Plant Cell, Tissue and Organ Culture **28**: 175–181, 1992.

The influence of ethylene on proliferation and growth of rose shoot cultures

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Received 10 February 1991; accepted in revised form 27 September 1991

Key words: axillary budding, gaseous atmosphere, micropropagation

Abstract

Ethylene accumulation in four different rose *in vitro* culture containers was evaluated. Multiplication rate was the highest, and axes most elongated, in the two containers where ethylene accumulation was limited. Pulse treatments of ethylene at various concentrations enhanced proliferation depending on concentration (5 ppm generally was the most favourable) and time of application, while reducing elongation of the shoots. An ethylene trap in the flask atmospheres of the cultures reduced rose shoot proliferation rate but increased elongation of the axes. Inhibitors of ethylene biosynthesis, aminoethoxyvinylglycine (AVG) and cobalt chloride ($CoCl_2$), increased multiplication rate by providing a higher number of axes of a suitable size for subculture. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) had a beneficial effect on multiplication rate, although reducing longitudinal growth of the axes.

Abbreviations: ACC – 1-aminocyclopropane-1-carboxylic acid, AVG – aminoethoxyvinylglycine, BA – benzyladenine, GA_3 – gibberellic acid, IBA – indolyl-3-butyric acid

Introduction

Ethylene is known to affect or control many growth and development processes including apical dominance (Abeles 1973; Hillman 1986), lateral bud growth (Yeang & Hillman 1982; Van Dijck et al. 1988) and adventitious bud formation (Huxter et al. 1981; Van Aartrijk et al. 1986), which constitute the basic phenomena of plant propagation by *in vitro* culture (Gaspar 1988). The role of ethylene biosynthesis in *in vitro* adventitious bud formation has been assessed (Huxter et al. 1981; Van Aartrijk et al. 1986; Robinson & Adams 1987; Kumar & Thorpe 1989) with some positive results. A clear distinction, however, has not always been established between the strict relation of ethylene biosynthesis with the developmental process and the effect of ethylene produced and accumulated in the atmosphere of the culture jar, retroacting exogenously. Ethylene indeed has been shown to accumulate within the enclosure to physiologically active levels (Jackson et al. 1987; Righetti et al. 1990).

The correlation between the rate of ethylene production and micropropagation through outgrowth of axillary buds has been less studied (Van Dijck et al. 1988; Gaspar et al. 1989). An increase in the ethylene production rate above a threshold seems to be necessary for the outgrowth of lateral buds in bromeliads cultured *in vitro* (Van Dijck et al. 1988). Our own results (Gaspar et al. 1989), on the contrary, provide evidence that there is no functional relationship

between axillary budding of rose shoot cultures and ethylene biosynthesis. Ethylene biosynthesis and evolution has been amplified in conditions where multiplication rate was unaffected or even nullified; and inhibitors of ethylene biosynthesis lowered the gas production without affecting shoot proliferation (Gaspar et al. 1989). That study was conducted under conditions that did not allow gas accumulation over 24-h periods: the air was renewed every day. The present investigation, on the contrary, examines the role of ethylene accumulated in, added to or removed from the flask atmospheres for longer periods, at different stages of the cultures in the multiplication cycle.

Materials and methods

Material and culture conditions

In vitro stock cultures of 'Madame Georges Delbard' greenhouse rose (*Rosa hybrida* L.) from the micropropagation unit of the nurseries Georges Delbard at Commentry, France, were used. They proliferated on a medium containing the macro- and micro-elements of Murashige & Skoog (1962), supplemented with glutamine and vitamins as proposed by Martin et al. (1981). Sucrose was added to all media at the rate of $30\,g\,l^{-1}$. Except where otherwise stated, growth regulators were IBA (0.49 μM), BA (6.67 μM) and GA_3 (0.29 μM). Media were adjusted to pH 5.5–5.6 with KOH and HCl prior to the addition of 'Touzard and Matignon' agar ($8\,g\,l^{-1}$) and subsequent autoclave sterilization for 20 min at 121°C and 118 kPa. For the purposes of the present work, shoot cultures were grown

(a) on 100 ml of medium in 600 ml cylinder (10 cm diameter) 'Le Parfait' glass jars closed with glass lids held in place with a sheet of transparent plastic Reynolds film;
(b) on 130 ml medium in 825 ml cylindrical (12 cm diameter) 'Delbard' glass jars closed with polycarbonate screw lids (sealed or not with a double sheet of transparent plastic Reynolds film); or
(c) on 60 ml medium in 300 ml rectangular (10.8 × 8.2 cm; 5.5 cm height) plastic containers, with plastic adjustable lids (from Reynolds Film Inc.)

