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Morphological differentiation in callus cultures of lavandin: a role of ethylene

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Abstract

The involvement of ethylene in shoot formation *in vitro* was studied in one year old lavandin (*Lavandula officinalis* Chaix × *Lavandula latifolia* Villars) callus. A peak in ethylene evolution characterized the non-regenerating leaf callus line, as compared to the shoot-forming calyx callus line, on the growth medium provided with 2,4-dichlorophenoxyacetic acid (1 mg dm^{-3}) and kinetin (0.5 mg dm^{-3}). After one year in culture, calyx callus attained the capacity to grow on auxin-reduced media, showing decreased ethylene production and faster shoot bud emergence, when transferred onto the regeneration medium, supplemented with 10 mg dm^{-3} benzyladenine. Shoot formation was also inhibited by addition of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, indicating an involvement of ethylene in the failure of regeneration.

Additional key words: *in vitro* cultures, *Lavandula officinalis* × *Lavandula latifolia*, shoot regeneration.

Introduction

Various studies have considered the role of ethylene in organogenesis in callus cultures. However, the data dealing with shoot regeneration are conflicting (Economou 1991). In tobacco (Huxter *et al.* 1981), a negative correlation was found between the rate of endogenous ethylene production and the differentiation of callus cells into shoot primordia. The impairing effect of ethylene on shoot regeneration from callus has been proved through the use of various inhibitors of ethylene

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Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; BA - benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GI - growth index.
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formation, such as silver nitrate, which improved organogenesis in different species (Purnhauser *et al.* 1987, Songstad *et al.* 1988, Williams 1990). In contrast, exogenously supplied ethylene was found to promote shoot regeneration in rice callus in the absence of other growth regulators (Cornejo-Martin *et al.* 1979). Ethylene also promoted xylogenesis in callus cultures of soybean (Miller *et al.* 1982) and lettuce pith explants (Miller *et al.* 1984). Previous studies have demonstrated the importance of ethylene in regulating adventitious bud regeneration (Panizza *et al.* 1988) and axillary shoot formation (Panizza *et al.* 1993) in *in vitro* cultures of a medicinal plant, lavandin.

The aim of the present research was to investigate the role of ethylene in lavandin shoot regeneration from callus cultures. To this purpose, calli with different organogenetic ability were characterized, both in the growth and the differentiation phase, as regards ethylene production and its modulation by means of exogenous ACC and 2,4-D.

Materials and methods

Plant material and tissue culture: Lavandin (*Lavandula officinalis* Chaix × *Lavandula latifolia* Villars) callus cultures were prepared using leaf and calyx explants according to Panizza and Tognoni (1988). Basal medium consisted of Linsmaier and Skoog (1965) medium with 3 % (m/v) saccharose and 0.75 % (m/v) *Difco Bacto* agar. The growth regulators were added to the basal medium and the pH was adjusted to 5.7 before autoclaving. Callus formation was induced on the basal medium supplemented with 1 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} kinetin (growth medium). One-year-old callus was transferred to the shoot regeneration medium, supplemented with 10 mg dm^{-3} BA. Calli were subcultured at 3-week intervals by transferring 300 mg pieces into 30 cm^3 glass bottles (*Pyrex*) closed with double aluminium foil. Each bottle contained 15 cm^3 of medium. Callus growth was expressed by a growth index (GI) = $(W-W_0)/W_0$, where W and W_0 were the final and initial callus fresh masses of each subculture, respectively.

It was not possible to use ethylene inhibitors, such as aminoethoxyvinylglycine (AVG) and $CoCl_2$, because preliminary studies had indicated that a drastic reduction of ethylene was associated with callus necrosis and premature death on the regeneration medium. Therefore the effect of a reduction in ethylene levels induced by lowered auxin concentrations was tested. One year old calyx callus was cultivated on lowered concentrations of 2,4-D (0.5 to 0.1 mg dm^{-3}) for two subcultures and subsequently transferred to the regeneration medium.

Sterile aqueous solutions of the ethylene precursor ACC (5, 15 or 25 μM) were added to the autoclaved regeneration medium before it solidified. The effect of the different treatments on parameters related to shoot regeneration (percentage of callus pieces with shoot buds, mean shoot number per callus piece) were determined at the end of the second subculture on the regeneration medium.

All cultures were grown at 25 °C under cool white fluorescent light (irradiance of 30 μmol m^{-2} s^{-1}) provided by *Philips TL 40 W/33 RS* lamps for 16 h d^{-1}.

