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Role of polyamines on *de novo* shoot morphogenesis from cotyledons of *Brassica campestris* ssp. *pekinensis* (Lour) Olsson *in vitro*

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Summary. The promotive effect of ethylene inhibitors (EIs), i.e. $AgNO_3$ and aminoethoxyvinylglycine (AVG) on *de novo* shoot regeneration from cultured cotyledonary explants of *Brassica campestris* ssp. *pekinensis* cv. Shantung in relation to polyamines (PAs) was investigated. The endogenous levels of free putrescine and spermidine in the explant decreased sharply after 1-3 days of culture, whereas endogenous spermine increased, irrespective of the absence or presence of EIs. $AgNO_3$ at 30 μM did not affect endogenous PAs during two weeks of culture. In contrast, explants grown on medium containing 5 μM AVG produced higher levels of free putrescine and spermine which increased rapidly after three days and reached a peak at 10 days. An exogenous application of 5 mM putrescine also resulted in a similar surge of endogenous free spermine of the explant. More strikingly, shoot regeneration from explants grown in the presence of 1-20 mM putrescine, 0.1-2.5 mM spermidine, or 0.1-1 mM spermine was enhanced after three weeks of culture. However, exogenous PAs generally did not affect ethylene production, and endogenous levels of 1-aminocyclopropane-1-carboxylate (ACC) synthase activity and ACC of the explant. This study shows the PA requirement for shoot regeneration from cotyledons of *B. campestris* ssp. *pekinensis in vitro*, and also indicates that the promotive effect of PAs on regeneration may not be due to an inhibition of ethylene biosynthesis.

Abbreviations: PAs, polyamines; AVG, aminoethoxyvinylglycine; SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylate; EIs, ethylene inhibitors

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Key words: *Brassica campestris* ssp. *pekinensis*, Chinese cabbage, ethylene biosynthesis, polyamine biosynthesis, shoot morphogenesis, tissue culture

Introduction

Polyamines (PAs) play an important role in cell growth and division in both prokaryotes and eukaryotes (Evans and Malmberg 1989; Heby and Persson 1990). In PA biosynthesis, ornithine and arginine form putrescine via ornithine decarboxylase and arginine decarboxylase. Putrescine is used as a precursor, together with the aminopropyl group derived from S-adenosylmethionine (SAM) after decarboxylation, to synthesize spermidine and spermine (Evans and Malmberg 1989; Smith 1990). In plants, the diamine putrescine, the triamine spermidine and tetramine spermine are always present in amounts varying from micromolar to more than millimolar (Galston and Sawhney 1990). Changes in PAs have been related to environmental stresses (Flores 1990; Kuehn et al. 1990) and various physiological processes including rhizogenesis (Jarvis et al. 1985), development of flowers (Gerats et al. 1988) and fruits (Glaston and Sawhney 1990), and senescence (Muhitch et al. 1983). Evidence from several lines of studies has indicated that PAs may be important for cell growth and somatic embryogenesis of *Daucus carota* (Montague et al. 1979; Feirer et al. 1984; Fienberg et al. 1984) and *Medicago sativa* (Meijer and Simminds 1988) and formation of floral buds from thin-layer explants of *Nicotiana tabacum* (Torrigiani et al. 1987). However, the role of PAs on plant morphogenesis *in vitro* is not clear.

PA biosynthesis is closely associated with ethylene biosynthesis, because both compete for the same precursor, SAM (Evans and Malmberg 1989). Ethylene is a gaseous plant hormone, which is involved in regulation of a wide range of physiological responses in plants (Reid 1987). In ethylene biosynthesis, the presursor methionine is converted to SAM via the catalysis of the enzyme SAM synthetase. The subsequent reactions of SAM→1-aminocyclopropane-1-carboxylate (ACC) and ACC→ethylene are catalyzed by ACC synthase and ACC oxidase (ethylene-forming enzyme), respectively (McKeon and Yang 1987).

