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Inhibition of Ethylene Production in Sunflower Cell Suspensions by the Plant Growth Retardant BAS 111..W: Possible Relations to Changes in Polyamine and Cytokinin Contents

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Abstract. At a concentration of 10^{-5} mol · L^{-1} the triazole-type growth retardant BAS 111..W completely inhibited the transiently elevated ethylene production in the exponential growth phase of heterotrophic sunflower cell suspensions. This effect, which could not be restored by adding gibberellin A_3, was accompanied by transiently increased levels of 1-aminocyclopropanecarboxylic acid (ACC) in the cells, which was increasingly converted to N-malonyl-ACC. Thus, the reactions from ACC to ethylene catalyzed by the ethylene-forming enzyme appeared to be blocked by the retardant. Concomitantly, higher endogenous levels of free spermidine and particularly spermine were found over control, whereas free putrescine, the direct precursor of both polyamines, simultaneously decreased. We assume that the remaining S-adenosylmethionine from ethylene biosynthesis was increasingly incorporated into spermidine and finally spermine. A further relation appears to exist between the reduced ethylene production and enhanced levels of cytokinins in the cells. The application of both BAS 111..W and aminoethoxyvinylglycine depressed ethylene formation while immunoreactive cytokinins from isopentenyladenosine-, trans-zeatin riboside-, and dihydrozeatin riboside-type increased. By additional treatment with ACC, the effects could partially be reversed. On the other hand, stimulation of ethylene production by ACC alone or ethephon considerably lowered cytokinin levels.

Undifferentiated, auxin-dependent sunflower cell suspensions produce peak levels of ethylene during the exponential growth phase (Sauerbrey et al. 1987). Data indicated that ethylene was synthesized by the cells via the ACC pathway (Sauerbrey et al. 1987). No functional relationship has been found between growth of the cultures and ethylene formation. Thus, sunflower cell suspensions offer an appropriate system for screening and characterizing the effects of compounds on cellular ethylene biosynthesis (Sauerbrey et al. 1987, 1988). Moreover, by influencing the production of ethylene, interrelations with other endogenous bioregulators can be elucidated. Here, the biosynthesis of polyamines, which is connected with ethylene formation by S-adenosylmethionine as a common precursor (Flores et al. 1989, Slocum et al. 1984), is of special interest. Emphasis should further be laid on the capacity of ethylene to decrease the level of cytokinins (Van Staden et al. 1987). Recently, plant growth retardants of the norbornanodiazetine (e.g., tetcyclacis) and triazole (e.g., LAB 150978, uniconazole) type were shown to inhibit ethylene formation in sunflower cell suspensions, leaf discs, and intact plants of different species (Hofstra et al. 1989, Sauerbrey et al. 1987, 1988). The conversion of 1-aminocyclopropanecarboxylic acid (ACC) to ethylene appeared to be affected (Grossmann 1990, Kraus et al. 1992, Sauerbrey et al. 1987). Initial results provided evidence that the triazole retardant BAS 111..W, which is currently being developed for agricultural use (Luib et al. 1987), influences ethylene biosynthesis similarly (Grossmann 1990).

This effect of N-heterocyclic retardants is in addition to their growth regulation induced by inhibition of cytochrome P-450-dependent oxidative reactions involved in gibberellin and sterol synthesis (Grossmann 1990, Rademacher 1992). It could be caused by directly influencing the ethylene-forming enzyme, which has also been suggested to involve a cytochrome P-450-mediated reaction (Kraus et al.

1992), or by an indirect effect on membrane properties (Sauerbrey et al. 1988). The aim of the present paper was to characterize BAS 111..W induced changes in ACC and N-malonyl-ACC (MACC) content and ethylene production of sunflower cell suspensions in more detail. In connexion with this, we have also studied the influence of the retardant on free polyamine titers and the levels of immunoreactive cytokinins of isopentenyladenosine (IPA)-, trans-zeatin riboside (ZR)-, and dihydrozeatin riboside (DZR)-type found in the cells. Aminoethoxyvinylglycine (AVG), an inhibitor of ACC synthase, and 2-chloroethyl-phosphonic acid (ethephon), which is spontaneously degraded to ethylene (Yang and Hoffman 1984), were included in the study for specific modulation of endogenous ethylene and analysis of its effect, particularly on cytokinin levels.