Jars in (a) and (b) were perforated and sealed with a serum cap to allow sampling of jar air with a syringe.

These containers (20 explants each) were maintained in a growth chamber at 24°C with 16-h photoperiod (from 6 h to 22 h) and light intensity of $3.2\,Wm^{-2}$ (Sylvania Gro-lux fluorescent lamps) at culture level inside the jar plus lid. Cultures were routinely subcultured every 3 weeks.

Ethylene measurement

Gas samples were taken directly from the jar atmosphere with a syringe through the serum cap, at 4 cm from the lid. Three samples of $1\,cm^3$ gas were taken from the bottles. Ethylene was quantified by GC, according to the technique used by Kevers & Gaspar (1985). A stainless steel column (3 m × 1.5 mm) filled with Porapack R (80–100 Mesh) was used. Column, injector, and flame ionization detector temperatures were 60, 90 and 90°C, respectively. The electrometer sensitivity was $1 \times 10^{-12}\,AmV^{-1}$. N_2 was used as carrier gas ($55\,cm^3\,min^{-1}$).

Effect of ethylene, of the ethylene precursor ACC, of inhibitors of ethylene biosynthesis (AVG and $CoCl_2$), and of a trap of ethylene

Ethylene was added to the cultures by direct injection of gas into the vessels at different times of the multiplication cycle. ACC, AVG and $CoCl_2$ were incorporated at different concentrations in the culture medium from the beginning of the culture. Ethylene was trapped in 0.25 M mercuric perchlorate, prepared by dissolving red mercuric oxide in perchloric acid (Abeles 1973), in a 2.5 ml open glass vial partly buried in the center of the culture medium or suspended from the lid.

Experiments were repeated at least three times and measurements also were replicated three times per experiment.

Results

Shoot proliferation and multiplication rate

Previous experiments with this rose had revealed that the most suitable way of multiplication consisted of using explants consisting of part of the main enlarged axis from the preceding subculture (with very short internodes, Fig. 1) bearing two newly formed side axes (formed either on the main or on the side axes) decapitated at two thirds of their length. On the multiplication medium, the morphological events were as follows: swelling of the main stem from day 1 on, swelling of the basal axillary buds from day 3 and progressive outgrowth from day 5, new visible leaves being formed on the newly developed axes from day 12 until day 21. During the last half of the cycle, outgrowth of some additional axillary buds will occur but they will not reach a sufficient size to be used. The cluster in total bore 6 to 9 new axillary shoots at the end of the multiplication cycle (Fig. 1), but only the 2 to 6 that were 2 to 2.5 cm high were used. Because the explant was made of 2 axes, a mean multiplication rate of 1.5 to 2.5 was reached.

Effect of container type on ethylene accumulation and rose proliferation

As seen in Fig. 2, ethylene accumulation in the culture container atmospheres of rose cultures greatly depended on the type of container. The amount of ethylene increased linearly during the first 9 days of culture in 'Le Parfait' and in 'Delbard' + film jars. In contrast ethylene remained at a low constant level in plastic containers during the whole multiplication cycle. Ethylene increased up to a certain degree in non-sealed 'Delbard' jars and remained relatively constant at that rather low level. The multiplication rate was highest and the stem axes were longest in the two last containers, where ethylene did not accumulate (Table 1). In the containers sealed with plastic film where ethylene accumulated, the rate of multiplication was lower, the shoots were shorter and leaves showed visible signs of epinasty.