Brassica campestris comprises various economically important vegetable and oilseed crops, but the plants are poorly responsive to tissue culture manipulations (Narasimhulu and Chopra 1988). In culture, *B. campestris* produced high levels of ethylene causing abnormal growth and development of the plant (Lentini et al. 1988), and also inhibiting *de novo* shoot regeneration *in vitro* (Chi et al. 1991; Pua 1993). Application of AVG and $AgNO_3$, which are the inhibitors of ethylene production and action, respectively (Beyer 1976; Yang and Hoffman 1984), have resulted in high frequency shoot regeneration (70-90%) from cultured explants of several recalcitrant genotypes of *B. campestris* (Chi and Pua 1989; Chi et al. 1990, 1991; Palmer 1992) and *B. juncea* (Pua and Chi 1993). The similar promotive effect of EIs on *de novo* shoot regeneration for other plant species including monocots, e.g. *Triticum aestivum* (Purnhauser et al. 1987) and *Zea mays* (Vain et al. 1989; Songstad et al. 1991), has also been demonstrated (Pua 1993). However, knowledge regarding the regulatory role of ethylene on shoot morphogenesis *in vitro* in relation to PA metabolism has been limited. In this study, we further investigated the effect of PAs and its relationship with ethylene on *de novo* shoot formation from cultured cotyledonary explants of *B. campestris* ssp. *pekinensis*.

Materials and methods

Plant tissue culture. Cotyledons excised from three day-old aseptically germinated seedlings of *Brassica campestris* ssp. *pekinensis* (Chinese cabbage) cv. Shantung were cultured as previously described (Chi et al. 1991). Briefly, explants were placed either in a 100x25 mm Petri dish containing 30 mL N1B2 medium (Murashige and Skoog medium (1962) containing 8.8 μM benzyladenine and 5.4 μM naphthaleneacetic acid) or in a 50 mL Erlenmeyer flask containing 25 mL medium. Flasks were sealed with serum rubber stoppers to facilitate gas sampling and ethylene measurement.

Effect of exogenous PAs on shoot regeneration and/or ethylene synthesis was investigated by culturing explants on N1B2 medium supplemented with (a) 0, 0.1, 0.5, 1, 2.5, 5, 10 and 20 mM putrescine, or 0, 0.1, 0.5, 2.5 and 5 mM spermidine or spermine in Petri dishes, and (b) 5 mM putrescine, 0.5 mM spermidine or 0.1 mM spermine in culture flasks. Explants for (a) were evaluated for percent shoot regeneration after three weeks of culture, while evaluation for (b) was conducted after 0, 3, 7, 10, 17 and 21 days of culture. Percent shoot regeneration was calculated based on the number of explants forming shoots as a percentage of the total number of explants. Each treatment consisted of three replicates each with 10 explants/dish or five explants/flask. All investigations were repeated three times and the results were pooled since the difference between experiments was negligible.

Exogenous PAs were filter-sterilized and then added to the medium after autoclaving at 1 kg/cm^2 and 121°C for 15 min.

PA analysis. Explants grown on N1B2 medium in the absence or presence of EIs (30 μM $AgNO_3$ and 5 μM AVG) were analysed for endogenous free PAs (putrescine, spermidine and spermine). For explants grown on medium supplemented with 5 mM putrescine, only endogenous free spermidine and spermine were analysed.

Frozen tissues were extracted with 10% perchloric acid according to Kumar and Thorpe (1989). Both the extract and PA standards, i.e. putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride (Sigma Chem Co, USA) each at 10 nM, was benzoylated as described by Flores and Galston (1982), except that 5 μL instead of 10 μL benzyol chloride was added to 250 μL extract. After benzoylation, the extract was air-dried in a fume hood and then stored at -20°C until used. The extract was dissolved in 100 μL methanol (HPLC grade) prior to PA analysis using the Waters HPLC system equipped with a pump (model 510) and a detector (model 484). The solvent system consisted of methanol:water (60%, v/v) run isocratically at a flow rate of 1.2 mL/min. Ten microliters of benzoylated extract was eluted at room temperature through a 3.9x30 mm reverse-phase column (Waters Delta Pak C18, 15 μm particle size) connected to a guard column (Waters Guard-Pak Nova-Pak C18). The benzoylated PAs in both the standards and extract were detected at 254 nm, with the retention times of 3.58, 5.50 and 8.45 min for the respective putrescine, spermidine and spermine. The benzoylated PAs in the extract was quantified by the Waters Maxima 820 system.

