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 purposes is the plant to plant variability, mainly due to genetic and biochemical heterogeneity.

Introduction

Sweet basil (*Ocimum 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, antihelminitic, 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 *Ocimum* 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., 1992; Banu and Bari, 2007) 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 and IAA 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 hypocotyl 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 BAP + 0.2 mg/l NAA. Kantia and Kothari, (2002) reported adventitious shoot bud formation achieved directly on the surface of the leaf explants in Dianthus chinensis. 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 250mM1/3 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.

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

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

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, positive relationships between increasing the...
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-inositol, 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 used. The cultures were kept in a growth chamber at 24±2°C under 16-hour photoperiod at 50 μmol m⁻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 plant agar (Duchefa, Netherlands) and were autoclaved at 121°C for 15 minutes. Cultures were then incubated at 24±2°C under 16-h photoperiod at 50 μmol m⁻² s⁻¹ irradiance using cool white fluorescent lights. 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.

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.

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Shoot regeneration (%)</th>
<th>Average number of shoots</th>
<th>Vitrification</th>
<th>Root formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conrol</td>
<td>0±0e</td>
<td>0±0f</td>
<td>0±0f</td>
<td>86.67±2abc</td>
</tr>
<tr>
<td>0.57 μM IAA</td>
<td>0±0e</td>
<td>0±0f</td>
<td>0±0f</td>
<td>90±0ab</td>
</tr>
<tr>
<td>2.85 μM IAA</td>
<td>0±0e</td>
<td>0±0f</td>
<td>0±0f</td>
<td>100±0a</td>
</tr>
<tr>
<td>11 μM BAP</td>
<td>96.67±0.33a</td>
<td>5.6±1.15a</td>
<td>13.3±1.33c</td>
<td>76.67±1.20bc</td>
</tr>
<tr>
<td>11 μM BAP+0.57 μM IAA</td>
<td>83.3±0.57b</td>
<td>4.1±2.08bc</td>
<td>33.3±0.57d</td>
<td>53.3±0.66d</td>
</tr>
<tr>
<td>11 μM BAP+2.85 μM IAA</td>
<td>80±0.88b</td>
<td>4.6±0.35b</td>
<td>10±0.57ef</td>
<td>83.3±0.33 abc</td>
</tr>
<tr>
<td>22 μM BAP</td>
<td>50±0.88d</td>
<td>3.4±1.22cd</td>
<td>50±1.15bc</td>
<td>36.67±0.66bc</td>
</tr>
<tr>
<td>22 μM BAP+0.57 μM IAA</td>
<td>76.67±0.66bc</td>
<td>3.3±3.60cd</td>
<td>43.3±1.20cd</td>
<td>36.67±0.33e</td>
</tr>
<tr>
<td>22 μM BAP+2.85 μM IAA</td>
<td>73.3±0.57bc</td>
<td>3.6±2.84cd</td>
<td>38±0.57cd</td>
<td>70±0.57c</td>
</tr>
<tr>
<td>33 μM BAP</td>
<td>43.3±1.20d</td>
<td>2.3±5.84e</td>
<td>75.3±0.57a</td>
<td>13.3±0.66f</td>
</tr>
<tr>
<td>33 μM BAP+0.57 μM IAA</td>
<td>66.67±0.33c</td>
<td>2.8±3.52de</td>
<td>70.3±0.33a</td>
<td>23.3±1.33f</td>
</tr>
<tr>
<td>33 μM BAP+2.85 μM IAA</td>
<td>53.3±0.88d</td>
<td>2.7±4.91de</td>
<td>55±4.33b</td>
<td>36.67±0.33e</td>
</tr>
</tbody>
</table>

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


Conclusions

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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References