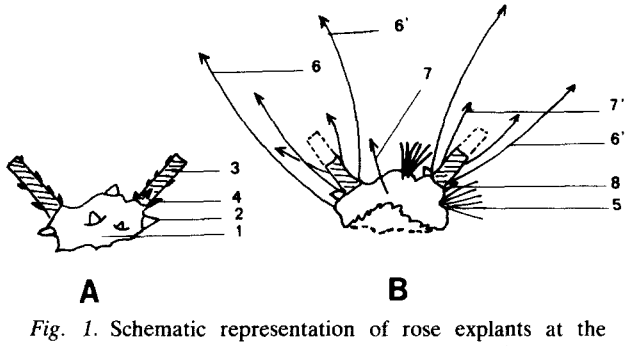

Fig. 1. Schematic representation of rose explants at the beginning (*A*) and at the end of subculture (*B*). 1: main enlarged basal stem; 2,8: buds on main enlarged axis; 3: decapitated side stem axis left on main stem with 4: shoot axillary bud; 5,6,7: respectively rosette-type, long and short shoots, developed from buds of the main enlarged axis; 6', 7': long and short shoot axes, respectively, developed from shoot axillary buds.

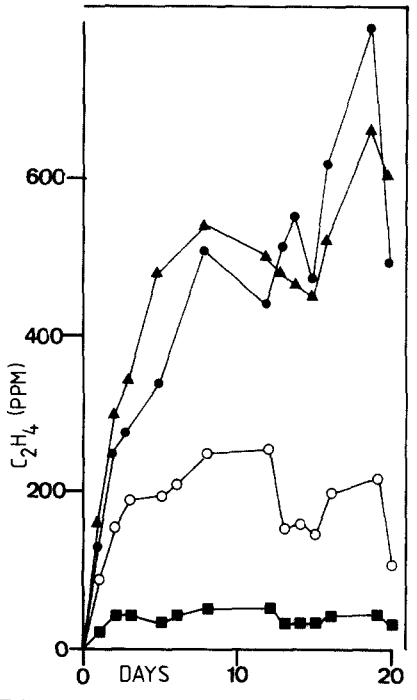

Fig. 2. Ethylene accumulation during the rose multiplication cycle on the same medium in four different containers: 'Delbard' jar plus (●) or minus (○) film, 'Le Parfait' jar plus film (▲) and plastic container minus film (■).

Effect of amount, moment and duration of application of added ethylene on multiplication rate and growth of stem axes

Ethylene was introduced at 1, 5, 20 or 100 ppm at days 0, 7 or 14 at the 21-day multiplication

Table 1. Multiplication rates and length of main axes of roses cultured in four different containers.

Containers	Multiplication rate	Length of main axes (cm)	Leaf epinasty
Delbard glass jar + polycarbonate lid	2.3 ± 0.3	1.5 ± 0.3	No
Delbard glass jar + polycarbonate lid + plastic film	1.8 ± 0.2	1.2 ± 0.2	Yes
Plastic jar + plastic cover	2.2 ± 0.2	1.8 ± 0.2	No
Le Parfait glass jar + glass lid + plastic film	1.5 ± 0.2	1.0 ± 0.3	Yes

cycle for periods varying from 21 to 7 days in 'Delbard' jars with plastic film in addition to the plastic lids. For periods longer than 7 days, injection was repeated every 7 days. A preliminary analysis of ethylene retention in 'Delbard' jars (in the absence of plants) without and with plastic films indicated that all of the injected ethylene was lost in the former after one day and lost more slowly in the latter (Fig. 3). Addition of ethylene to the flask atmospheres of rose cultures increased multiplication under different conditions (Table 2). Ethylene at 1 ppm was efficient only if applied early in and maintained during the whole multiplication cycle. Ethylene at 5 ppm was the most efficient and could be added with about the same efficiency at days 0 (and maintained during the cycle), 7 or 14. Twenty ppm of ethylene slightly improved multiplication when added for 7 days at day 14, had no affect when added for 7 days at day 0, and decreased multiplication in all other cases. One hundred ppm was generally unfavourable to multiplication except for a pulse at day 0. None of the C_2H_4 concentrations used stimulated growth of the stem axes (Table 2). One hundred ppm C_2H_4 was the most growth inhibitory. Proliferation enhancement through 5 ppm C_2H_4 apparently was at the expense of the growth of the axes for further multiplication.

