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Influence of ACC and Ethephon on Cell Growth in Etiolated Lupin Hypocotyls. Dependence on Cell Growth State

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Abstract. The possible implication of ethylene on the growth regulation of etiolated lupin hypocotyls was investigated. Excised hypocotyl sections from actively growing seedlings produced ethylene at a rate of 3 nmol h^{-1} g^{-1} min^{-1}. The rate of ethylene production was increased about 7 times when sections were treated with 10 mM 1-aminocyclopropane-1-carboxylic acid (ACC). Measurement of endogenous ACC showed that 95 % of total ACC (64.2 nmol g^{-1} min^{-1}) corresponded to conjugated ACC.

Treatments to intact seedlings with the ethylene precursor ACC, and the ethylene generating compound, 2-chloroethyl phosphonic acid (ethephon) during the cell elongation phase of the hypocotyl (from 7 to 21 dage), modified the cell growth of the organ. ACC (1 or 5 mM) or low concentrations of ethephon (0.66 mM) produced a transient decrease in the growth rate without modifying the final length of the hypocotyls. Higher concentrations of ethephon reduced the final length ; the younger the seedlings were, the greater the reduction. Simultaneously to inhibition of cell elongation, ethephon produced stimulation of the radial expansion of cells in pith and cortex.

The growth inhibition period, which lasted for 2 days after the treatments, was followed by another period in which the growth rate of treated plants surpassed that of the control. In both cases differences were observed along the hypocotyls due to the different growth status of the cells. It is suggested that the sensitivity to ethylene and the metabolism of ethylene depend on the growth status of the cells.

During the growth of etiolated hypocotyls of lupin (*Lupinus albus* L.) the longitudinal distribution of endogenous IAA has been positively correlated with the growth gradient along the hypocotyl (Sanchez-Bravo *et al.* 1986). In the age range studied (from 7 to 21 d) a correlations was noted between cell elongation and growth of the hypocotyl; for this reason, IAA has been proposed as a growth-controlling factor in this organ (Ortuño *et al.* 1988). The participation of phytohormones other than auxin in the control of etiolated lupin hypocotyl growth has not yet been

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demostrated. However, some data lead one to suspect that ethylene is involved in this control: for example, the discovery of ethylene as a plant hormone was based on its effects on the growth of etiolated seedlings (the "triple response" of legume plants, Neljubow 1901); the control of cell growth and cell differentiation by a dual regulation of ethylene and IAA has already been suggested (Sargent *et al.* 1973, Osborne 1976); ethylene is synthesized in etiolated seedlings as in other plant tissues from the precursor ACC (Jones and Kende 1979, Satoh and Esashi 1983, Taylor 1988); and, asymmetric distribution of ACC has been proposed as being responsible for the differential growth which maintained the hypocotyl hook (Schierle *et al.* 1988).

Contrary to the above it has been found that normal growth continued when the action of ethylene was strongly inhibited by application of Ag to different plant species (Cameron and Reid 1983).

The important role played by ethylene in some plant responses such as fruit ripening is now clear. However, doubts have been formulated about the importance of ethylene as a component in the balance of growth regulators that maintain normal vegetative growth (Reid 1988).

In the present paper we investigate the possible participation of ethylene in the control of growth of etiolated lupin hypocotyls. For this: *i)* A preliminary study is carried out in which the capacity of the etiolated lupin hypocotyls to produce ethylene in the presence or absence of exogenous ACC is established, and the level of endogenous ACC is measured. *ii)* The growth response of etiolated lupin hypocotyls to the application of the ethylene precursor, ACC, and the ethylene generating compound, ethephon, is studied. *iii)* Since a longitudinal growth gradient exists (Ortuño *et al.* 1985), the response of hypocotyl regions at different localities along the organ is investigated.

MATERIAL AND METHODS

Growth Conditions and Treatments

Lupin seeds (*Lupinus albus* L. from Bari, Italy) were immersed for 24 h in water and germinated in moist vermiculite at 25 °C in darkness, in a growth chamber. The hypocotyls were sprayed (0.2 ml cm^{-1} of hypocotyl) with aqueous solutions of ethephon (0.66, 3.3 or 6.6 mM) or ACC (1 or 5 mM) at different ages. In a parallel experiment, the seeds were only incubated in aqueous solution of ethephon (6.6 mM) for 24 h.