Ethylene measurement. Ethylene produced by explants grown in a culture flask containing N1B2 medium supplemented with 5 mM putrescine, 0.5 mM spermidine or 0.1 mM spermine was measured as previously described (Pua and Chi 1993). In brief, prior to ethylene measurement, the serum rubber stopper was removed from the flask which was allowed to stand in a laminar flow hood for 1 h. The flask was then resealed and allowed to stand for additional 3 h. A 1 mL gas sample was withdrawn from the flask with a hypodermic syringe at different intervals (0, 3, 7, 10, 14, 17 and 21 days after culture) and measured for the ethylene content with gas chromatography.

ACC and ACC synthase assays. Explants grown in the absence or presence of exogenous PAs (5 mM putrescine, 0.5 mM spermidine and 0.1 mM spermine) for 0, 7 and 14 days were harvested, treated with liquid nitrogen, and stored at -80°C until homogenization. The frozen tissues were homogenized and extracted in a potassium phosphate buffer (100 mM Na_2HPO_4 + 100 mM NaH_2PO_4) at 4°C. The extract was then assayed for ACC synthase activity and ACC, which are the key enzyme and intermediate of ethylene biosynthesis, respectively, based on the method of Lizada and Yang (1979) with slight modification according to Pua and Chi (1993).

Results

Cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung grown in the presence of EI (30 μM $AgNO_3$ or 5 μM AVG) generally began to form shoots at the cut end of the petiole after 10 days of culture and reached maximum shoot formation (70-80%) after 14-21 days. Explants grown in the absence of EIs were poorly regenerative (20-30%). Tissues analysed for endogenous levels of free PAs, i.e. putrescine, spermidine and spermine, in explants during two weeks of culture showed that both putrescine and spermidine declined sharply after 1-3 days, whereas spermine increased, despite the presence of EIs (Fig. 1). However, accumulation of endogenous free PAs was differential in response to EIs. Explants grown on AVG medium produced 33-72% higher levels of free putrescine during 1-10 days of culture, whereas free spermine level increase rapidly after three days and reached a peak at 10 days. In contrast, $AgNO_3$ did not affect endogenous free PAs of the explants (Fig. 1). was lower (35-54%) than that of control explants during the first three days of culture, but began to

Shoot regeneration of explants was affected by exogenous application of PAs (Table 1). Explants grew vigorously at all putrescine concentrations tested (0.1-20 mM). However, spermidine and spermine at concentrations higher than 2.5 mM were generally inhibitory to growth of the explant, which became brown in color and subsequently died after three weeks. Apart from explant growth, the most striking effect of exogenous PAs was the enhanced *de novo* shoot regeneration from explants *in vitro*. The highest regeneration frequency, with each explant forming 5-20 shoots, occurred at 1-20 mM putrescine, 0.1-2.5 mM spermidine or 0.1-1 mM spermine after three weeks of culture (Table 1). PA analysis showed that explants grown in the presence of 5 mM putrescine responded similarly to AVG-treated explants, with respect to endogenous free PAs, in which spermidine decreased after 1-3 days of culture, whereas spermine increased and peaked at 10 days (Fig. 2). Nevertheless, the pattern of shoot regeneration response was generally similar

