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Studies on Lavandin Callus Cultures: Ethylene Production in Relation to the Growth

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Abstract. Ethylene production and growth of callus cultures of lavandin (*Lavandula officinalis* CHAIX × *Lavandula latifolia* VILLARS) cv. Grosso were examined. Callus lines, derived from various kinds of primary explants (shoot tip, leaf and calyx), exhibited differences in ethylene production patterns independent of callus growth. Moreover these differences could not be ascribed to the explant source. Within a line, ethylene pattern paralleled callus growth curve. Variations in ethylene evolution were induced in shoot tip callus by means of ACC, AVG and varied amounts of 2,4-D in the culture medium. Following all these treatments callus growth was not altered. The decrease in 2,4-D concentration caused changes in Chl *a* and water content of the tissues.

Additional index words: Lavandula officinalis × *Lavandula latifolia.*

Lavandin is a sterile hybrid between *Lavandula officinalis* CHAIX and *Lavandula latifolia* VILLARS; the essential oil of this plant is composed mainly of monoterpenes with important applications in pharmacy and cosmetics. Tissue cultures of lavandin cv. Grosso were established to study *in vitro* propagation of selected clones and the biochemical production of secondary metabolites by undifferentiated cells. While studying the parameters affecting growth and differentiation of callus cultures, ethylene production was examined (PANIZZA *et al.* 1988). It is now well established that callus cells synthesize ethylene during the growth cycle in response to various cultural conditions (HUXTER *et al.* 1979, MILLER and PENGELLY 1984). However, the role of ethylene in growth has not yet been clarified and *in vitro* systems need to be explored in this respect. The aim of this work is to study growth and morphology changes in lavandin callus in response to factors modifying ethylene evolution.

MATERIALS AND METHODS

Shoot tips and leaves of greenhouse grown lavandin (*Lavandula officinalis* CHAIX × *Lavandula latifolia* VILLARS) cv. Grosso were obtained from actively growing plants; calyces were excised from flowers after anthesis.

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Plant material was washed in a solution of 0.1 % (v/v) Tween-20 in water for 30 min. Excised plant parts were surface sterilized with 10 % sodium hypochlorite (7 ± 2 % chloro active) for 10 min and then rinsed three times with sterile water.

Sterile explants were placed in 25 ml Erlenmeyer flasks containing 20 ml LINSMAIER and SKOOG medium (1965) supplemented with 30 g l^{-1} sucrose, 7.5 g l^{-1} Difco Bacto Agar, 0.5 mg l^{-1} kinetin and 1 (standard medium), 0.75, 0.50, 0.25, 0.10 mg l^{-1} 2,4-D. Hormones were added to the medium before autoclaving and the pH adjusted to 5.7. ACC (25 μM) and AVG (10 μM) were added to the autoclaved medium (while the medium was still warm) after sterile filtration. Flasks were closed with double aluminium foil. The cultures were grown at 25 ± 1 °C under cool white fluorescent light (10 W m^{-2}) provided by Philips TL 40 W/33 RS lamps with 16 h photoperiod.

Callus cultures were subscultured every 21–28 days by transferring pieces with average initial fresh mass of 400 ± 16 mg.

Callus growth was expressed as growth index (GI):

$$GI = (M - Mo) / Mo$$

where M and Mo are the final and the initial mass, respectively. Weekly GI was calculated with the final and the initial mass within each week. Both dry and fresh masses were measured.

To measure ethylene production the culture flasks were uncapped and flushed with sterile air to remove any residual ethylene. Each assay was performed 6 h after sealing the flasks with silicon caps. Preliminary experiments showed that ethylene was neither lost nor released by this material in a six hour period. For ethylene determination 1 ml samples were withdrawn with a gas tight syringe and injected into a gas chromatograph equipped with a dual flame ionization detector and a metal column (150 × 0.4 cm ID) packed with alumina (70–230 mesh). Column and detector temperatures were 70 °C and 350 °C, respectively. N_2 was used as a carrier at flow rate of 40 ml min^{-1}. The rate of ethylene production was calculated on a per hour basis and expressed as μl l^{-1} (callus piece)$^{-1}$.