Materials and Methods

Chemicals

The growth retardant used was 1-phenoxy-3-(1H-1,2,4-triazole-1-yl)-4-hydroxy-5,5-dimethyl-hexane(BAS 111..W, racemic mixture, from BASF Aktiengesellschaft, Ludwigshafen, Germany). Ethephon and ACC were obtained from Calbiochem (La Jolla, CA, USA) and AVG from Fluka AG (Buchs, Switzerland).

Cell Culture

Heterotrophic cell suspensions of sunflower (*Helianthus annuus* L. cv. Spanners Allzweck), derived from shoots, were cultivated in a modified Murashige-Skoog medium (Seitz and Richter 1970) with amino acids (including 0.05 mg · L^{-1} DL-methionine), vitamins, 2 mg · L^{-1} indole-3-acetic acid, 0.2 mg · L^{-1} kinetin, and 0.1 mg · L^{-1} 2,4-D. The cells, grown in culture for 8 years, were subcultured at intervals of 7 days in the exponential growth phase under the following conditions: 250 ml Erlenmeyer flasks containing 80 ml of cell suspension (with 15 ml of inoculum) were shaken on a rotary shaker at 110 rpm in the dark at 25°C. Stock solutions of the chemical compounds 1000-fold-concentrated (to the final concentration in the experiment) were prepared in acetone. The solutions were pipetted into the flasks and the solvent allowed to evaporate before adding freshly subcultured cells from the exponential growth phase. At various times after treatment, aliquots of cell suspensions were removed from four replicate flasks for determination of ethylene production. The remaining cell material was harvested by filtration and copiously washed with 200 ml bidistilled water. The total fresh weight was determined and the cell material was frozen at −80°C, then homogenized with a mortar and pestle under liquid N_2. All experiments were repeated at least twice and proved to be reproducible. The results of a representative experiment are shown.

Determination of Ethylene

Suspended cells (2 ml with ~0.1–0.5 g cells dependent on the growth stage) were incubated for 6 h in sterile plastic tubes (17 mm in diameter, 100 mm in height, 15.5 ml volume; Greiner, Frickenhausen, Germany; three replicate tubes per flask) capped with a rubber septum and shaken at 400 rpm and 25°C in the dark. A 1 ml gas sample of head space was withdrawn with a syringe, and ethylene was quantified by gas chromatography (alumina column, flame ionization detector; see Sauerbrey et al. 1987). Ethylene production was linear during the 6 h incubation period and with regard to the quantity of cell mass.

Determination of ACC and MACC

For determination of ACC and ACC conjugate, 100 mg of powdered cell material were extracted overnight in 5 ml 70% ethanol. After centrifugation, a 3 ml aliquot of the supernatant was evaporated to dryness in vacuo, then brought to a volume of 2.75 ml with 2 ml double-distilled water and 0.75 ml 2 N KOH. The ACC content was assayed by converting it to ethylene according to Lizada and Yang (1979). Ethylene was measured by gas chromatography. MACC was quantified by hydrolyzing it to ACC by treatment with 2 N HCl at 110°C for 4 h. The concentration of ACC was similarly assayed (Lizada and Yang 1979). Recovery was about 50%, as checked at various levels of added standard ACC in the same ethanolic extract used for ACC determination. The amount of MACC was determined by subtracting the value of ACC in the nonhydrolyzed sample from that in the hydrolyzed sample.

