP free medium was unfavorable for shoot multiplication. The concentration of BAP alone or in combination with IAA had a significant effect on the number of shoots produced from nodal segments (Table 2). Comparison of the different culture media for shoot formation revealed that the culture media containing 11 µM BAP gave the best results for shoot regeneration. The lowest adventitious bud induction frequency from nodal explants (43.33%) was induced by 33µM BAP. Moreover, the concentration of BAP affected the average number of shoots per regenerating nodal segments. Results showed that 11 µM BAP + 0 µM IAA produced the maximum average number of shoots. Apart from IAA concentration, shoot regeneration and average number of shoots decreased as the BAP concentration in the culture medium increased.

Vitrification

Results showed that increasing the BAP concentration in media induced vitrification in regenerated shoots. In the first experiment, this disorder was recorded only in cotyledon explant and the highest vitrification (43.33%) was observed on the medium containing 10 µM BAP (Table 1).

Rooting

Our results showed that in all media, where the shoots were inoculated, root formation was achieved. Root primordial emerged from the shoot based on first week of culture on hormone free medium or medium supplemented with different concentrations of BAP and IAA. In the first experiment, different explant types showed different responses to increasing BAP in the culture medium. In cotyledon and nodal explants, root formation decreased as the BAP concentration increased. Whereas in hypocotyle explant, rooting percentage increased by increasing of BAP concentration in culture medium (Table 1). In the second experiment, the highest root formation was observed in the culture media containing 2.85 µM IAA. Similarly in the second experiment, increasing BAP concentration decreased rooting (Table 2). Maximum number of roots (10) was formed on 2.85 µM IAA, 0.57 µM IAA and hormone free medium, (Figure1F).

Discussion

The explants source has been proved to be an important factor for *in vitro* growth and development of plant species, affecting callus induction and adventitious bud induction as well as shoot regeneration. Statistical analysis revealed that there were significant differences between the three tested explants in the present study. Cotyledonary explants produced maximum shoots per explant and per media. Bicca Dode et al. (2003) obtained a high rate of shoot regeneration per explant (66.7%) and a higher number of shoots per explant (3.46) from cotyledon leaves of *Ocimum basilicum* L. grown in MS culture medium supplemented with 5 mg/l^{-1} BAP + 0.2 mg/ l^{-1} NAA. Kantia and Kothari, (2002) reported adventitious shoot bud formation achieved directly on the surface of the leaf explants in *Dianthus chinesis*. The different responses of the explant types are probably due to the endogenous hormonal balance in plant tissues (Grattapaglia and Machado, 1998). These differences in the three explants can be explained by changes in the levels of endogenous hormones and the expression of genes encoding hormone receptors, as proposed by Close and Gallagher-Ludeman, (1989). On the other hand, BAP concentration had a significant effect on shoot formation. In another word, shoot formation increased as BAP concentration in culture medium increased. Depending on species or cultivars, the most important achievement obtained in the propagation of many plant materials through tissue cultures has been frequently based on the successful adjustment of the type and combination of plant growth regulators (Tran, 1981; Murashige, 1990; Gürel and Gürel, 1996). Elizabeth et al. (2008) found that maximum average number of shoots (8.9±1.3) was observed on the medium containing 8.87 µM BAP and 4.83 µM NAA in the 'white Albatross' varieties of chrysanthemum. It has been shown a significantly larger average number of orange mint leaf disks regenerated shoots on basal medium containing 44.4 µM benzyladenine (BA) and 250mll^{-1} coconut water (CW) (Van Eck and Kitto, 1992). In the present study, the concentration of BAP alone or in combination with IAA had a significant effect on the number of shoots produced from nodal segments. These results confirmed the positive effect of hormones on adventitious bud induction. The cytokinin type and concentration are key factors for successful *in vitro* multiplication. According to Grattapaglia and Machado, (1998), the cytokinin 6-benzylaminopurine and KIN are very effective in promoting proliferation. Cytokinins participate in the regulation of many plant processes that induce callus cell division in the presence of auxin, leading to bud or root formation directly on the explant or from calli, (Taiz and Zeiger, 2004). According to Galiba et al. (1986), a polygenic system may be involved in *in vitro* regeneration. Therefore, their results indicated that the presence of cytokinin in the culture medium might have been essential for shoot development in Basil. Cytokinins play a primary role in cell division and also break the apical dominance and influence shoot induction and growth (Preece, 1995). Our results are consistent with those of the previous reports regarding the positive effects of BAP on shoot regeneration (Laskar et al.,

Table 1. Effect of different explant type and BAP combination on multiple shoot induction in *Ocimum basilicum* L.