Ethylene determination: For ethylene determination, the containers were sealed with holed plastic screw caps provided with rubber septa. After 1 h accumulation, 1 cm^3 samples were withdrawn with a hypodermic syringe and injected into a gas chromatograph equipped with a dual flame ionization detector (FID) and a metal column (i.d. 150 × 0.4 cm) packed with aluminum mesh (70 - 230). Column and detector temperatures were 70 and 350 °C, respectively. N_2 was used as a carrier at a flow rate of 30 cm^3 min^{-1}. Ethylene identification was performed by coinjection with pure standard. Quantification was achieved by the external standard technique. Ethylene production from the explants was estimated according to Mensuali-Sodi *et al.* (1992) quantifying ethylene losses and abiotic contributions to ethylene accumulation in the culture system.

Statistical analysis: Each treatment was replicated five times and data are means of duplicate experiments. Analysis of variance was chosen to test which means were statistically different (the least statistical difference was used at $P \leq 0.05$).

Results

Callus growth and shoot regeneration: Ethylene production (Fig. 1*A*) was monitored through a subculture on the growth medium in one-year-old callus lines, calyx callus which regenerated shoots, and leaf callus which never showed shoot forming ability. A peak in ethylene evolution at day 14 in culture was characteristic of leaf callus, at a level significantly higher than calyx callus on the same day. Notwithstanding this difference in ethylene formation, the two lines exhibited no statistically significant difference in their growth over the culture period (GI = 17.8 and 15.1, for calyx and leaf callus, respectively).

After transfer to the regeneration medium, leaf callus showed severe necrosis associated with the cessation of ethylene release (Fig. 1*B*) and died within a few days. On the contrary, calyx callus exhibited highly pigmented green areas on the outer surface during the first subculture on the regeneration medium. Shoot buds became visible on the green spots by the end of the second subculture. On BA-containing medium (Fig. 1*B*) ethylene production by calyx callus decreased in the first subculture, with an initial value five times lower than in the growth medium. This trend was much more pronounced during the second subculture (Fig. 1*C*), when ethylene evolution was near zero. In the first subculture, calyx callus growth was reduced in the regeneration medium (GI = 6.51), but GI values (17.48) were comparable to those on the growth medium (17.8) by the end of the second subculture.

Modulation of ethylene levels: To test the hypothesis that auxin-induced ethylene on callus growth medium might affect subsequent shoot formation, the regeneration ability of calli pre-cultured on media with reduced amounts of 2,4-D was analyzed. Exploratory experiments had demonstrated that 1 mg dm^{-3} 2,4-D was critical to the survival in culture of the non-regenerating leaf callus. On the contrary calyx callus,

although not completely auxin autotrophic, was able to grow in the presence of decreased concentrations of 2,4-D showing greening of the tissue. On the regeneration medium (Fig. 2*A*), calyx callus pre-cultured on 0.1 mg dm^{-3} 2,4-D evolved significantly lower ethylene than the control (1 mg dm^{-3} 2,4-D pre-treatment), but the production was equal to the control value by the end of the first subculture. The reduction in ethylene levels was accompanied by an acceleration of the regeneration process (Fig. 2*B*) although the number of regenerated shoots was unaffected.

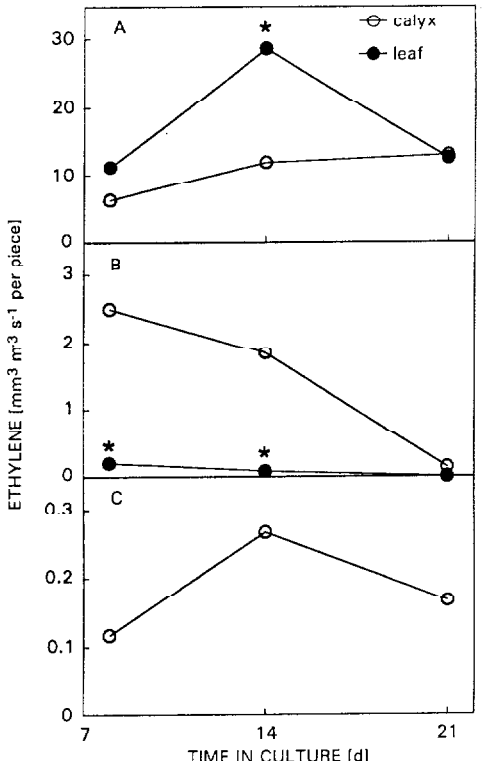

Fig. 1. Ethylene production by 1-year-old calyx and leaf callus on the growth medium (*A*), and during the first (*B*) and the second (*C*) subculture on the regeneration medium. Values marked by an *asterisk* differ significantly from the control (calyx callus) at $P \leq 0.05$.