Determination of Cytokinins

Cytokinins were extracted and analyzed immunologically as described previously (Grossmann et al. 1991). Powdered cell material (2 g) was extracted four times for 1 h in 8 ml 80% aqueous methanol (containing 10 mg · L^{-1} butylated hydroxytoluene) at 4°C. After centrifugation, the supernatants were combined, concentrated by rotary evaporation to dryness, and redissolved in 1 ml of double-distilled water. Further purification and separation of the cytokinins in the aqueous extract was carried out by HPLC (Grossmann et al. 1991). Then, 0.8 ml of the cleared sample was injected onto a reversed-phase Nucleosyl 120–5 μm C_{18} column (Macherey-Nagel, Düren, Germany), which had been previously equilibrated with 100 mmol · L^{-1} acetic acid. The mobile phase consisted of a linear gradient from 100 mmol · L^{-1} acetic acid to 100% methanol. Gradient sweep time was 30 min at a flow rate of 3 min^{-1}. The fractions containing IPA (25.8 min), DZR (19.6 min), and ZR (19.4 min) were collected, evaporated to dryness, and dissolved in 50 mmol · L^{-1} tris(hydroxymethyl)aminoethane-buffered-saline, pH 7.8, 10% methanol for enzyme-immunoassay using monoclonal antibodies (mcAB) for IPA (Weiler and Spanier 1981), ZR, and DZR (Eberle et al. 1986). [For cross-reactivities of the mcAB against IPA-, ZR-(clon J3-I-B3), and DZR-(clon J23-II-B1) type cytokinins, see Weiler and Spanier (1981) and Eberle et al. (1986).] No interference with the phytohormonal determinations resulted from significant cross-reactivities of the mcAB against cytokinin-O and N-glucosides [e.g., isopentenyl adenine-9-glucoside (154% for IPA-mcAB), zeatin-9-glucoside (19% for ZR-mcAB), dihydrozeatin-9-glucoside (63% for DZR-mcAB); J. Kwiatkowski and K. Grossmann, unpublished data] because the fractions containing these glucosides were well separated by the HPLC-gradient used. The detection limit was 1 pmol as estimated from standard curves. All samples were assayed at least in triplicate and the concentrations were expressed as the equivalents of cytokinins in pmol · g^{-1} fresh weight. In immunohistograms of the HPLC

separations of the cell extracts, no immunoreactive material was detected other than that eluting with the standards. No indication of interference was detected at various levels of added standard hormone in dilution analysis of the final purified extract fractions. Recovery, as checked with internal radiolabeled standards in the same extract used for immunoassay, was above 70% for all cytokinins.

Free Polyamine Extraction and Analysis

Two hundred milligrams of powdered cell material were extracted in 5 ml of 10% (vol/vol) perchloric acid. Free polyamines were determined in the supernatant after centrifugation (20,000 *g* for 20 min) and dansylation according to Langebartels et al. (1991). Dansylated products were extracted into toluene and separated by HPLC on a Spherisorb ODS II 5 μm column (250 × 4.6 mm) with a methanol-water gradient (65–95% methanol, 25 min) at a flow rate of 1 ml min^{-1}. Polyamine contents were determined using a spectrofluorimeter at 360 nm (excitation) and 510 nm (emission). Putrescine, spermidine, and spermine standards were run each time polyamine levels were analyzed.

Results

Transiently elevated ethylene production in heterotrophic sunflower cell suspensions has been found in the exponential growth phase (Fig. 1). As previously shown for tetcyclacis and LAB 150978 (Sauerbrey et al. 1987), the triazole-retardant BAS 111..W completely abolished this peak in ethylene formation at a concentration of 10^{-5} mol · L^{-1} (Fig. 1). The effect could not be overcome by the addition of gibberellin A_3 (not shown). Concomitantly, the endogenous ACC content transiently increased with maximum levels between days 5 and 7 after subculture (Fig. 1). Then, ACC levels sharply declined while MACC accumulated (Fig. 1). Thus, ACC appeared to be increasingly converted to MACC. This is in contrast to the effects of tetcyclacis and LAB 150978, both of which seem to be inhibitors of the conjugation of ACC by malonyltransferase (Grossmann 1990). However, as a common effect of these N-heterocyclic retardants, BAS 111..W also effectively blocked the conversion of ACC to ethylene. This conclusion was further supported by dose–response curves with the retardant showing that the decrease in ethylene formation was closely correlated with rising ACC and MACC levels in the cells (Fig. 2). Thereby, growth reduction occurred only at higher retardant concentrations, which suggests that the effects on ethylene formation are not functionally related to cell culture growth.