Extracts were prepared by grinding 1 g of callus tissue in 8 ml 96 % ethanol and centrifuging it at 1000 × *g* for 3 min. All operations were carried out under a green safelight. The absorbances of the extracts at 665 and 649 nm were measured spectrophotometrically. These readings were converted to Chl *a* content as described by WINTERMANS and DEMOTS (1965).

All data are presented as means ± SE of five replicate flasks. Analysis of variance procedure was chosen in order to test the significance of observed results: to determine which means were statistically different, least statistical difference was used at α values of 0.01 and 0.05.

Abbreviations used: ACC = 1-aminocyclopropane-1-carboxylic acid; AVG = aminoethoxyvinylglycine; Chl = chlorophyll; 2,4-D = 2,4-dichlorophenoxyacetic acid; dry m. = dry mass; fr. m. = fresh mass; GI = growth index.

A linear correlation coefficient (r) was calculated at $\alpha = 0.05$ between growth rate and ethylene production.

RESULTS

Differences were found in ethylene levels between callus lines (Table 1) independently of callus growth. Moreover, these variations in ethylene formation could not be attributed to callus source, as shown by average values of each origin (Table 1). We observed that ethylene evolution increased from the first day and reached a maximum the 14th–21st day, depending on callus line.

TABLE 1

Ethylene production and growth index (calculated on fr. m. values) of lavandin calli derived from various kinds of explant: S = shoot tip; L = leaf; C = calyx. Data are means of five replicates ± SE. Within each column, values followed by different letters are statistically different, $\alpha = 0.01$ (small letters are used for callus lines, capital letters for average values of each explant source)

	Ethylene production [10^{-2} µl l^{-1} h^{-1} (callus piece)$^{-1}$]				GI
Day	1	7	14	21	
Callus					
S1	30.1 ± 7.3a	60.3 ± 7.9a	259.2 ± 25.5a	170.5 ± 12.1ab	24.9 ± 1.3a
S2	12.5 ± 7.7ab	48.7 ± 17.4a	128.6 ± 6.3bcd	90.9 ± 11.9bc	18.4 ± 2.5ab
S3	1.7 ± 0.3b	39.3 ± 9.3a	127.1 ± 39.8bcd	127.5 ± 35.3bc	15.7 ± 1.9ab
$\bar{x}$	14.8 ± 4.8A	49.4 ± 6.9A	171.6 ± 23.6A	129.7 ± 15.3A	19.7 ± 1.9A
L1	12.2 ± 0.7ab	66.8 ± 32.3a	172.2 ± 22.2ab	75.3 ± 28.1c	15.1 ± 5.2ab
L2	2.9 ± 0.2b	20.5 ± 3.2a	51.5 ± 8.2d	63.6 ± 4.7c	14.9 ± 2.0ab
L3	6.3 ± 0.8b	39.9 ± 5.1a	145.6 ± 24.0bc	140.5 ± 23.2bc	8.4 ± 1.1b
$\bar{x}$	7.1 ± 1.2A	42.4 ± 11.4A	123.1 ± 18.6A	93.1 ± 15.1A	12.8 ± 2.8A
C1	27.5 ± 9.1a	37.3 ± 8.9a	71.0 ± 10.6cd	78.4 ± 14.4bc	17.8 ± 3.7ab
C2	12.0 ± 3.7ab	37.9 ± 23.7a	158.0 ± 33.8bc	242.2 ± 40.1a	10.2 ± 1.3b
C3	12.8 ± 3.1ab	23.7 ± 3.7a	76.8 ± 13.4cd	101.7 ± 19.9bc	8.3 ± 1.6b
$\bar{x}$	17.4 ± 3.8A	32.9 ± 8.0A	101.9 ± 16.6A	140.7 ± 26.0A	12.1 ± 2.2A

To verify the presence of possible connections between ethylene pattern and callus growth curve, ethylene evolution and weekly GI were measured at the same time points in a shoot tip line (Fig. 1). Maximum ethylene production (Fig. 1A) coincided

with maximum dry mass GI and preceded maximum fresh mass GI by a week (Fig. 1B). Ethylene and dry mass GI values were significantly and positively correlated ($r = 0.94$, $\alpha = 0.05$). This was true in calli of any tested origin (data not shown).