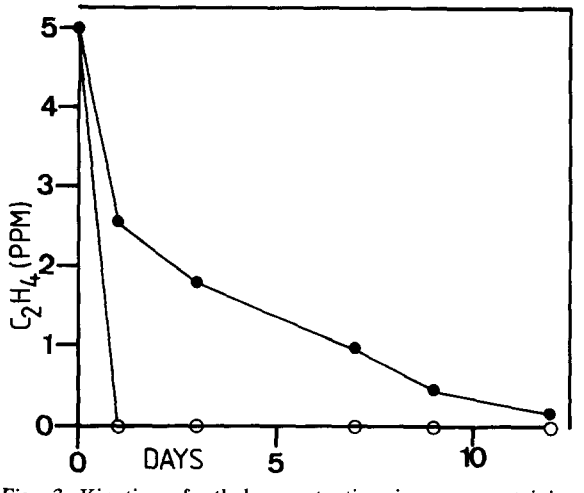

Fig. 3. Kinetics of ethylene retention in agar containing (without plants) 'Delbard' jars (plus lids) plus (●) or minus (○) plastic film. Forty ml of 100 ppm ethylene were injected at zero time.

Effect of ethylene removal from the container atmosphere

Ethylene trapping in 'Delbard' jars began at days 0, 7 or 14 for periods varying from 21 to 7 days (Table 3). In all the cases examined, removal of ethylene from the flask atmospheres decreased the rate of multiplication. Removal of ethylene during the first week of the cycle also limited growth of the axes; in all other cases, on the contrary, it increased growth by as much as 100% (Table 3).

Effect of ACC (ethylene precursor), AVG and $CoCl_2$ (inhibitors of ethylene biosynthesis)

AVG and $CoCl_2$ did not greatly affect the rate of multiplication of the roses (Table 4) in 'Le Parfait' glass jars. The slight increase could be attributed to the enhanced growth of the axillary shoots in the presence of these compounds. ACC somehow enhanced axillary budding, which

Table 2. Effect of different durations of ethylene (varying concentrations) application from different times (days 0, 7 and 14) after subculture on rose multiplication rate and shoot height.

Day treatment started	Ethylene injected concentration (ppm)	Duration of treatment (days)		
		7	14	21
		Multiplication rate		
0	1	2.4 ± 0.3	2.2 ± 0.1	2.8 ± 0.3
	5	2.3 ± 0.3	2.1 ± 0.2	3.7 ± 0.5
	20	2.6 ± 0.4	1.9 ± 0.2	1.5 ± 0.2
	100	2.7 ± 0.2	1.7 ± 0.1	1.4 ± 0.1
7	1	2.3 ± 0.2	2.3 ± 0.4	
	5	4.4 ± 0.5	4.4 ± 0.3	
	20	1.7 ± 0.2	1.8 ± 0.2	
	100	1.5 ± 0.3	0.7 ± 0.1	
14	1	–		
	5	4.6 ± 0.6		
	20	2.7 ± 0.2		
	100	1.6 ± 0.2		
		Shoot height (mm)		
0	1	14 ± 2	12 ± 1	11 ± 1
	5	14 ± 2	12 ± 1	10 ± 1
	20	12 ± 1	10 ± 2	9 ± 1
	100	0	11 ± 1	9 ± 2
7	1	15 ± 2	14 ± 2	
	5	11 ± 1	11 ± 1	
	20	12 ± 2	10 ± 1	
	100	15 ± 3	10 ± 1	
14	1	–		
	5	10 ± 1		
	20	12 ± 1		
	100	10 ± 1		

Control multiplication rate was 2.3 ± 0.3 and shoot height was 15 ± 2. Means ± standard error.

Table 3. Effect of an ethylene trap (with respect to beginning and duration of treatment) on rose multiplication rate and shoot height.