Growth Measurements

At 8^{th} day a uniform sample of seedlings was marked with ink in the hypocotyl and ten zones (A–J) of 0.5 cm length were delimited.

The growth of seedlings, expressed as elongation of the hypocotyl, was measured

as indicated in a previous report (Ortuño *et al.* 1988). The cell size was measured in microscopic preparations using an ocular micrometer (Ortuño *et al.* 1985).

Determination of Ethylene Production and EFE Capacity

Hypocotyls from 8 day old seedlings of uniform length (5 ± 0.5 cm) were cut into sections of 0.5 cm and immediately enclosed in a 15 ml glass vial to determine ethylene production. To estimate the EFE capacity, 10 µl of 10 mM ACC were placed on the uppermost cut surface of each section and then infiltrated three times in vacuum for 5 min periods. In a parallel experiment the sections were infiltrated with 10 µl of 1 mM cycloheximide previous to the ACC infiltration. The infiltrated sections were transferred to 15 ml glass vials. The vials were sealed and kept at 25 °C in darkness. At different times 1 ml gas samples were withdrawn and measured by GC. To avoid low pressure conditions 1 ml ethylene-free air was injected into the vials just before withdrawing each sample.

Determination of ACC

The method of Atta–Aly *et al.* (1987) was used for the ACC extraction with trichloroacetic acid. The free ACC content was determined directly in the extract. To measure the conjugated ACC (presumed to be 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC)), the extract was hydrolyzed with 2 N HCl as described by Hoffman *et al.* (1982). The content of conjugated ACC was calculated by subtracting the free ACC content (non hydrolyzed extracts) from the total ACC content (hydrolyzed extracts). The ACC content in the extracts was determined by measuring the amount of ethylene converted from ACC according to the method of Lizada and Yang (1979).

Measurement of Ethylene

A Cromatix KNK–2000 gas chromatograph equipped with a flame ionization detector and a stainless steel column (3 m length, 1/8" inner diameter) filled with alumina was used to measure ethylene. Ethylene concentration was calculated from a calibration plot of known concentrations of standard ethylene samples.

Chemicals

The following compounds were used: ACC from Sigma Chemical Company (USA), Ethephon (commercial Ethrel containing 48 % 2-chloroethylphosphonic acid) from Amchem Products Inc. (USA). All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

An actively growing lupin hypocotyl of 8 d showed a capacity to convert exogenous ACC to ethylene (EFE capacity), at a rate of about 20 nmol of ethylene h^{-1} g^{-1} d.m. (Table 1), equivalent to 33.3 nl h^{-1} g^{-1} f.m. or 2 nmol h^{-1} g^{-1} f.m. The EFE capacity was insensitive to cycloheximide, in accordance with previous reports (Yu *et al.* 1979, Taylor *et al.* 1988). On the other hand, the production of ethylene by hypocotyl sections in the absence of ACC was linear during a period of 4.5 h, the rate of emission being 3 nmol h^{-1} g^{-1} d.m. (Table 1). This rate of ethylene production corresponds to 5.0 nl h^{-1} g^{-1} f.m. or 0.3 nmol h^{-1} g^{-1} f.m.

The level of total ACC in an 8 d old hypocotyl was measured as being 64.2 nmol ACC g^{-1} d.m. (SE = ± 3.9). The amount of free ACC was scarce (1.8 ± 0.2 nmol g^{-1}

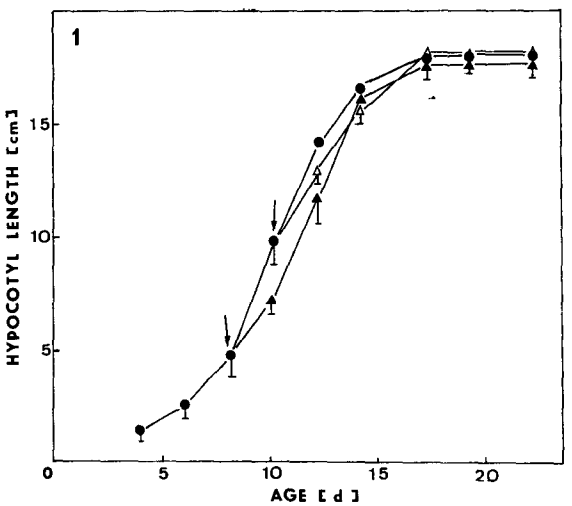

Fig. 1. Influence of ACC on the growth of lupin hypocotyls. The hypocotyl length at different ages in the control (•) and in the seedlings sprayed with 1 mM (△) or 5 mM (▲) ACC is indicated. The age at which treatment (arrow) was carried out was 8 and 10 d for 5 and 1 mM ACC respectively. Data correspond to the mean values of 10 plants. Bars denote ± SE when larger than symbols.

d.m.). Thus the conjugated ACC represented the greatest part of the ACC in the etiolated lupin hypocotyl at this age.

