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Scientia Horticulturae 86 (2000) 81–88
SCIENTIA HORTICULTURÆ
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Efficient plant regeneration from multiple shoots formed in the leaf-derived callus of *Lavandula vera*, using the "open culture system"

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Accepted 8 February 2000

Abstract

To develop an efficient procedure for plant regeneration from leaf-derived callus of *Lavandula vera* DC, the production of multiple shoots and the formation of roots from these shoots were studied. When calli were cultured in a medium with 4.0×10^{-7} M *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU), urea-type cytokinin, multiple shoots were obtained from the greenish surface of the callus efficiently at a rate of 52.2%. For multiple shoot development, the shoots were divided into 7–20 shoot clusters with 2–3 shoots and subcultured every 2 weeks. Two modified culture-systems were employed for subculture; a "closed culture system" and an "open culture system". In the open-system, shoot clusters grew and elongated vigorously. In the closed-system, most of the clusters showed vitrification and did not elongate. In root induction culture, shoot clusters in the open-system were well rooted and grew vigorously, the highest rate of root formation being 74.0%, in the $\frac{1}{2}$ MS medium supplemented with 1.0×10^{-6} M indoleacetic acid (IAA). On the other hand, shoot clusters in the closed-system showed vitrification and did not root well. About seven times more regenerants were obtained from the multiple shoots than from normal shoots treated with 4.4×10^{-6} M 6-benzylamino purine (BA), a purine-type cytokinin. These results indicate that efficient plant regeneration in *L. vera* from callus using multiple shoots is possible by means of an open-system. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Multiple shoot; Open culture system; CPPU

Abbreviations: BA, 6-benzylamino purine; CPPU, *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indoleacetic acid; i^6Ade, N^6-(Δ^2-isopentenyl) adenine; IBA, 3-indolebutanoic acid; MS, Murashige and Skoog medium; NAA, 1-naphthaleneacetic acid; TDZ, thidiazuron

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PII: S0304-4238(00)00140-0

1. Introduction

The use of multiple shoots in mass propagation is now standard for several plants, e.g. strawberry (Yoshihara and Hanyu, 1992), sweet potato (Gosukonda et al., 1995), and carnation (Roest and Bokelmann, 1981).

Lavandula sp. (lavender) is one of the most important aromatic plants and is useful for perfumes, cosmetics and medicines. Several *Lavandula* species are cultivated worldwide. They are propagated vegetatively (e.g. by stem cutting) and have a low propagation rate because of their poor rooting ability (Gras and Calvo, 1996).

Tissue culture techniques have been applied to *Lavandula* plants. Direct shoot formation from different tissue-explants of *L. latifolia* Med. (Calvo and Segura, 1989a; Gras and Calvo, 1996), and shoot formation from callus of *L. latifolia* (Calvo and Segura, 1988, 1989b; Jordan et al., 1990), *L. angustifolia* Mill. (Quazi, 1980) and Lavandin (*L. officinalis*×*L. latifolia*) (Panizza and Tognoni, 1988) were reported. Also in *L. vera* DC, we observed normal shoot formation from leaf-derived callus cultured in medium supplemented with 4.4×10^{-6} M 6-benzylamino purine (BA), a purine-type cytokinin, and succeeded in root induction and plant regeneration. On the other hand, we found multiple shoot formation on culture in medium with thidiazuron (TDZ) and *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU), an urea-type cytokinin, although all of the multiple shoots failed to develop and died during rooting culture (Tsuro et al., 1999). Multiple shoots have about 20–40 adventitious shoots, so, many shoots can easily be obtained by division. Therefore, the use of multiple shoots is essential for efficient plant regeneration.

In this experiment, in order to establish a method for the efficient regeneration of plants from leaf-derived calli of *L. vera*, we tried to use multiple shoots and modified open and closed culture systems.