Compared with ethylene production, free putrescine levels in control cultures of sunflower showed similar, but less pronounced changes during the growth cycle (Fig. 3). A slight peak on day 5 was subsequently followed by a gradual decline. A greater decline in concentration with the age of the cells was found for spermidine and, particularly, spermine. However, their contribution to the total free polyamine levels in the cells amounted to only 10% and 0.5%, respectively (Fig. 3). Treatment with BAS 111..W significantly decreased the putrescine content of the cells during incubation (Fig. 3). Conversely, gradually elevated titers of spermidine and spermine to approximately 30% and 130%, respectively, over control levels were observed, particularly in the second half of the growth cycle (Fig. 3).

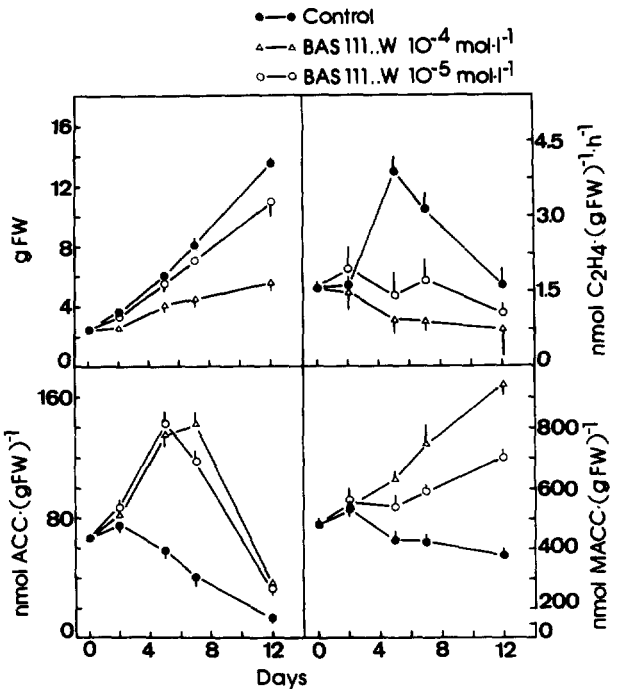

Fig. 1. Effect of BAS 111..W on growth (measured by fresh weight) and production of ethylene and content of ACC and MACC in sunflower cell suspensions. Vertical bars represent SE of the mean.

In another set of experiments designed to either decrease or increase ethylene formation in sunflower cell suspensions, we investigated possible interactions with changes in the endogenous cytokinin content. The reduction in ethylene production by both BAS 111..W and AVG, an inhibitor of ACC-synthase (Yang and Hoffman 1984), was accompanied by enhanced levels of cytokinins of the DZR-, ZR-, and IPA-type (Tables 1 and 2). For example, 10^{-5} mol · L^{-1} AVG led to an approximately 90% decrease in ethylene formation and ACC content, whereas cytokinin-like levels increased by more than 100% compared to control.

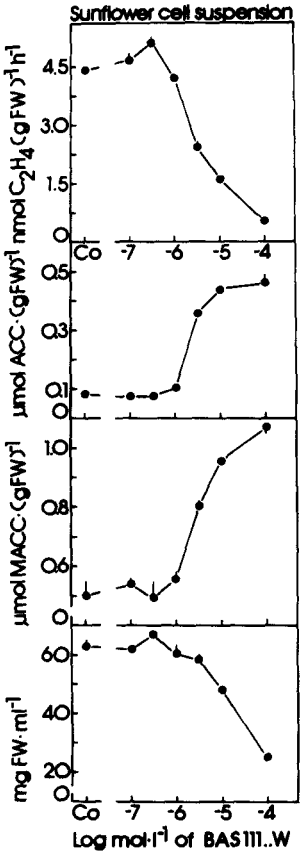

Fig. 2. Dose–response curves of the effect of BAS 111..W on ethylene formation, ACC and MACC content, and growth (measured by fresh weight) in sunflower cell suspensions 6 days after treatment. Vertical bars represent SE of the mean.