The values measured for EFE capacity, endogenous ACC level and ethylene production were comparable to those reported in other vegetative plant tissues where ethylene has been proposed as playing a physiological role (Schierle and Schwark 1988).

From 7 to 21 d, the growth of etiolated lupin hypocotyls is basically due to cell elongation (Ortuño *et al.* 1988). During this period, the application of exogenous ACC produced a transient inhibition of the hypocotyl growth during a two-day period after the treatment, although in the later growth period the same final

Table 1

EFE capacity and ethylene production in etiolated lupin hypocotyl sections. Ethylene evolved from sections in presence of ACC (EFE capacity) or in absence of ACC (ethylene production) at different times of incubation is indicated. The mean values ± SE of three replicates are shown.

Time [h]	Ethylene [nmol g^{-1} d.m.]	
	− ACC	+ ACC
1.5	4.3 ± 0.2	17.5 ± 1.2
3.0	9.1 ± 0.3	61.2 ± 2.4
4.5	13.6 ± 0.2	97.3 ± 9.8

hypocotyl length as in control was attained (Fig. 1). The existence of EFE activity in the hypocotyls (Table 1) suggests that this growth inhibition was probably due to the ethylene produced from exogenous ACC.

In ethephon-sprayed seedlings, the effects on hypocotyl growth varied according to both the age at which the treatment was carried out and the concentration of ethephon (Fig. 2). Similarly to the ACC treatment, the lowest ethephon concentration assayed (0.66 mM) produced only a transient reduction in growth, but higher concentrations reduced the final length of the hypocotyl in relation to the control. In seedlings sprayed at 8th day, a second spray at 14th day produced a new inhibition of hypocotyl growth (Fig. 2).

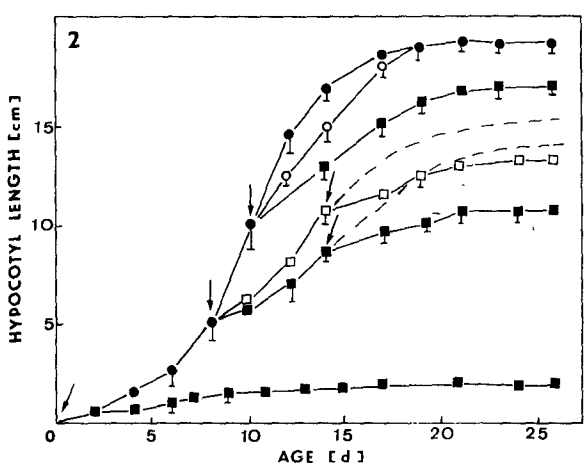

Fig. 2. Influence of ethephon on the growth of lupin hypocotyls. At different ages the hypocotyl length of control (•) and ethephon treated plants (0.66 mM (○), 3.3 mM (□) or 6.6 mM (■) is represented. Arrows indicate the ages at which treatments were carried out: incubation of seeds (0 d); sprays to the seedlings (8 or 10 d). Some seedlings sprayed at 8th d were treated again at 14th d; discontinuous lines (– – –) represent the growth kinetics of plants treated only at 8th d. Data correspond to the mean values of 10 plants. Bars denote ± SE when larger than symbols.

When the effects of ACC (Fig. 1) were compared with those of ethephon (Fig. 2), it was demonstrated that the latter inhibited the growth more efficiently. Thus, a low concentration of ethephon (0.66 mM) produced a similar growth response to that obtáined with a higher concentration of ACC. Furthermore, 3.3 mM ethephon produced a higher growth inhibition than 5 mM ACC, in 8 d old seedlings.