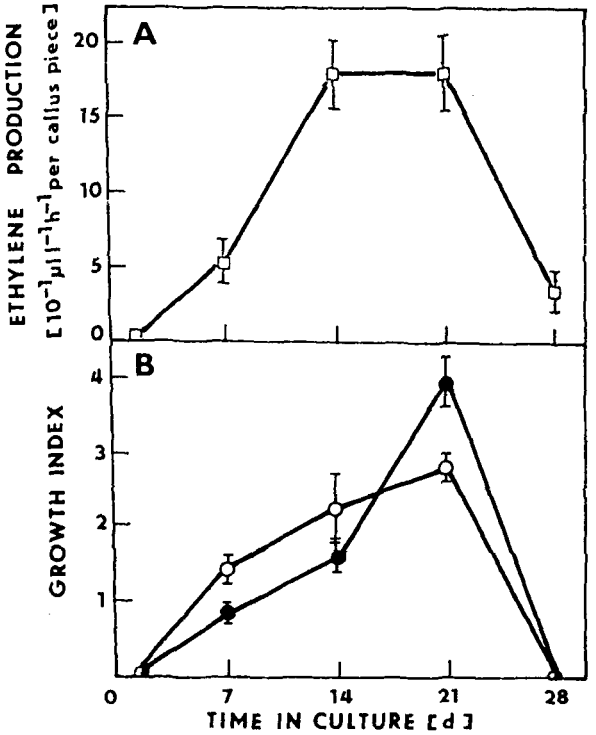

Fig. 1. Ethylene production (A) in comparison with weekly growth index curves (B) expressed in both dry (○) and fresh masses (●). Data as means ± SE for five replicate flasks.

Ethylene evolution in shoot tip callus on the standard medium was compared with ethylene on media containing decreasing amounts of 2,4-D. Following transfer onto media containing 0.25 and 0.10 mg l^{-1} 2,4-D (Experiment I), ethylene declined to a lower level than the control, particularly after the 14th day in culture (Table 2). After two months of subculture (Experiment II), decreased ethylene production occurred during the whole culture period in every medium but the control, most pronouncedly on 0.10 mg l^{-1}–2,4-D medium (Table 2). In the first experiment GI of calli on 2,4-D-decreased media grew less as compared with the control, however in the second experiment GI values went back to the control levels (Table 2).

After two months of culture, morphological changes occurred in callus on 2,4-D-reduced media: tissues appeared hard, compact and green whereas the control tissue was friable and light yellow. Table 3 shows that fr. m./dry m. ratio was lower than the control at the three lower 2,4-D concentrations, whereas Chl *a* was higher only on 0.10 mg l^{-1}-2,4-D medium.

Growth of shoot tip callus was not affected by ACC addition to both the standard

TABLE 2

Ethylene production and growth index (calculated on fr. m. values) of shoot tip callus on media provided with different 2,4-D concentrations and the same amount of kinetin (0.5 mg l^{-1}). Data are means of five replicates ± SE. In each experiment (I and II) values followed by different letters are statistically different, $\alpha = 0.05$, within each column