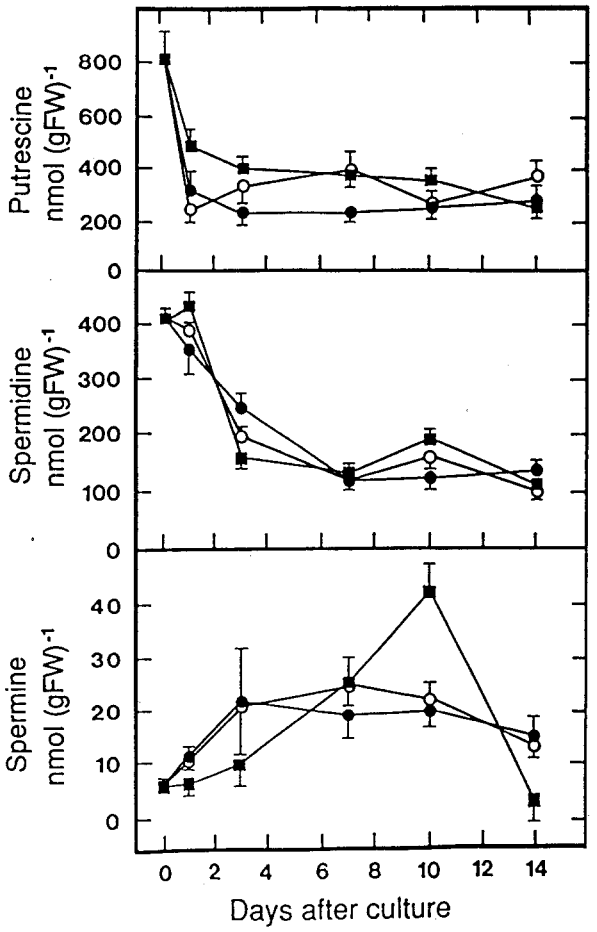

Fig.1. Effect of ethylene inhibitors on endogenous levels of free PAs of cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung during two weeks of culture. Explants were grown on N1B2 medium in the absence (●) or presence of 5 μM AVG (■) or 30 μM $AgNO_3$ (O). Vertical bars indicate SE of three measurements

Table 1. Shoot regeneration from cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung in response to exogenous PAs

Concn (mM)	Shoot regeneration (%)[a]		
	Putrescine	Spermidine	Spermine
0	37 (37 ± 3)[b]	37 (37 ± 3)	37 (37 ± 3)
0.05	nd	nd	65 (52 ± 3)
0.1	nd	70 (58 ± 7)	63 (52 ± 4)
0.5	69 (57 ± 4)	73 (60 ± 3)	61 (52 ± 4)
1	62 (52 ± 2)	66 (54 ± 2)	50 (45 ± 4)
2.5	76 (63 ± 5)	66 (55 ± 4)	2 (2 ± 2)
5	81 (68 ± 6)	47 (43 ± 4)	nd
10	84 (72 ± 7)	nd	nd
20	77 (64 ± 5)	nd	nd

[a] Explants were grown on N1B2 medium with or without exogenous PAs after three weeks of culture. Shoot regeneration percentage was calculated based on the number of explants forming shoots as a percentage of the total number of explants

[b] Each datum not in the parenthesis is the mean of the non-transformed value, while each datum in the parenthesis is mean ± SE of arsine transformed value of three replications each with 6-9 explants. nd, not determined

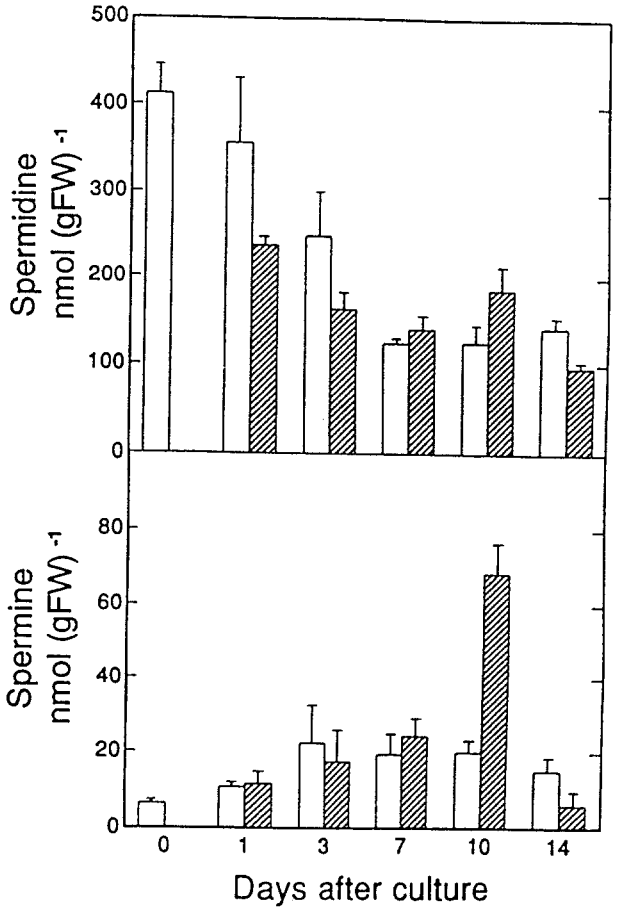

Fig. 2. Effect of exogenous putrescine on endogenous levels of free spermidine and spermine of cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung after three weeks of culture. Explants were grown on N1B2 medium in the absence (□) or presence of 5 mM putrescine (▨). Vertical bars indicate SE of three measurements