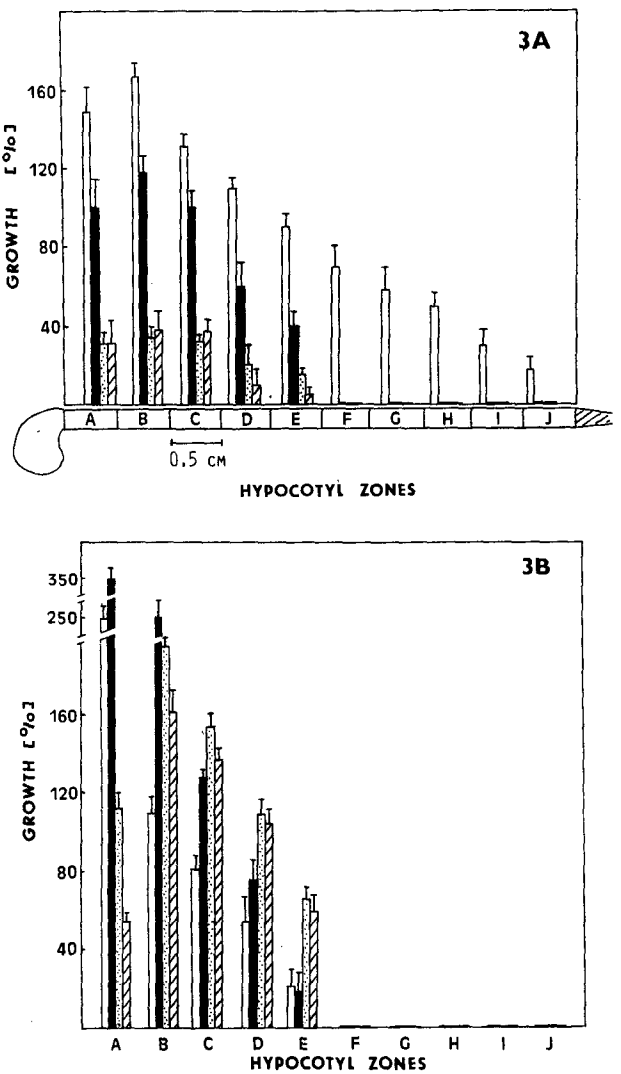

Fig. 3. Effect of ACC and ethephon on the distribution of growth along the lupin hypocotyl. The relative growth rate in two growth periods (3A 8–10 d, 3B 10–14 d) is indicated for the different zones in the hypocotyl (A–J). The treatments were carried out on 8 d old seedlings (Fig. 1 and 2). At this age, ten zones of 0.5 cm length were marked along the hypocotyl.
Control – empty columns, 5 mM ACC – full columns, 6.6 mM – dashed columns and 3.3 mM – dotted columns – ethephon. Data correspond to the mean values of 10 plants. Bars denote ± SE.

Considering that 95 % of endogenous ACC was conjugated ACC, it is reasonable to assume that the etiolated lupin hypocotyls contained an active malonyl ACC synthase which conjugated the main part of the exogenous ACC added to intact plants. In fact, according to the EFE activity measured in sections (Table 1), only 2.9 % of added ACC can be transformed into ethylene after a 48 h period, if the conversion rate remains constant. So, the difference in the growth responses between the ACC and the ethephon treatments can be imputed to the different level of ethylene produced by these treatments.

The inhibition produced by ethephon increased as the age of the seedlings diminished from 10 to 8 d (Fig. 2). This can be explained by comparing Figs. 3a and 3b, which show that the number of zones exhibiting growth in the control was greater at 8 d and consequently the number of elongating cells also greater. The highest inhibition of growth was obtained when seeds were incubated in ethephon (Fig. 2). Here, inhibition of cell division, in addition to that of cell elongation, can be suspected.

A thickening of the hypocotyl was observed after the double treatment with ethephon as well as in the ethephon-incubated seed assay. The cell diameter in both pith and cortex increased after the double treatment with 6.6 mM ethephon, mainly in the apical zone of the hypocotyl (Table 2). Similar effects on cell division and cell growth have been described for ethylene-treated plants (Neljubow 1901, Sauerbrey *et al.* 1987).