2. Materials and methods

2.1. Callus induction

Lateral branches with fully developed leaves, about 5 cm in length, were excised in May and June from *L. vera* in a greenhouse at 25–30°C with natural daylength and light intensity (about 500–700 μmol^{-2} s^{-1}). They were immersed in 70% ethanol for 1 min, and soaked in sodium hypochloride solution (about 2% active chlorine) for 20 min for sterilization. They were then washed three times with sterilized distilled water. After sterilization, the leaves were detached from the branches and cut into small segments 3 mm×5 mm long. They were placed on 10 ml of Murashige and Skoog (MS) (1962) basal medium containing 3%

sucrose and 0.3% gellan gum, supplemented with 1.0×10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0×10^{-6} M kinetin in a test tube (Ø 18 mm×L 180 mm), and cultured in the dark at 25°C for 40 days. The medium was adjusted to pH 5.8 and autoclaved at 121°C for 20 min.

2.2. Induction and development of multiple shoots

For multiple shoot induction, calli (about 7 mm×7 mm in size) were transferred to MS basal medium supplemented with 2.3×10^{-6}, 4.5×10^{-6} and 2.3×10^{-5} M TDZ and 2.0×10^{-7}, 4.0×10^{-7}, 2.0×10^{-6} and 4.0×10^{-6} M CPPU. At the same time, calli were cultured on MS basal medium supplemented with 4.4×10^{-6} M BA. The culture was performed under continuous cool-white fluorescent light (70 μmol^{-2} s^{-1}) at 25°C. Fifty calli were cultured for multiple shoot induction and the experiments were replicated six times. After 10 weeks, the numbers of multiple and normal shoots were counted and shoot formation rates, number of shoots or multiple shoots obtained/number of cultured calli were determined. Shoot formation rates were analyzed statistically by Fisher's exact probability test ($p<0.05$), using StatView Ver. 4.0 (Sato and Konishi, 1994).

Multiple shoots obtained in medium with CPPU of 4.0×10^{-7} M were transferred to 50 ml of the same medium for multiple shoot induction in 200 ml culture flasks and cultured under continuous light (70 μmol^{-2} s^{-1}) at 25°C. After 2 weeks, propagated multiple shoots (about 3–3.5 cm in diameter) were divided into 7–20 shoot clusters with 2–3 shoots (about 7 mm×7 mm in size). Then, they were subcultured on the medium used for multiple shoot induction every 2 weeks till shoots grew to 1 cm in height. Two modified culture systems were employed in subculture. One was a "closed culture system", where the culture flask was sealed with aluminum-foil, and the other was an "open culture system", where the flask was sealed with aluminum-foil having a small hole (7 mm×7 mm) covered with a membrane filter (0.25 μm pore size, Iwaki, Japan). In the open-system, gas and vapor generated in the flask could be exchanged through the membrane filter. Subculture conditions were the same as for multiple shoot induction.

2.3. Root induction and acclimatization

Shoot clusters with a shoot 1 cm or longer were transferred to root induction medium: $\frac{1}{2}$ MS solid medium (MS basal medium with macro nutrients at half-strength) supplemented with or without 1.0×10^{-6} M of indoleacetic acid (IAA), 1.0×10^{-6} M of 1-naphthaleneacetic acid (NAA) and 1.0×10^{-6} M of 3-indolebutanoic acid (IBA), respectively. The medium with or without NAA and IBA was sterilized by autoclaving. Only IAA was filter-sterilized and added to the autoclaved medium just before gelling. Root induction culture was carried out

under cool-white fluorescent light (70 μmol^{-2} s^{-1}) at 25°C. Both closed- and open-systems were also employed in root induction culture. After 4 weeks, the number of shoots having roots over 1 cm in length was counted, and the root formation rate was calculated. Experiments on root induction was carried out six times.

An analysis of variance (ANOVA) (SAS Institute, 1990) was conducted for the root formation rate in each medium.

For acclimatization, regenerated shoots having roots were transplanted to vermiculite moistened with Hyponex® solution ($\times\frac{1}{2000}$) in top-sealed plastic bottles. The humidity in the bottle was decreased by opening the seals within 2 weeks. After acclimatization, plantlets were transferred to soil in pots and maintained in a greenhouse at 25–30°C with natural daylength and light intensity (about 500–700 μmol m^{-2} s^{-1}).