to that of EI-treated explants (Fig. 3). Explants grown in Petri dishes (Table 1) tended to be more regenerative than those grown in culture flasks (Fig. 3), but both types of explants showed similar shoot regeneration response to exogenous PAs. However, shoot regeneration could not be promoted by exogenous application of adenine (0.01-5 mM), a precursor of PA biosynthesis, which was detrimental to explants particularly at high concentrations (≥ 5 mM) (data not shown).

The effects of exogenous application of PAs on ethylene production and endogenous levels of ACC synthase activity and ACC of the explants were also investigated. Ethylene production rates increased rapidly after three days of culture, irrespective of the presence or absence of exogenous PAs (Fig. 4). For control explants and those grown in the presence of spermidine and spermine, ethylene levels remained fairly constant during 3-14 days of culture but after which began to decline, while putrescine-grown explants increased ethylene production by 1.5-2-fold and reached a peak after seven days (Fig. 4). The level of ethylene production generally corresponded to that of endogenous ACC synthase activity and ACC levels of the explants, which showed 5-9-fold increase of endogenous ACC synthase activity and 10-15-fold increase of ACC after seven days of culture (Table 2). However, while the ACC synthase activity remained stable, ACC accumulation declined sharply by 14 days (Table 2).

Discussion

We have peviously shown that the use of 10-30 μM $AgNO_3$ or 5-10 μM AVG can enhance *in vitro* shoot regeneration from seedling explants of several recalcitrant genotypes of *B. campestris* and *B. juncea* (Chi and Pua 1989; Chi et al. 1990) as well as shoot and plant regeneration from hypocotyl protoplast-derived somatic embryos of *B. juncea* (Pua 1990). However, both EIs exerted

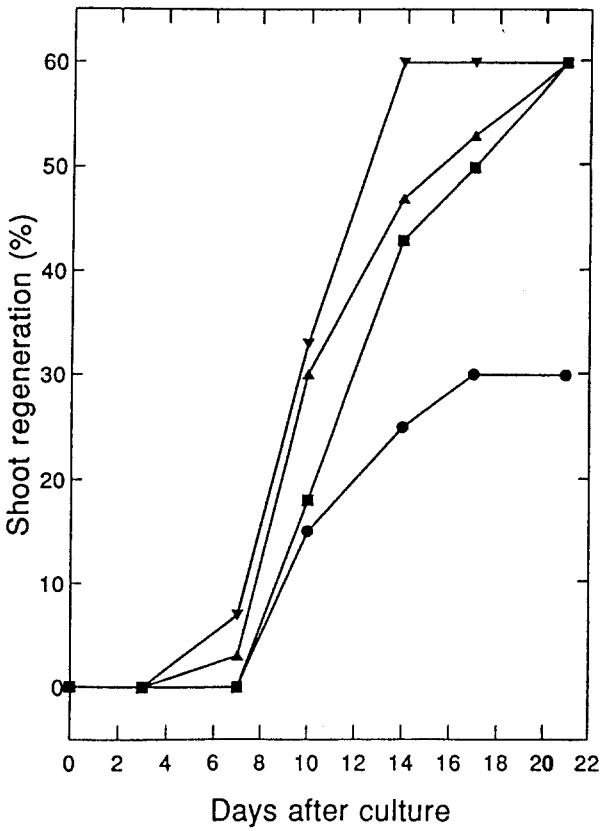

Fig. 3. Shoot regeneration from cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung in resonse to exogenous PAs during three weeks of culture. Explants were grown on N1B2 medium in the absence (●) or presence of 5 mM putrescine (■), 0.5 mM spermidine (▲) or 0.1 mM spermine (▼). Percentage of shoot regeneration was calculated based on the number of explants forming shoots as a percentage of the total number of explants. Each treatment consisted of six replicate flasks each with five explants

a distinct effect on ethylene synthesis in cultured cotyledons of *B. campestris* ssp. *pekinensis* (Chi et al. 1991) and *B. juncea* (Pua and Chi 1993).