When the growth rate of different zones marked in the hypocotyls was measured, it was noted that the growth inhibition produced by ACC or ethephon varied along the hypocotyl depending on the localization of the zones (Fig. 3). After treatment at 8th day, the growth in the subsequent 2-d period (from 8th to 10th day) ways completely inhibited in the basal zones (F to J) by both ACC and ethephon (Fig. 3a). These zones exhibit small growth rates in the control, thus indicating that cells were also growing slowly (Ortuño *et al.* 1988). The remaining zones (A to E) show a partial inhibition, the effect of ethephon being higher than that of ACC. These results suggest that the sensitivity of the cells to ethylene was dependent on the cell growth

Table 2

Effect of ethephon on the cellular diameter. The measurements were made at 21 d of age after a double treatment with 6.6 mM ethephon (Fig. 2) in cortex and pith cells (second layer near the vascular cylinder) from different zones of the hypocotyl (Apical = zone B, and Basal = zone J). The data are expressed in μM and correspond to the mean of 10 measurements ± SD

	Cortex	Pith	Treatment
Apical	64 ± 13	59 ± 13	Control
Basal	71 ± 8	57 ± 9	
Apical	107 ± 13	125 ± 25	Ethephon
Basal	78 ± 11	77 ± 7	

Table 3

Final length of the different zones of the hypocotyls. In 8 day old seedlings, ten zones of 5 mm length were marked (Fig. 1 and 2). Sprayings with ACC (5 mM) or ethephon (3.3 or 6.6 mM) were carried out at 8th d and repeated at 14th d (Fig. 2). Data represent the mean length (mm) ± SD of the zones in control or treated seedlings when hypocotyl growth ceased (21 d age). The zones not shown (F to J) maintained the initial length (5 mm) in the treated seedlings. In control seedlings the length of these zones varied from 9 mm (zone F) to 6 mm (zone J).

	Treatment			
Zone	Control	ACC	Ethephon 3.3	Ethephon 6.6
A	55 ± 4	59 ± 7	29 ± 5	20 ± 2
B	36 ± 3	46 ± 5	23 ± 6	26 ± 4
C	24 ± 2	26 ± 5	18 ± 4	16 ± 3
D	17 ± 4	16 ± 3	14 ± 4	10 ± 2
E	11 ± 3	8 ± 2	10 ± 2	8 ± 2

status. According to Fig. 3a the cells had maximum sensitivity to ethylene at the end of their elongation period, since low ethylene concentration (as presumably produced by the ACC treatment) was sufficient to nullify the elongation. Young cells from the apical zones seem to have less sensitivity since higher concentrations of ethylene were needed to increase the growth inhibition.

In the period from 10th to 14th day (Fig. 3b), the growth rate was greater in 5 mM ACC-treated plants than in the control, mainly in the apical zones (zones A and B). In the treatment of 3.3 and 6.6 mM ethephon, the growth rate was also greater than in the control except in zone A. So, the growth inhibition period in treated seedlings (8th to 10th day, Fig. 3a) was followed by a period (from 10th to 14th day) in which the growth rates surpassed those of the control (Fig. 3b). This phenomenon is reminiscent of the so-called "overshoot" described by Miller and Kramer (1965): pine seedlings submitted to water stress exhibit, when rewatered, higher growth rates than controls. The "overshoot" phenomenon has been related to the hormonal response to water stress (Itai and Benzioni 1976). The participation of ethylene in this response has been frequently reported (McMichael *et al.* 1972, Ben-Yehoshua and Aloni 1974, Apelbaum and Yang 1981). Our results show that the "overshoot" depends on ethylene concentration and the localization along the hypocotyl (Fig. 3b). Lowest concentrations of ethylene (ACC treatment) produced the highest increases in growth rates. The phenomenon did not occur in the basal zones F to J.

From observing the final lengths of the hypocotyl zones (Table 3) it can be deduced that ethephon reduces the cell length regardless of localization, whereas ACC reduces the cell length in the basal zones but maintains or even increases the length in the apical zones, as compared to the control. This last effect of ACC was due to the "overshoot" in these zones.

From Table 3 it can be deduced that final length of the apical cells was dramatically reduced as a result of the double treatment with ethephon. However, the cell

diameter was greatly increased in these cells (Table 2). Although the young cells were less sensitive to the ethylene action, they were exposed to ethylene during a longer period of time and consequently the effects of ethylene on both the cell elongation and the radial expansion, were greater than in older cells.

The present results suggest that the differences in the cell growth response against the ethylene are related to the different growth status of the cell along the hypocotyl at the moment of treatment. Depending on the cell growth status, changes in both the sensitivity to ethylene and the capacity to metabolize ACC, ethephon and ethylene can occur.

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