Effect of benzyladenine and indolebutyric acid on ultrastructure, glands formation, and essential oil accumulation in *Lavandula dentata* plantlets

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Abstract

Lavandin (*Lavandula dentata*) axillary buds were grown in Linsmaier-Skoog (LS) medium solidified with 10 % bactoagar (control) and supplemented with 0.1 mg dm^{-3} benzyladenine (BA), 0.1 mg dm^{-3} indolebutyric acid (IBA) or both plant growth regulators. In the studied conditions the axillary buds developed into plantlets. The addition of BA inhibited the formation of glands by 44 % as compared with the control plantlets and also inhibited their development: these plantlets had the highest number of unbroken glands (in pre-secretory state) when compared with plantlets grown in the other conditions. The presence of BA stimulated chloroplast formation, and increased the content of essential oils by 150 % with respect to the control plantlets. It also increased their secretion, and the number of lipid droplets in the chloroplasts, cytosol and plasmalemma. On the contrary, the presence of IBA decreased the essential oil concentration in plantlets by 31 % when compared with the control ones and inhibited their secretion capacity.

Additional key words: axillary buds, chloroplasts, growth regulators, lavandin.

Introduction

Lavandula species are of great interest due to their content of essential oils, which are important in the perfume, cosmetic, flavouring and pharmaceutical industries. These plant species can be vegetatively propagated from woody stem cuttings, but this process is subject to great variations. Grown from seeds, the plants also exhibit very high variation in growth rate and essential oil production. The use of *in vitro* propagation of efficient clones has been investigated (Jordan *et al.* 1998). Among the existing techniques, enhanced axillary-branching culture has become an important method for the rapid and large-scale propagation of several plant species (Quazi 1980).

The essential oils of *L. dentata* mainly consist of monoterpenes and sesquiterpenes, which are synthesized in the chloroplast and cytosol, respectively (Suga and Endo 1991, Croteau 1984). These two terpenes can be secreted from the producing cells and combined in intercellular spaces. They are mainly formed and stored in specialized glands, which are abundant on the leaf surface (Bosabalidis and Tsekos 1982, Croteau 1981). Increased essential oil content could be the result of the enhanced biosynthesis of these compounds or reduced catabolism.

The presence of plant growth regulators in the culture medium controls several morphogenic responses, such as the glandular density of the leaf surface, as well as certain histological changes: cell volume (depending on the water content), the vascular system differentiation, and the trichome formation (Davies 1987). Metabolic responses, such as the amount and composition of essential oils are also dependent on the auxin/cytokinin ratio (Charlwood *et al.* 1989), which stimulates or inhibits certain enzymes responsible for the different steps of terpenoid biosynthesis.

In this paper we describe the effect of cytokinin (BA), or auxin (IBA) or both together, on growth, morphology, density of glands, and essential oil production in *Lavandula dentata* plantlets.

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Abbreviations: BA - benzyladenine; IBA - indolebutyric acid; LS medium - Linsmaier-Skoog medium; SEM - scanning electron microscopy; TEM - transmission electron microscopy.

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Materials and methods

Micropropagation of apical shoots: Axillary buds (1 mm) were excised in the prefloral state from one-year old *Lavandula dentata* L. plants cultured in the Mediterranean region. They were surface-sterilised by immersion in 50 % ethanol for 1 min and in 20 % sodium hypochlorite for 15 min, followed by 5 rinses in sterile water.

The explants were placed in sterile conditions, in a vertical position on a paper bridge, in tubes (150 × 25 mm) containing 10 cm^3 of Linsmaier and Skoog (1965; LS) liquid medium. They were grown for 30 d in a growth chamber at a temperature of 25 °C. Cool "white light" (irradiance 10 W m^{-2}) was provided by *Philips TL W/33 RS* fluorescent lamps for 16 h per day. Replications were made in *Magenta* flasks on the same LS medium solidified with 10 % bactoagar (control), and on this medium supplemented with 0.1 mg dm^{-3} BA, or 0.1 mg dm^{-3} IBA, or 0.1 mg dm^{-3} IBA and 0.1 mg dm^{-3} BA together. Bajaj *et al.* (1988) showed that IBA facilitates the micropropagation and essential oil formation in a large quantity of aromatic plants. We had found previously that concentrations of IBA and/or BA lower than 0.1 mg dm^{-3} had no demonstrable effects on the morphology of *Lavandula* plantlets, but higher concentrations of BA frequently caused vitrification of the plantlets.

Explants grown on the different media were subcultured every 8 weeks. Plantlet length and fresh and dry masses were determined every week for a 8-week period.