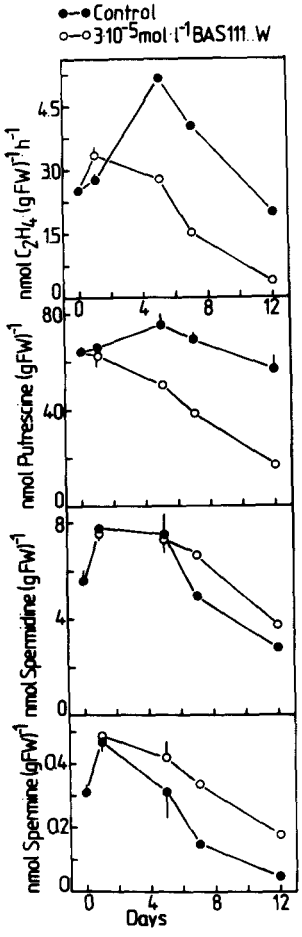

Fig. 3. Effect of BAS 111..W on ethylene formation and on free polyamine titers in suspension cells of sunflower. Vertical bars represent SE of the mean.

This rise in cytokinin content appeared to be correlated with the intensity of ethylene inhibition. On the other hand, according to their presumed site of action in ethylene synthesis, BAS 111..W stimulated ACC and MACC levels, while AVG caused their decrease (Tables 1 and 2). Thus, it may be concluded that both compounds exerted their effect on cytokinin contents by blocking ethylene formation. This hypothesis was supported when ACC was applied in addition to BAS 111..W and AVG (Table 1). Here, ACC was able to partially neutralize both ethylene inhibition and cytokinin accumulation. This result also demonstrated that no correlation exists between the reduction of cell fresh weight and the induced levels of cytokinins. In the case of AVG, the inhibition of cell fresh weight accumulation might be explained by cell-damaging effects of the compound (Grossmann et al. 1991).

The application of ACC alone or ethephon generating an up to threefold increase in ethylene emanation reduced titers of DZR- and IPA-type cytokinins by ~40% and 80% of control, respectively (Table 1).

Discussion

In addition to their effects on cell division and elongation, N-heterocyclic growth retardants have been reported to cause a variety of other physiological effects, including delayed senescence and enhanced resistance to fungal infections (Davies et al. 1988, Fletcher and Hofstra 1988, Grossmann 1990, 1992). For example, in oilseed rape, the lowering of seed losses due to pod shatter, the enhanced translocation of assimilates into the growing seed, and the reduced susceptibility of the pod wall to fungal infections have been associated with a retardant-induced delay in pod senescence (Luib et al. 1987). The extended longevity has been attributed to a

Table 1. Influence of BAS 111..W, AVG, ACC, and ethephon on growth (measured by fresh weight) and production of ethylene and endogenous content of ACC, MACC, and immunoreactive cytokinins in sunflower cell suspensions determined after 6 days of incubation.