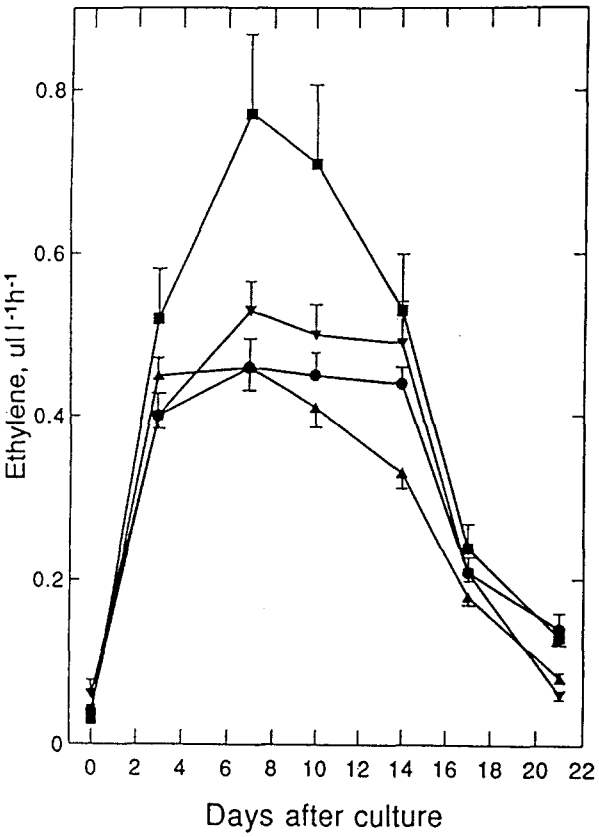

Fig. 4. Ethylene production rates in cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung in response to exogenous PAs during three weeks of culture. Explants were grown on N1B2 medium in the absence (●) or presence of 5 mM putrescine (■), 0.5 mM spermidine (▲) or 0.1 mM spermine (▼). Each treatment consisted of six replicate flasks each with five explants. Vertical bars indicate SE of six measurements

AVG treatment had resulted in a reduction of ethylene production, concomitant with a decrease of endogenous ACC synthase activity and ACC accumulation, while $AgNO_3$ was stimulatory. AVG is a potent inhibitor of ethylene biosynthesis by inhibiting the activity of ACC synthase (Yang and Hoffman 1984), while $AgNO_3$ is believed to inhibit ethylene action, but not ethylene production, by competing with ethylene for binding sites located predominantly at the intracellular membrane (Beyer 1976; Veen and Overbeek 1989). We therefore assume that the presence of AVG may have resulted in an accumulation of SAM, which may be used as a precursor for PA biosynthesis via decarboxylated SAM and spermidine intermediates. But in the case of $AgNO_3$, SAM may have been utilized for enhanced ethylene production, thereby decreasing PA synthesis. In this study, AVG treatment has resulted in higher levels of endogenous free putrescine during 1-10 days of culture, and a surge of endogenous free spermine at 10 days, in cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung. This appears to be in line with the above assumption. The effect of PAs in *in vitro* shoot regeneration is further supported by the study of exogenous PAs, in which shoot regeneration can be enhanced by the use of 1-20 mM putrescine, 0.1-2.5 mM spermidine or 0.1-1 mM spermine. Furthermore, tissue analysis shows a three-fold increase of free spermine in explants grown on putrescine medium at 10 days, as compared with control explants. A similar response has also been obtained in AVG-grown explants, in which the endogenous level of free spermine peaks at 10 days of culture. These results indicate that enhanced shoot regeneration

Table 2. Effect of exogenous PAs on endogenous levels of ACC synthase activity and ACC of cotyledonary explants of *B. campestris* ssp. *pekinensis* cv. Shantung during two weeks of culture