TEM and SEM: After 35 d of culture, a samples of leaves from the fourth internode was taken from each medium and fixed in 45 % glutaraldehyde in 0.1 M cacodylate acetate buffer (pH 7.2) for 3 h at room temperature. After 10 min rinses with buffer solution, samples were postfixed in 2 % osmium tetroxide in the same buffer for 1 h and rinsed three times with distilled water. The samples were dehydrated in a graded acetone series, infiltrated and embedded in freshly prepared epoxy resin (Spurr 1969), and polymerized at 70° C for 8 h. Ultra-thin sections (20 - 40 nm) were cut with a glass knife in a *Reichert Ultracut Ultramicrotome* (Wien, Austria), stained with 2 % uranyl acetate for 1 h and lead citrate for 5 min, and finally, examined using a transmission electron microscope (*Philips EM-200*, Einhoven, The Netherlands).

For SEM, samples were dehydrated with graded concentrations of ethanol, transferred to liquid amyl acetate, and then to liquid CO_2 for drying. Samples were mounted on a support, covered with coloidal silver and gold sputter coater (*Polaron E5000*, Eat-Stgrinftead, UK), and were examined under a scanning electron microscope (*Hitachi S-2300*, Tokyo, Japan), using 15 kV acceleration.

Number of glands: Gland number was obtained from SEM micrographs of leaves. Ten micrographs per section and two sections per specimen and three specimens from each treatment were analysed.

Chloroplastic and cytoplasmic volume fractions: Chloroplastic and cytoplasmic volume was determined on TEM micrographs by superimposing a morphometry grid on each micrograph and dividing the number of points on these particular organelles by the total number of intersections falling on the corresponding cell, as described by Weibel and Bolender (1973). Five micrographs per section, two sections per specimen, and three specimens from each treatment were analysed.

Determination of essential oil content: 1 g of plant material dried at room temperature was grinded to a coarse powder and steam distilled for 4 h as described by Gamez *et al.* (1990). The isolated oil was dried over anhydrous Na_2SO_4 stored at 4 - 6 °C, and weighed.

Results and discussion

Growth: During 8-week culture, *Lavandula dentata* plantlets increased their length, and fresh and dry masses according to growth regulator used (Fig. 1). Plantlets grown on medium supplemented with 0.1 mg dm^{-3} BA had higher number of leaves, and the leaves were more intense green than leaves of control plantlets. BA also delayed the leaf ageing and consequently, the leaves of treated plantlets were younger in appearance than leaves of controls. Plantlets grown on medium supplemented with 0.1 mg dm^{-3} BA showed the highest length and fresh mass, but the highest dry mass was found in plantlets grown on 0.1 mg dm^{-3} IBA.

Scanning electron microscopy: The common characteristic of control and treated plantlets was the presence of glands in both epidermal surfaces, especially on the abaxial surface, which also had a profusion of bifurcated non-glandular trichomes (Fig. 2). Since 78 % of glands in control plantlets were surface-broken, we can infer that after 35 d of growth the glandular trichomes were mostly in a post-secretory stage (Lüttge 1971). The addition of BA to the culture medium in general inhibited the formation of trichomes in the plantlets (44 % lower than in control ones); therefore, the number of glands was lower, although most of them were unbroken (159 %

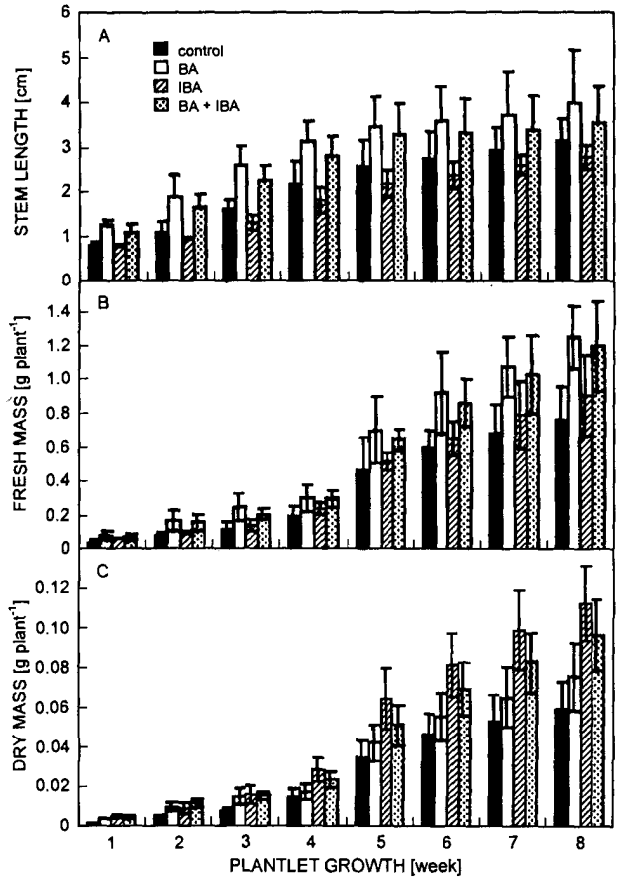

Fig. 1. Stem length (*A*), fresh mass (*B*), and dry mass (*C*) of *Lavandula dentata* plantlets grown 8 weeks on the media with BA, IBA or BA + IBA [1 mg dm^{-3}]. Each value is the average of 20 determinations ± SE. Values are significantly different according to LSD test, $P = 0.05$.