		Ethylene production [10^{-2} µl l^{-1} h^{-1} (callus piece)$^{-1}$]				GI
	Day	1	7	14	21	
	2,4-D [mg l^{-1}]					
Exp. I	1.00*	2.6 ± 0.5a	56.3 ± 8.0a	181.1 ± 29.3a	179.8 ± 30.5a	22.0 ± 1.7a
	0.75	0.0 ± 0.0b	52.4 ± 21.1a	91.2 ± 25.8b	152.3 ± 13.1a	12.1 ± 1.7b
	0.50	0.0 ± 0.0b	45.2 ± 9.7a	177.7 ± 10.7a	123.2 ± 31.0a	11.8 ± 3.0b
	0.25	0.0 ± 0.0b	51.2 ± 11.0a	83.4 ± 8.6b	54.8 ± 11.9b	10.5 ± 0.2b
	0.10	0.0 ± 0.0b	38.7 ± 10.1a	48.9 ± 5.5b	8.6 ± 2.5b	7.3 ± 1.7b
Exp. II	1.00*	2.9 ± 0.5a	60.2 ± 11.1a	259.1 ± 36.1a	170.5 17.0a	24.9 1.7a
	0.75	0.0 ± 0.0b	15.8 ± 2.7b	31.2 ± 3.8c	54.6 10.0b	47.2 2.8a
	0.50	0.0 ± 0.0b	26.7 ± 3.3b	135.2 ± 7.1b	76.5 24.8b	38.9 29.3a
	0.25	0.0 ± 0.0b	19.1 ± 6.0b	81.2 ± 12.4bc	42.9 16.3b	28.5 0.7a
	0.10	0.0 ± 0.0b	17.0 ± 8.6b	8.9 ± 3.4d	2.0 1.3c	19.8 8.9a

* Control medium.

TABLE 3

Chlorophyll *a* content and fr. m./dry m. ratio in shoot tip callus on media provided with different concentrations of 2,4-D and the same amount of kinetin (0.5 mg l^{-1}). Data are means of five replicates ± SE. Within each column, values followed by different letters are statistically different, α 0.05

2,4-D [mg l^{-1}]	Chl *a* [µg g^{-1} (dry m.)]	fr. m./dry m.
1.00*	445 53b	42.0 ± 2.7a
0.75	231 31b	40.8 ± 5.5ab
0.50	388 26b	23.7 ± 4.8c
0.25	542 35b	27.3 ± 1.9bc
0.10	1418 ± 200a	26.0 ± 6.1c

* Control medium.

and 0.10 mg l^{-1}-2,4-D medium although ethylene production was stimulated on 0.10 mg l^{-1}-2,4-D medium (Fig. 2A).

AVG was provided to shoot tip callus on the standard medium since it showed the highest ethylene production. Ten µM AVG strongly reduced ethylene levels during the whole culture period (Fig. 2B) without modifying callus growth.

DISCUSSION

From our results it is clear that each callus line is characterized by specific ethylene production. Modifications in ethylene levels, induced by various treatment, did not alter callus growth. HUXTER *et al.* (1979) suggest that most ethylene production may be caused by metabolic changes in response to auxin treatment, nevertheless they do not exclude that very low ethylene levels may regulate callus growth when released by tissues. We were not able to induce still lower ethylene levels by means of AVG,

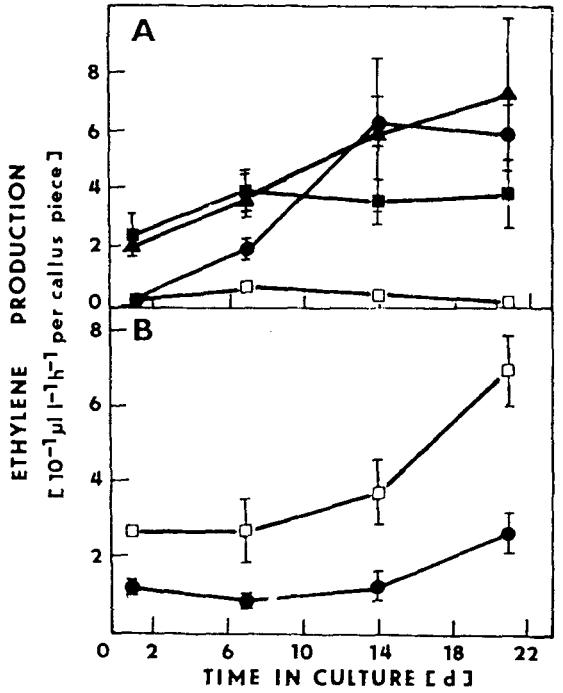

Fig. 2. Ethylene production by lavandin callus on (A) the standard medium with (▲) and without (●) 25 µM ACC, on 0.10 mg l^{-1} 2,4-D medium with (▪) and without (□) 25 µM ACC, or (B) on the standard medium with (▪) and without (□) 10 µM AVG. Data as means ± SE for five replicate flasks.