3. Results and discussion

3.1. Induction and development of multiple shoots in the two culture systems

Leaf-derived calli showed two morphogenic features, resulting from the culture with purine- and urea-type cytokinin in shoot induction. In BA, normal shoots were differentiated from green spots, which appeared on the surface of the calli. On the other hand, only multiple shoots were differentiated from the greenish surface of calli cultured in medium with TDZ and CPPU. Table 1 shows the shoot

Table 1
Effect of different types of cytokinin on shoot formation

Cytokinin[a]	Concentration (M)	Number of calli cultured	Number of calli with shoot(s)	Shoot formation rate (%)
BA	4.4×10^{-6}	299	169	56.5 a[b]
TDZ	2.3×10^{-6}	210	7	3.3 d
	4.5×10^{-6}	300	98	32.7 b
	2.3×10^{-5}	300	72	24.0 b
CPPU	2.0×10^{-7}	209	5	2.4 d
	4.0×10^{-7}	295	154	52.2 a
	2.0×10^{-6}	300	31	10.3 c
	4.0×10^{-6}	300	12	4.0 d

[a] Only normal shoot was differentiated in BA, and multiple shoot was differentiated in TDZ and CPPU.

[b] Different letters within a column represent significant differences at the 5% level by Fisher's exact probability test.

formation rates in different cytokinins after 10 weeks of culture. The maximum normal shoot formation percentage (56.5) was observed in BA 4.4×10^{-6} M. A significantly higher percentage for multiple shoots, 52.2%, was found in CPPU 4.0×10^{-7} M. Urea-type cytokinin has much higher activity for shoot multiplication than purine-type cytokinin. In the shoot induction culture of broccoli, Mok et al. (1987) showed that multiple shoots were formed by TDZ, while normal shoots were differentiated on application of N^6-(Δ^2-isopentenyl) adenine (i^6 Ade) as a purine-type cytokinin. Furthermore, in the shoot primordia culture of *Rhododendron pentaphyllum* Maxim., multiple shoots were found in the medium with 0.1 μM CPPU, whereas only one adventitious shoot was differentiated in medium with zeatin, a purine-type cytokinin, at a concentration of 1 μM (Yoshizawa et al., 1998). These results suggested that the feature of shoots differentiation might depend on the type of cytokinin.

Shoots obtained in BA were easily rooted in the subculture medium with or without 1.0×10^{-6} M IBA and most were successfully acclimatized and transplanted to pots in a greenhouse. However, multiple shoots formed in TDZ or CPPU could not root and died during the subculture for root induction. To establish rooting conditions for multiple shoots, we tried two modified culture systems, closed and open. Multiple shoots obtained from the medium with 4.0×10^{-7} M of CPPU were used in subsequent experiments. For multiple shoot development, fully propagated multiple shoots (about 3–3.5 cm in diameter) were divided into 7–20 shoot clusters with 2–3 shoots (about 7 mm×7 mm in size), and subcultured in the medium used for multiple shoot induction. When shoot clusters were cultured in the closed-system, they became vitrified and had little elongation. On the other hand, in the open-system, vitrification did not occur and shoots grew vigorously. After 1 month of subculture, a difference of multiple shoot-development was observed in the closed- and open-system. In shoot culture of *Magnolia soulangeana* Soul., a higher level of CO_2 was detected in the hermetically closed flasks, and the growth rate of shoot was reduced (Proft et al., 1985). Dalton and Street (1976) concluded that CO_2 accumulation (>5%) in the tightly closed flask was responsible for the reduction in growth of cells in *Spinacia oleracea* L. The results suggested that efficient CO_2 reduction in the flask was essential for the development of the shoot. Additionally, in the open-system, no condensation was formed in the flask. In the culture of *Mentha piperita* L., Sato et al. (1994) recognized that vitrified multiple shoots began to develop vigorously on "Milli Seal" sealing with culture vessels. Thus, a membrane filter might be available for efficient gas exchange and reducing the internal humidity in the culture flask. Furthermore, shoot clusters divided from multiple shoots, developed more vigorously than the non-divided multiple shoot (data not shown). From the results mentioned above, we suggested that both dividing multiple shoots and employing an open-system were necessary for the vigorous development of multiple shoots.