Beginning of treatment (days after subculture)	Duration of treatment (days)		
	7	14	21
	Multiplication rate		
0	1.6 ± 0.2	2.0 ± 0.2	1.4 ± 0.1
7	1.5 ± 0.1	2.1 ± 0.3	
14	1.8 ± 0.2		
	Shoot height (mm)		
0	12 ± 1	20 ± 2	30 ± 4
7	25 ± 3	21 ± 3	
14	18 ± 2		

Control multiplication rate was 2.3 ± 0.3 and shoot height was 15 ± 2). Means ± standard error.

could not be attributed to a greater number of more rapidly growing shoots, since height of these shoots on the contrary was considerably reduced (Table 4). Shoots grown in the presence of ACC were thicker as if they were treated by exogenous ethylene.

Discussion

The present results confirm that significant concentrations of ethylene often accumulate in the gas phase within vessels used for *in vitro* cultures and that the actual amount will vary according to the volume of the culture vessel and the manner in which it was sealed (Huxter et al. 1981;

Table 4. Effect of ACC, AVG and $CoCl_2$, incorporated in the multiplication medium in 'Le Parfait' jars, on the multiplication rate and the length of the main shoots, at the end of the multiplication cycle.

Compound tested	Multiplication rate	Main shoot length (mm)
0	2.0 ± 0.3	18 ± 3
ACC		
5×10^{-6} M	2.4 ± 0.4	15 ± 2
1×10^{-5} M	2.2 ± 0.3	13 ± 1
5×10^{-5} M	2.0 ± 0.2	8 ± 1
AVG		
2.5 to 50, mg l^{-1}	2.3 ± 0.2	23 ± 3
100 mg l	2.0 ± 0.1	23 ± 2
$CoCl_2$		
5×10^{-6} M	2.2 ± 0.3	20 ± 2
1×10^{-5} M	2.2 ± 0.2	21 ± 1
1×10^{-4} M	2.2 ± 0.3	23 ± 3

Means ± standard error.

Gavinlertvatana et al. 1982; De Proft et al. 1985; Kevers & Gaspar 1985; McClelland & Smith 1990). The present results in total also confirm George & Sherrington's (1984) review and conclusion that the concentrations of ethylene that develop in culture flasks (particularly those that are tightly closed) can be sufficient to modify organogenesis and morphogenesis and plantlet development. Adventitious shoot formation might be either inhibited or stimulated through ethylene of the culture vessel, depending on the concentration and the period of accumulation (Ringe 1972; Huxter et al. 1981). The role of ethylene in multiplication through axillary budding is by no means clear. AVG, as an inhibitor of ethylene biosynthesis, has been shown to significantly reduce senescence of roses *in vitro*, which resulted in a better growth and propagation rate (Mekers et al. 1984), but no relation has been made with the accumulation of ethylene in the flask atmospheres. Ethephon on the other hand has been used to overcome the inhibition of buds at the basal region of rose plants, thus promoting the production of vigorous renewal canes (Halevy 1986). Our results shed additional light on these apparently contradictory results. Indeed our data reveal that exogenous ethylene promote axillary budding under certain concentrations and depending on the time and duration of application. This apparently occurs at the expense of shoot growth. On the other hand, reduction of ethylene production through the use of inhibitors of its synthesis, like AVG, favour shoot growth, which may offer a greater number of suitable axes for further multiplication. As already shown previously (Gaspar et al. 1989), AVG thus should not influence axillary budding directly.

It has also been shown that ethylene and CO_2 interacted on the metabolism of the other (Cheverry et al. 1988; Sisler & Wood 1988; Taylor & Gunderson 1988) with resulting changes in their ratios in the flask atmospheres (Cornejo-Martin et al. 1979; De Proft et al. 1985; Woltering 1986; Kumar & Thorpe 1989) and interference with differentiation of shoot buds (Kumar & Thorpe 1989). The organogenic changes due to reduction or enhancement of ethylene level in the flask atmospheres of cultures thus should not be automatically attributed solely to ethylene.

Acknowledgements

This work was carried out at the request and with the financial aid of the society Moët-Hennessy for the nurseries G. Delbard. The authors thank Drs J.P. Barbe and Y. Mazière who provided the plant material and participated in the examination of the results.

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