Endogenous ethylene levels were increased when ACC was supplied to calyx callus on the regeneration medium. The highest concentration of ACC was effective at increasing ethylene production after 14 d in culture (Fig. 3*A*), but an increase in ethylene levels was evident in all treatments by the end of the first subculture (Fig. 3*A*, *B*). However, it must be stressed that the ability of calyx callus to convert exogenous ACC to ethylene was much lower during the second subculture on BA containing regeneration medium. The addition of ACC to the regeneration medium improved neither leaf callus survival nor differentiation and could only induce a

slight and temporary increase in ethylene evolution at the highest concentration (Fig. 3C). ACC treatments to calyx callus significantly reduced both regeneration percentage and shoots per callus (Fig. 4).

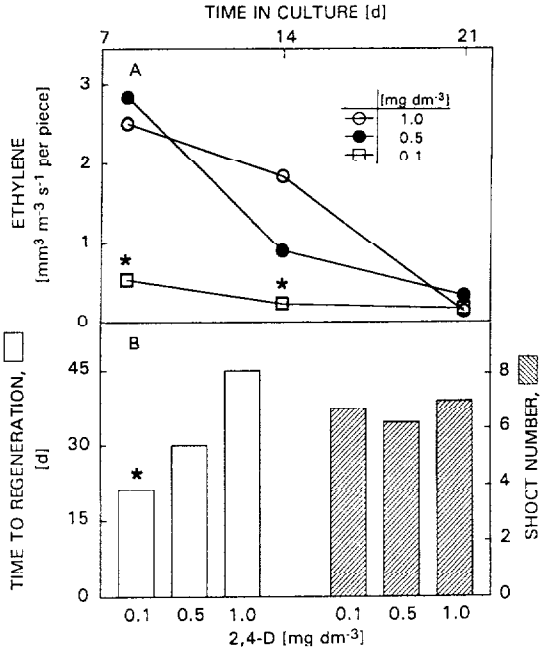

Fig. 2. Ethylene production on the regeneration medium (*A*), and mean shoot number per callus piece and days to shoot formation (*B*) in calyx callus pre-cultured on media with decreasing amounts of 2,4-D. Values marked by an *asterisk* differ significantly from the control (1 mg dm^{-3} 2,4-D) at $P \leq 0.05$.

Discussion

The present data indicate a close relationship between ethylene production and the regeneration process. The analysis of endogenous ethylene levels in a shoot-forming and a non-shoot-forming callus line seems sufficient to characterize the regeneration behaviour of the callus. The importance of ethylene production in the maintenance of the dedifferentiation phase in tobacco tissue was also supported elsewhere (Jackson *et al.* 1990). Regenerating tissues produce less ethylene than non-regenerating tissues, for example during shoot formation in tobacco callus (Grady and Bassham 1982, Huxter *et al.* 1981) and somatic embryogenesis in alfalfa cell cultures (Cvikrová *et al.* 1991). However, in the above papers the decrease in ethylene evolution could be ascribed to the lack of auxin in the regeneration medium. On the contrary, in the present study the discrepancy in ethylene production between the two callus lines was observed on the same culture medium during the phase of undifferentiated growth. Despite the difference in ethylene emanation, leaf and calyx

callus exhibited analogous growth patterns on 2,4-D supplemented medium. Huxter *et al.* (1979) concluded that there was a close correlation between the rate of ethylene production and tobacco callus growth. Conversely, the present data indicate that, both on the growth and on the regeneration medium, ethylene evolution is not connected with the growth of lavandin callus, in accordance with previous findings (Mensuali-Sodi *et al.* 1989). No correlation between ethylene evolution and growth was also reported in habituated tissues of tobacco (Köves and Szabó 1987).