higher than in control plantlets) (Table 1). This fact is probably due to delay of general differentiation, which conditioned the pre-secretory state of glands. Goldthwaite (1987) and Bruni and Modesi (1983) obtained similar results working with leaves of *Thymus vulgaris*.

Fig. 2. Scanning microphotograph of the leaf surface of *Lavandula dentata* plantlets after 5 weeks of growth on the control medium; *bar* = 500 μm.

The addition of IBA also inhibited the gland formation, especially on the adaxial surface of leaves (Table 1), but in this case the glands presented a post-secretory stage, because many of them were surface broken, as in the control plantlets.

When both BA and IBA were added to the growth medium, the formation of glandular trichomes was highly inhibited in all plantlets (63 % lower than those of control plantlets), but the number of broken glands was also lower than those of control plantlets by 70 % (Table 1).

It is remarkable that IBA operated in a similar mode to BA, when considering the gland formation process, but the auxin effect on the secretory stage of glands was opposite to that of BA. As we mentioned previously, the number of glands in a pre-secretory stage was lower in plantlets grown on culture medium supplemented with IBA than in controls (Table 1). Taking these results into account, we suggest that the auxin/cytokinin ratio is an important factor in the development stage of glands as also stated by Charlwood *et al.* (1989).

Table 1. Number of glandular (GT) and non-glandular (NGT) trichomes, and percentage of unbroken glands (USG) in adaxial and abaxial surfaces of leaves of *L. dentata* plantlets grown on control LS medium or LS medium containing BA, IBA or both growth regulators. Values are significantly different according to LSD test, $P = 0.05$, $n = 12$.

Culture medium	Adaxial surface GT [mm^{-2}]	NGT [mm^{-2}]	USG [%]	Abaxial surface GT [mm^{-2}]	NGT [mm^{-2}]	USG [%]
Control	90.6 ± 9.1	10.9 ± 2.1	22	105.3 ± 11.1	1.4 ± 0.1	21
BA	51.1 ± 7.6	2.9 ± 0.6	57	58.2 ± 6.9	-	58
IBA	64.7 ± 8.6	21.7 ± 3.8	19	94.2 ± 7.3	14.5 ± 2.7	19
BA + IBA	42.5 ± 4.8	16.4 ± 3.2	45	82.1 ± 9.1	11.1 ± 2.6	45

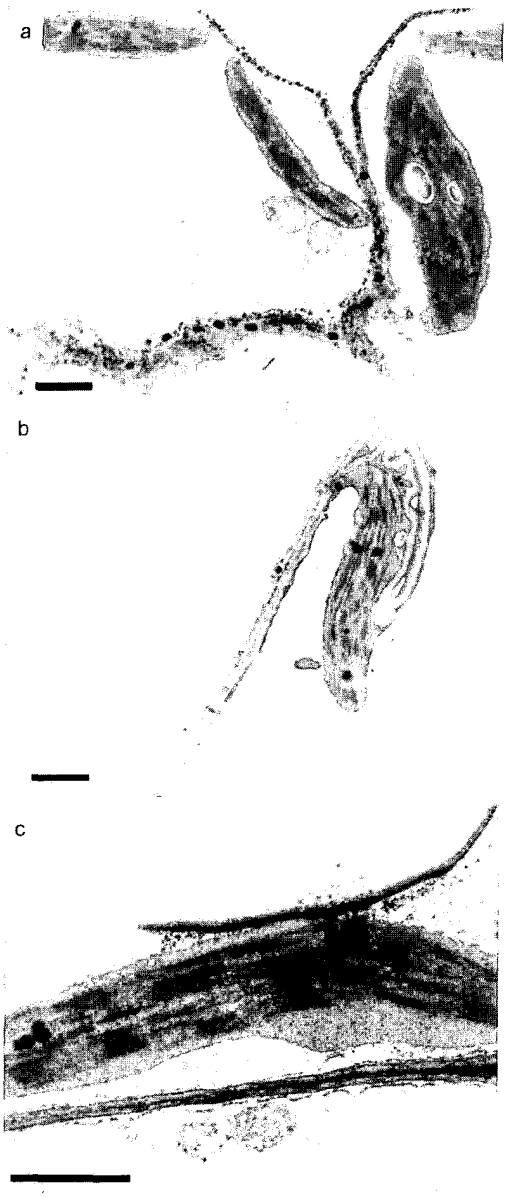

Fig. 3. Electronic microphotograph of mesophyll cells of *Lavandula dentata* plantlets after 5 weeks of growth on control medium (*a*), medium with 0.1 mg dm^{-3} BA (*b*), or 0.1 mg dm^{-3} IBA (*c*); *bar* = 1 μm.