3.2. Root induction and acclimatization

For root induction, shoot clusters having a shoot over 1 cm in length were transferred to the root induction medium. Closed- and open-systems were also employed in root induction culture. Root differentiation was observed after about 2.5–3 weeks of culture. When shoot clusters were cultured in the closed-system, they showed vitrification and only 1–5 roots per shoot, which looked like fine threads, were differentiated. On the other hand, in the open-system, shoot clusters were not vitrified and many roots were differentiated and elongated vigorously. Fig. 1 shows the root formation rates after 4 weeks of root induction culture. The open-system has significantly higher rates than the closed-system, regardless of the plant growth regulators. In the medium with IAA in the open-system, the maximum rate of root formation was 74.0%. These results suggested that the open-system was effective for not only the development of multiple shoots but also root induction.

For acclimatization, regenerated shoots having roots over 1 cm in length were transferred to vermiculite moistened with Hyponex® solution ($\times\frac{1}{2000}$) in top-sealed plastic bottles. Acclimatization was carried out for 2 weeks. More than 95.0% of regenerants obtained from the open-system were acclimatized and continued to grow vigorously. On the other hand, about two-thirds of the regenerants obtained from the closed-system did not survive and died during acclimatization. After acclimatization, regenerated plantlets were transferred to soil in pots and they continued to grow actively in a greenhouse. Table 2 shows the plant regeneration rates obtained from normal shoots using the closed-system

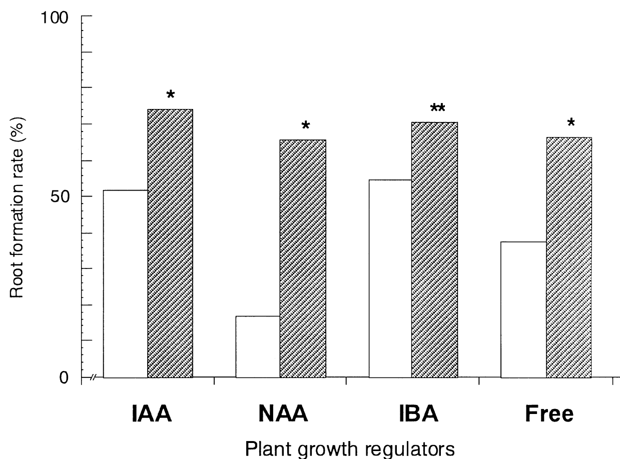

Fig. 1. Root formation rates in the closed- and open-system. (Empty region), closed-system; (shaded region), open-system. ☆ and ☆☆ within a growth regulator indicate significantly different by ANOVA at the 0.01 and 0.1% level.

Table 2
Plant regeneration rates in normal shoot and multiple shoot culture

Shoot induction medium	No. of Calli induced	No. of calli forming shoot	No. of shoot clusters divided from multiple shoots[a]	No. of shoots forming root[b]	No. of regenerants obtained	Plant regeneration rate[c]
4.4×10^{-6} M BA	299	169	–	165	165	0.55
4.0×10^{-7} M CPPU	295	154	1642	1215	1172	3.97

[a] Multiple shoots induced by 4.0×10^{-7} M of CPPU were divided into 7–20 shoot clusters, and subcultured in the open-system.

[b] Normal shoots induced by BA were cultured in medium with 1.0×10^{-6} M of IBA in the closed-system, and shoot clusters from multiple shoots were cultured in 1.0×10^{-6} M of IAA in the open-system.

[c] No. of regenerated plantlets obtained/No. of calli cultured.

and multiple shoots using the open-system. When calli were cultured in the normal shoot culture series, using 4.4×10^{-6} M BA and 1.0×10^{-6} M IBA in the closed-system, 165 regenerated plantlets were obtained from 299 calli. In the multiple shoot culture series, using 4.0×10^{-7} M CPPU and 1.0×10^{-6} M IAA and the open-system, 1172 plantlets were regenerated from 295 calli. Consequently, the plant regeneration rate was seven times higher in the multiple than normal shoot culture series.

In this study, we achieved the efficient plant regeneration from leaf-derived callus of *L. vera* using multiple shoots and an open-culture system. All the regenerated plantlets obtained in this experiment have been transferred to the field to evaluate growth habits and morphological characteristics.

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