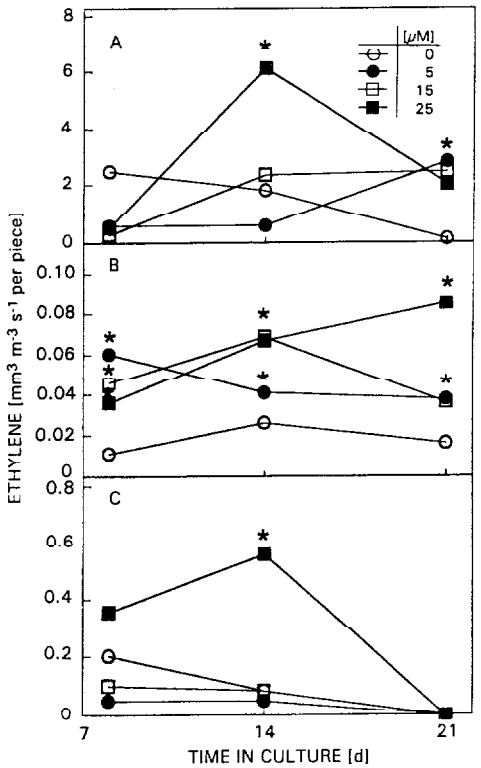

Fig. 3. Ethylene production by calyx callus in the first (*A*) and the second (*B*) subculture on the regeneration medium and by leaf callus in the first subculture (*C*) on the regeneration medium after treatment with different concentrations of ACC. Values marked by an *asterisk* differ significantly from the control (without ACC) at $P \leq 0.05$.

The survival of the non-regenerating leaf callus was strictly dependent on a critical amount of 2,4-D in culture medium, both in the presence and absence of ACC. Conversely, calyx callus, able to undergo shoot regeneration, attained the capacity to grow on 2,4-D reduced media. This characteristic was evident after one year in culture and suggests a tendency to achieve auxin habituation. In tobacco callus (Szabó *et al.* 1994) habituation was a gradual process, observed after 8 - 10 subcultures, characterized by greening of the tissue and maintenance of the organogenetic capacity. Similarly, lavandin calyx callus retained cellular totipotency after more than 20 subcultures. Previous studies on lavandin callus (Mensuali-Sodi

et al. 1989) had demonstrated that the reduction of 2,4-D in the growth medium induced low ethylene emanation associated with increased chlorophyll content, suggesting cell differentiation. This trend was confirmed by the present data on calli pre-cultured on 0.1 mg dm^{-3} 2,4-D which showed faster shoot initiation and lower rates of ethylene evolution during the first two weeks in culture on the regeneration medium. In the regeneration of sunflower plants from callus (Paterson Robinson and Adams 1987), pre-treatments of the source explants with ethylene inhibitors also showed that the ethylene control of shoot formation took place during the early stages of culture.

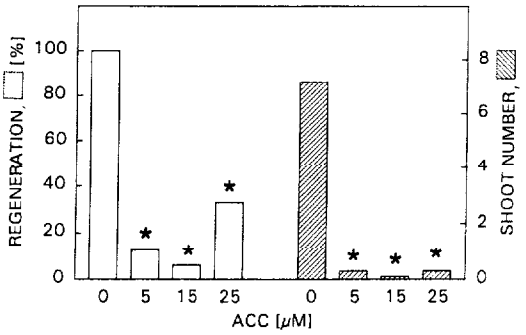

Fig. 4. Shoot formation percentage and mean shoot number per callus piece in calyx callus after treatment with different concentrations of ACC. Values marked by an *asterisk* differ significantly from the control (without ACC) at $P \leq 0.05$.

As a confirmation of the inhibitory role of ethylene in callus differentiation, treatments with the precursor ACC strongly impaired shoot formation. Analogously the increase in endogenous ethylene levels inhibited shoot production in *Brassica* (Sethi *et al.* 1990) and maize cultures (Songstad *et al.* 1988). The ability of calyx callus to convert exogenous ACC to ethylene was strongly reduced in the second subculture on the regeneration medium, indicating that the last step of ethylene biosynthesis, *i.e.* ACC oxidation, was impaired as the regeneration process took place.

It is worth noting that previous results (Panizza *et al.* 1993) had indicated a promoting role for ethylene in axillary shoot formation in lavandin cultures. This picture is further complicated by the results (Panizza *et al.* 1994) showing that ethylene can either promote or unaffect lavandin axillary budding *in vitro* according to its biotic or abiotic origin, respectively. Biddington (1992) stated that the controversial action of ethylene in the morphogenetic processes *in vitro*, extensively reported in literature, might be attributed to the different species analyzed. On the contrary, we report such an opposite effect for ethylene within the same plant cultivar which may be explained in terms of the regeneration pattern induced by different exogenous growth regulators, likely resulting in changed sensitivity to the hormone. Thus our results suggest that great caution should be given to draw general conclusions on the effect of ethylene on *in vitro* morphogenesis and various regeneration modes should be taken into consideration.

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