Explants	BAP concentration (μM)	Shoot regeneration (%)	Average number of shoots	Vitrification	Root formation (%)
Cotyledon	0	0f	0e	0c	90a
	1	20de	3.3c	0c	60cd
	5	60b	6.03b	3.33c	40e
	10	93.33a	10.53a	43.33a	33.33ef
Nodal	0	0f	0e	0c	86.67ab
	1	16.67e	1.3d	0c	76.67ab
	5	36.67c	3.93c	0c	73.33bc
	10	56.67b	5.86b	0c	56.67d
Hypocotyl	0	0f	0e	0c	3.33h
	1	13.33e	0.16e	0c	16.67gh
	5	20de	0.23e	0c	23.33fg
	10	30cd	0.36e	0c	36.67ef

* Means in each column followed by the same letters are not significantly different at 5% level using DMRT

Fig 1. Effect of PGRs on shoot formation, Vitrification and Root induction of *Ocimum basilicum* L. A) Nodal explants of *ocimum basilicum* after three weeks, B,C) Early shoot regeneration from nodal explants after 4 weeks, D) Vitrificated shoots on 22 μM BAP medium, E) Regenerated normal shoots on MS medium supplemented with 11 μM BAP and 0.57 μM IAA, (F) Root induction after transfer of *Ocimum basilicum* L. regenerated shoot to MS basal medium without PGRs.

2005; Bicca Dode et al., 2003).Moreover, addition of growth regulators such as auxins to the culture is extremely important, since they are able to start cell division and control the growth processes and cell elongation. According to Mendoza and Kaeppler, (2002), the use of auxin in combination with cytokinin, leads to rapid cell division, forming a large number of relatively small and undifferentiated cells. In the present study, the highest average number of shoots per explants was obtained when the nodal segments were grown in culture medium with 11 μM BAP + 0 μM IAA. Narayanaswamy (1977) reported that the toxicity caused by an excess of growth regulators in the culture medium, or the extended period of time in which the culture was exposed to them, might lead to genetic, physiological and morphological changes, resulting in a reduction of the proliferation rate *in vitro*. The decreasing of shoot formation because of increasing BAP concentration observed in the second experiment may be related to the toxicity of BAP at higher concentrations. Concerning root formation, capacity of different media analysis showed that BAP free medium was the superior treatment. According to Barceló Coll et al. (1988), shoots are sites of intense auxin production, which when translocated to the stem base, stimulate rooting. Nevertheless, the quality of shoots at the propagation stage generally determines the success of rooting (Grattapaglia and Machado, 1998). Vitrification is a morphological and physiological disorder frequently affecting both herbaceous and woody plants during *in vitro* vegetative regeneration (Leshem, 1983; Meira et al., 1983). The role of growth factor imbalance as an inducer of vitrification has been discussed. It has been shown that, in carnation, high concentration of NAA in the culture medium increases the proportion of shoots that turn into vitrified plantlets, while BAP has the opposite effect (Leshem et al., 1988). Kevers et al. (1987) have found that BAP availability in the culture medium induces vitrification in apple. In the present study, posiive relationships between increasing the

Table 2. Effect of different combination of BAP and IAA hormones in MS medium for multiple shoot induction from nodal explants of *Ocimum basilicum* L.

Culture medium	Shoot regeneration (%)	Average number of shoots	Vitrification	Root formation (%)
Conrol	0±0e	0±0f	0±0f	86.67±0abc
0.57 µM IAA	0±0e	0±0f	0±0f	90±0ab
2.85 µM IAA	0±0e	0±0f	0±0f	100±0a
11 µM BAP	96.67±0.33a	5.6±1.15a	13.33±1.33e	76.67±1.20bc
11 µM BAP+0.57 µM IAA	83.33±0.57b	4.1±2.08bc	33.33±0.57d	53.3±0.66d
11 µM BAP+2.85 µM IAA	80±0.88b	4.6±5.03b	10±0.57ef	83.3±0.33abc
22 µM BAP	50±0.88d	3.4±9.82cd	50±1.15bc	36.67.3±0.66e
22 µM BAP+0.57 µM IAA	76.67±0.66bc	3.33±3.60cd	43.3±1.20cd	36.67±0.33e
22 µM BAP+2.85 µM IAA	73.33±0.57bc	3.6±2.84cd	38±0.57cd	70±0.57c
33 µM BAP	43.33±1.20d	2.33±5.84e	75.33±0.57a	13.33±0.66f
33 µM BAP+0.57 µM IAA	66.67±0.33c	2.8±3.52de	70.33±0.33a	23.33±1.33ef
33 µM BAP+2.85 µM IAA	53.33±0.88d	2.7±4.91de	55.5±0.33b	36.67±0.33e

*Means in each column followed by the same letters are not significantly different at 5% level using DMRT

BAP level in culture media and vitrification of regenerated shoots were observed. The lowest and the highest vitrification were achieved in media containing 0 and 33 µm BAP, respectively. High BAP levels caused the shoots to turn greenish-yellow with some vitrification in regenerated shoots. However, when low concentration of BAP was used, the shoots remained green and healthy, and no vitrification was observed. This may indicate that vitrification can be reduced by lowering BAP doses in the culture medium as it has already been reported that high BAP levels cause vitrification in several plant species (Constantine, 1986; Hussey, 1986). During vitrification or hyperhydricity, some shoots developed *in vitro* appeared brittle, glassy and water-soaked. In many species, vitrification may be represented by symptoms not visible to the naked eye, e.g., poorly developed vascular bundles, abnormal wax quality, abnormal functioning stomata, etc. As a consequence, vitrification is the consequence of culture conditions, and leads to losses of plantlets (Genkov and Ivanova, 1995).