Compound, concentration (mol × L^{-1})	Fresh weight (g)	Ethylene formation (nmol × g FW^{-1} × h^{-1})	ACC content (nmol × g FW^{-1})	MACC content (nmol × g FW^{-1})	DZR content (pmol × g FW^{-1})	IPA content (pmol × g FW^{-1})
Control	11.17 ± 0.28a	1.84 ± 0.05 (100)a	7.56 ± 0.53c	74.4 ± 3.0c	40 ± 9 (100)c	59 ± 2 (100)b
BAS 111..W, 10^{-5}	8.68 ± 0.05b	1.61 ± 0.07 (88)a	38.45 ± 2.25b	157.0 ± 9.5b	69 ± 6 (173)c	87 ± 6 (148)b
AVG, 10^{-5}	7.13 ± 0.27c	0.20 ± 0.01 (11)c	0.78 ± 0.01e	31.4 ± 0.9d	109 ± 13 (273)b	146 ± 38 (248)a
BAS 111..W, 10^{-5} + AVG, 10^{-5}	6.09 ± 0.08d	0.25 ± 0.01 (14)c	3.36 ± 0.09d	39.1 ± 1.3d	149 ± 8 (373)a	77 ± 9 (131)b
BAS 111..W, 10^{-5} + AVG, 10^{-5} + ACC, 10^{-4}	5.96 ± 0.09d	1.13 ± 0.03 (61)b	201.42 ± 12.73a	737.8 ± 5.4a	110 ± 18 (275)b	64 ± 4 (109)b
Control	7.27 ± 0.11b	1.40 ± 0.06 (100)c	29.9 ± 1.6b	215.2 ± 5.4b	21 ± 0.8 (100)a	43 ± 1.3 (100)a
ACC, 10^{-4}	8.04 ± 0.07a	2.65 ± 0.19 (189)b	230.0 ± 12.3a	732.1 ± 10.7a	13 ± 0.3 (62)b	9 ± 1.5 (21)b
Ethephon, 10^{-4}	8.05 ± 0.05a	4.22 ± 0.19 (301)a	24.8 ± 1.3b	173.2 ± 3.6c	14 ± 1.3 (67)b	10 ± 0.8 (23)b

Data are means ± SE. Values followed by a common letter are not significantly different (P = 0.05; in the case of DZR, P = 0.1, Duncan's multiple range test). ZR contents were below the detection limit. Values in brackets represent percent of control.

Table 2. Influence of AVG on growth (measured by fresh weight) and production of ethylene and endogenous content of ACC, MACC, and immunoreactive cytokinins in sunflower cell suspensions determined after 6 days of incubation.

Compound concentration (mol × L^{-1})	Fresh weight (g)	Ethylene formation (nmol × g FW^{-1} × h^{-1})	ACC content (nmol × g FW^{-1})	MACC content (nmol × g FW^{-1})	DZR content (pmol × g FW^{-1})	ZR content (pmol × g FW^{-1})
Control	7.84 ± 0.13a	3.77 ± 0.05 (100)a	72.4 ± 2.9a	345.9 ± 7.3a	34.9 ± 0.7 (100)b	9.9 ± 0.4 (100)c
AVG, 10^{-6}	6.43 ± 0.12b	2.56 ± 0.04 (68)b	31.0 ± 1.5b	182.0 ± 2.1b	41.6 ± 2.1 (119)b	22.6 ± 0.4 (228)b
AVG, 10^{-5}	3.71 ± 0.08c	0.07 ± 0.01 (2)c	5.5 ± 1.3c	170.6 ± 10.9b	67.7 ± 5.8 (194)a	25.0 ± 0.5 (253)a

Data are means ± SE. Values followed by a common letter are not significantly different (P = 0.05, Duncan's multiple range test). IPA contents were not determined. Values in brackets represent percent of control.

shift in the plant's hormonal status, favoring increases in the senescence-delaying cytokinins against the senescence hormones ethylene and ABA (Grossmann 1992, Grossmann et al. 1991). Polyamines also exhibit antisenescence properties, the order of activity being spermine > spermidine > putrescine (Slocum et al. 1984). This effect may be based on their potential to stabilize membranes, to act as antioxidants, and to inhibit ethylene formation (Bors et al. 1989, Flores et al. 1989).