Ultrastructure of leaf cells: Although glands are the main structures for the biosynthesis and accumulation of essential oils (Charlwood *et al.* 1989), mesophyll cells of leaves also show the capacity to produce these secondary compounds.

The addition of BA to the growth medium decreased the size of mesophyll cells and increased the number of chloroplasts (Bhatnagar *et al.* 1963). Consequently, the chloroplast area/cell area ratio increased when compared with that of control cells (Table 2). Higgins and Jacobsen (1978) working with tobacco cell cultures also observed this effect Lew and Tsuji (1982) working with cotyledons of cucumber treated with BA, found increased number of chloroplasts and chlorophyll content.

In cells of control leaves we observed a high number of lipidic droplets inside the chloroplasts, probably terpenic compounds (Piñol and Palazón 1993, Reil *et al.* 1994), because monoterpenes are synthesized in these organelles. We also observed some droplets in the cytosol, plasmalemma and cell wall (Fig. 3*a*) in the cells adjacent to the epidermis or large intercellular spaces, but not in cells in contact with one another. Lüttge (1971) showed that this fact could be related to biosynthesis of essential oils, because it begins in the chloroplasts, afterwards they are biochemically diversified in the cytosol endoplasmatic reticulum (ER) and finally, actively secreted across the plasmalemna and cell wall.

The highest number of lipidic droplets in the chloroplasts and cytosol was observed in leaves of plants grown on medium supplemented with 0.1 mg dm^{-3} BA. A remarkable fact was the presence of a considerable number of cell wall protuberances, which were always associated with the plasmalemma (Fig. 3*b*). The function of this enlarged cytoplasmatic membrane surface is probably to facilitate the export of substances to intercellular or subcuticular space (Lüttge 1971).

Leaf cells of plantlets grown on a medium supplemented with IBA were similar to the control cells when considering the size and the number of chloroplasts with lipidic droplets. Consequently, their volume chloroplast/volume cell ratio is very similar to that of control plantlets (only 10 % lower), but they contained large intracellular lipidic drops, especially in the epidermal and glandular cells.

Finally, leaf cells of plantlets grown on a culture medium supplemented with both phytohormones showed a very similar ultrastructure to mesophyll cells of plantlets grown only in presence of IBA (Fig. 3*c*).

Since leaves of plantlets grown in medium with IBA and BA + IBA did not have any protuberances in plasmalemma and cell walls, we can infer that IBA, at the concentration used, inhibited the essential oil secretion from mesophyll cells to intercellular spaces and determined the capacity of mesophyll cells to secrete essential oils.

Table 2. Chloroplasts area/cell area ratio and essential oil content in plantlets of *L. dentata*, grown for 5 weeks on the control LS medium or LS medium containing BA, IBA or both growth regulators. Means ± SE, n = 12. Values are significantly different according to LSD test, $P = 0.05$.

	Control	BA	IBA	BA + IBA
Chloroplast area/cell area	0.10 ± 0.04	0.14 ± 0.02	0.09 ± 0.03	0.10 ± 0.04
Oil content [$\times 10^{-2}$ cm^3 g^{-1}]	0.26 ± 0.01	0.65 ± 0.02	0.18 ± 0.02	0.50 ± 0.02

Essential oil content: The content of essential oil in the leaves of control plantlets was relatively low but similar to other *in vitro* grown plantlets. (Webb *et al.* 1984). The addition of BA increased the essential oil content by 150 % when compared with the control plantlets (Table 2). On the contrary, plantlets supplemented with IBA showed low essential oil concentration, 72 % and 31 % of the content in control and BA plantlets, respectively (Table 2). When both growth regulators were added to the growth medium, accumulated essential oil levels were lower than those observed in BA grown plantlets, but 100 % higher than those achieved by the control ones (Table 2).

In conclusion, our results suggest that the inhibitory effect of IBA on essential oil accumulation was not dependent on the growth rate but it was closely related to the number of glands (differentiation processes) and their integrity. Obviously, the integrity of glands had a great influence on essential oil content. Plantlets grown with BA showed the highest number of unbroken glands. These results agree with the very high content of essential oil.

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