since concentrations higher than 10 µM caused the callus to die within a few days. Even the decrease in ethylene production, achieved by lowering 2,4-D in the culture medium (Table 2), did not affect callus growth. In fact, after an initial decline, GI values were back to the control level: this result indicates that a certain time is needed to restore normal growth following variations in hormonal levels. Unfortunately, no callus was able to grow on 2,4-D-deprived medium.

The changes in water and Chl *a* content of the tissue (Table 3), may be ascribed to the decrease in both 2,4-D and ethylene levels. DE PROFT *et al.* (1985) found that high CO_2 and ethylene concentrations corresponded with low Chl *a* content in *Magnolia* tissue cultures. Ethylene involvement in loss of Chl has also been reviewed by LIEBERMANN (1979). It is significant to note that variations in tissue greening can

be related to monoterpene production as reported by WATTS *et al.* (1985) in celery tissue cultures.

We conclude that lavandin callus growth is not affected by ethylene production at tested levels. Nevertheless it is important to stress that ethylene evolution parallels callus growth (Fig. 1), and they both sharply decrease by the end of the culture. It is well known that an inverse relationship exists between secondary metabolite production and growth rate of cell cultures, suggesting the presence of a switch from primary to secondary metabolism (YEOMAN *et al.* 1980). The observed differences in ethylene evolution between callus lines may indicate different levels of metabolic activity. Further studies, now in progress, on monoterpene production by lavandin callus may confirm our hypothesis.

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REFERENCES

DE PROFT, M. P., MAENE, L. J., DEBERGH, P. C.: Carbon dioxide evolution in the culture atmosphere of *Magnolia* cultures *in vitro*. – Physiol. Plant. **65** : 375–379. 1985.

HUXTER, T. J., REID, D. M., THORPE, T. A.: Ethylene production by tobacco callus. – Physiol. Plant. **46** : 374–380, 1979.

LIEBERMANN, M.: Biosynthesis and action of ethylene. – Annu. Rev. Plant Physiol. **30** : 533–591, 1979.

LINSMAIER, E. M., SKOOG, F.: Organic growth factor requirements of tobacco tissue cultures. – Physiol. Plant. **18** : 100–127, 1965.

MILLER, A. R., PENGELLY, W. L.: Ethylene production by shoot-forming and unorganized crown-gall tumour tissue of *Nicotiana* and *Lycopersicon* cultured *in vitro*. – Planta **161** : 418-424, 1984.

PANIZZA, M., MENSUALI SODI, A., TOGNONI, F.: *In vitro* propagation of lavandin : ethylene production during plant development. – Acta Horticult. **227** : 334–339, 1988.

WATTS, M. J., GALPIN, I. J., COLLIN, H. A.: The effect of greening on flavour production in celery tissue cultures. – New Phytol. **100** : 45–56, 1985.

WINTERMANS, J. F. G. M., DEMOTS, A.: Spectrophotometric characteristics of chlorophyll *a* and *b* and their phenophytins in ethanol. – Biochim. biophys. Acta **109** : 448–453, 1965.

YEOMAN, M. M., MIEDZYBRODZKA, M. B., LINDSEY, K., MCLAUCHLAN, W. R.: The synthetic potential of cultured plant cells. – In: SALA, F., PARISI, B., CELLA, R., CIFERRI, O. (ed.): Plant Cell Cultures: Results and Perspectives. Pp. 327–343. Elsevier/Nort-Holland Biomedical Press, Amsterdam 1980.