Days after culture	Control[a]	Putrescine (5 mM)	Spermidine (0.5 mM)	Spermine (0.1 mM)
		ACC synthase, units (mg protein)$^{-1}$		
0	0.3 ± 0.1[b]	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
7	2.1 ± 0.2	2.7 ± 0.7	2.4 ± 0.3	1.5 ± 0.2
14	1.9 ± 0.4	2.2 ± 0.3	2.2 ± 0.3	1.2 ± 0.4
		ACC, nmol (gFW)$^{-1}$		
0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
7	3.7 ± 1.0	6.2 ± 1.4	4.6.± 1.1	4.1 ± 1.1
14	1.4 ± 0.5	0.8 ± 0.1	1.5 ± 0.1	1.1 ± 0.2

[a] Explants were cultured on N1B2 medium in the absence of exogenous PAs
[b] Each value represents mean ± SE of three replications each with 10 explants

of cv. Shantung may be associated with an elevated level of endogenous free spermine, although the mechanism is not clear.

Results of this study also indicate that shoot regeneration may not be related to the endogenous level of free spermidine *per se,* although spermidine has been associated with flower bud initiation in *N. tabacum* (Kaur-Sawhney et al. 1988) and embryoid differentiation in *D. carota* (Feirer et al. 1985). The promotive effect of exogenous spermidine on shoot regeneration in this study may be explained by the conversion of spermidine to spermine, since the former is a precursor of the latter (Smith 1990). With respect to $AgNO_3$, endogenous free PAs of the explants are not affected during two weeks of culture. It indicates that shoot regeneration enhanced by $AgNO_3$ may not be related to PA metabolism. Although changes of the metabolic processes leading to shoot formation in the presence of $AgNO_3$ are not clear, the finding of this study suggests that it may differ from that induced by AVG. Further study is needed to clarify this point.

Ample evidence indicates that ethylene is implicated in some plant physiological responses regulated by PAs. The use of PA synthesis inhibitors has been shown to promote ethylene production and flower senescence, e.g. *Dianthus caryophyllus* (Roberts et al. 1983), while exogenous application of PAs are inhibitory to ethylene synthesis of several types of plant tissues including *Malus* fruits, *Phaseolus vulgaris* and *N. tabacum* (Apelbaum et al. 1981) and *Tradescantia* petals and *Vigna radiata* hypocotyls (Suttle 1981). This mutually antagonistic relationship between PAs and ethylene has been attributed to the competition for the precursor, SAM. However, results of this study did not support the precursor competition hypothesis, in view of the similar pattern of ethylene synthesis, i.e. ethylene production, endogenous ACC synthase activity and ACC levels, between control and PA treated explants. Enhanced ethylene synthesis in explants grown with exogenous putrescine after 7-10 days is intriguing, but PA-stimulated ethylene production has also been reported in *Malus* fruits (Wang and Kramer 1990) and senescence of *D. caryophyllus* (Downs and Lovell 1986). Nevertheless, our results suggest that the promotive effect of PAs on *in vitro* shoot regeneration of cv. Shantung may not be related to ethylene metabolism.

An increase of the endogenous levels of free putrescine and spermine in the presence of AVG, together with the promotive effect of exogenous PAs on shoot regeneration in this study suggest that cell differentiation leading to shoot formation from cotyledons of *B. campestris* ssp. *pekinensis* cv. Shantung *in vitro* may be associated with PA metabolism. Although the mechanism of *in vitro* shoot regeneration regulated by PAs is not known, our results indicate that the promotive effect of PAs in regeneration is not due to an inhibition of ethylene biosynthesis. In plants, free PAs can form conjugates with various secondary metabolites, e.g. hydroxycinnamic acid (Evans and Malmberg 1989), and the bound PAs have been associated with floral initiation (Torrigiani et al. 1987) and fruit development (Biasi et al. 1988) and also utilized as a taxonomic marker in the Butulaceae (Meurer-Grimes et al. 1989). Therefore, apart from the free PAs, the possible role of bound PAs in *in vitro* shoot morphogenesis cannot be ruled out.

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