Material and methods

Plant material

Seeds of *Ocimum basilicum* cv. Hamadany were obtained from the gene bank of Agriculture Research Center of West Azerbaijan, Urmia, Iran. The seeds were first sterilized by soaking in a solution of 70% (v/v) ethanol for one minute and 5% (v/v) sodium hypochlorite for 10 minutes followed by rinsing three times with sterile distilled water. These surface-sterilized seeds germinated on MS (Murashige and Skoog, 1962) medium supplemented with 3% (v/v) sucrose, 100 $mg.l^{-1}$ myo-inosytol, and 2 $mg.l^{-1}$ glycine. Media were solidified with 0.7% agar (Duchefa, Netherlands) and their pH was adjusted at 5.8±1 before autoclaving for 15 minutes at 121°C. Moreover, 25 seeds per flask and 20 flasks in each experiment for explants preparation were cultured. The cultures were kept in a growth chamber at 24±2°C under 16-hour photoperiod at 50 $\mu mol^{-2}s^{-1}$ irradiance provided by cool white fluorescent tube (Philips, India) with 60-65% relative humidity. Two sets of experiments were carried out on shoot induction and proliferation. Seeds were germinated after one week of culturing with normal roots, shoots and leaves.

Evaluation of the effect of different explants on adventitious bud induction

In the first experiment, three types of explants including nodal segments, hypocotyls and cotyledonary leaves were used. Explants from *in vitro* grown seedlings were inoculated on MS media (Duchefa, Nederland) with B5 vitamins, supplemented with 30 gl^{-1} sucrose and different concentrations of BAP (0, 1, 5 and 10 µM). The pH of all media was adjusted at 5.8 prior to the addition of plant agar (Duchefa, Nederland) and were autoclaved at 121°C for 15 minutes. Cultures were then incubated at 24±2°C under 16-h photoperiod at 50 $\mu mol^{-2}s^{-1}$ irradiance using cool white fluorescent lights. The induced shoots were subcultured onto fresh media every two weeks for 8 weeks.

Evaluation of the effect of different hormonal combinations on adventitious bud induction

Nodal segments (5-10 mm) from two week-old seedlings germinated *in vitro* were excised and used as explants. MS basal media (Duchefa, Netherlands), containing B5 vitamins, 30 g/l^{-1} sucrose was supplemented with 6-benzylaminopurine (BAP) at concentration of 0, 11, 22 and 33 µM alone or in combination with IAA (0, 0.57 and 2.85 µM) and prepared in Petri plates (90 x 20 mm containing 20 ml medium) for multiple adventitious bud induction. The pH of all media was adjusted at 5.8 prior to the addition of $7gl^{-1}$ plant agar (Duchefa, Netherlands) and the plates were finally autoclaved at 121°C for 20 minutes. The induced shoots were subcultured onto fresh media once every two weeks for 8 weeks. The culturing conditions were the same as above. Cultures were evaluated 8 weeks after inoculation for the mean number of shoots, roots and vitrification.

Root development of in vitro propagated shoots

After 3 to 4 weeks, when regenerated shoots were as long as more than 4 cm, they were separated and transferred into MS basal medium with or without IAA.

Statistical analyses

All experiments were set up in a factorial experiment based on completely randomized design. Three replicates per treatment with 10 explants for each replicate were used. The number of explants with adventitious buds and the number of shoots per explants, as well as vitrification and rooting percentages were calculated. Data were subjected to Analysis of Variance (ANOVA) for testing the differences among treatments using MATATC software and Duncan's multiple range test (DMRT). After rooting of all regenerated shoots, the plants were transferred to pots containing garden soil and grown in a greenhouse in order to grow into normal plants.

Conclusion

In this study, a simple and reliable regeneration protocol has been presented. The highest frequency of shoot bud induction was observed in cotyledon explants followed by nodal and hypocotyls. The maximum shoot regeneration and shoot number were obtained on media with 10 µM BAP. This protocol can be found very advantageous for a variety of purposes, including mass multiplication of ocimum species, medicinal plant breeding studies and transgenic plant production. These results showed that high concentrations of BAP can increase vitrification in *in vitro* regenerated shoots.

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