Furthermore, polyamines are known to respond to adverse environmental conditions. External application of polyamines reduced, for example, leaf damage in tobacco caused by ozone fumigation (Bors et al. 1989, Langebartels et al. 1991). The levels of polyamines are also influenced by N-heterocyclic retardants (Grossmann 1990). Increases in spermidine and spermine content induced by the pyrimidine-type retardant ancymidol and the triazole paclobutrazol have been detected in roots of apple seedlings (Wang and Faust 1986) and in uniconazole-treated mung bean seedlings (Hofstra et al. 1989). Preliminary data from our laboratory derived from studies with seedlings of barley and soybean and cell suspensions of oilseed rape treated with BAS 111..W also pointed in this direction (H. Koehle and K. Grossmann, unpublished data). In this context, Roberts et al. (1984) have demonstrated that an inhibition of ACC production in ethylene biosynthesis resulted in an enhanced flux of methionine-derived S-adenosylmethionine into polyamines. Decarboxylated S-adenosylmethionine donates aminopropyl groups to putrescine, subsequently forming the triamine spermidine and the tetramine spermine (Slocum et al. 1984). Thus, Hofstra et al. (1989) hypothesized that uniconazole increases spermine levels in etiolated mung bean seedlings by inhibiting ethylene synthesis.

The use of sunflower cell suspensions provides a more detailed insight into the influence of a N-heterocyclic retardant on ethylene and polyamine synthesis. When the conversion of ACC to ethylene

was blocked by treatment with BAS 111..W, the levels of spermidine and, more markedly, spermine increased relative to controls. We propose that S-adenosylmethionine diverted from ethylene biosynthesis was increasingly incorporated into spermidine and finally spermine. Consequently, free putrescine in the cells, as the direct precursor of these biologically more active polyamines, simultaneously decreased. On a molar basis, the reduction in free putrescine content was more pronounced than the increases in spermidine and spermine. Thus, additional influences on polyamine conjugation or putrescine synthesis cannot be ruled out. However, this does not detract from the proposal that enhanced spermidine and spermine titers could significantly contribute to the regularly observed antisenescence effects of N-heterocyclic retardants. Furthermore, polyamines are known to reduce ethylene levels both by inhibition of ACC synthase and by conversion of ACC to ethylene (Flores et al. 1989, Li et al. 1992). Once accumulated, polyamines may therefore amplify the effects of growth retardants in inhibiting ethylene biosynthesis.

In plants, however, cytokinins are thought to be the dominant senescence-retarding factors (Nooden and Leopold 1988). The content of this group of phytohormones in various plant material has also been found to be considerably increased by N-heterocyclic retardants (Fletcher and Arnold 1986, Grossmann et al. 1987, 1991, Izumi et al. 1988). In particular, after soil treatment of pumpkin seedlings with BAS 111..W, a close correlation was observed between a reduced loss in total chlorophyll and increasing levels of DZR- and ZR-type cytokinins in the senescing cotyledons (Grossmann et al. 1991). Both types of cytokinin also accumulated in intact cotyledons of oilseed rape when their chlorophyll synthesis was stimulated by BAS 111..W (Grossmann et al. 1991). In these cases, there may also be a causal relationship between retardant-caused inhibition of ethylene biosynthesis and enhanced levels of cytokinins. The results reported here give support to this hypothesis. In sunflower cell suspensions, the application of both BAS 111..W and AVG depressed ethylene formation, while cellular cytokinin levels increased. By additional treatment with ACC, the effects could be reversed. Thus, it seems that BAS 111..W does not directly interfere with cytokinin biosynthesis or metabolism but acts rather indirectly by inhibiting ethylene formation. A possible explanation of this effect may be the capacity of ethylene to decrease the level of cytokinins in plant tissues (Van Staden et al. 1987), possibly by accelerating their degradation (Bollmark and Eliasson 1990). On the other hand, an ethylene-mediated alteration in the membrane permeability of the suspension cells leading to reduced uptake of kinetin from culture medium might also result in changed contents of cellular cytokinins. It will be the aim of further studies to investigate the biochemical role of ethylene in controlling endogenous polyamine and cytokinin levels. Here, certain N-heterocyclic growth retardants can be favorably used to manipulate ethylene synthesis.

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