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The Influence of Polyamines on Androgenesis of *Cucumis sativus* L.

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Summary

The influence of polyamines (putrescine and spermidine; 5–1000 μM) was studied on androgenesis of *Cucumis sativus* L. cvs 'Calypso' and 'Green Long'. Addition of polyamines (putrescine or spermidine; 5–200 μM) to the embryo induction medium [B5 (GAMBORG et al. 1968) containing 2.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 μM 6-benzyladenine (BA) and 0.25 M sucrose] enhances the rate of embryogenesis and spermidine showed optimum response (90.66 and 100.33 embryos per 60 anthers of 'Calypso' and 'Green Long' respectively) at 200 μM. Higher concentrations (500 or 1000 μM) of putrescine and spermidine were not beneficial for embryogenesis. Embryo differentiation was achieved on B5 medium supplemented with 0.25 μM of α-naphthaleneacetic acid (NAA), 0.25 μM kinetin (Kn) and 0.09 M sucrose. Transfer of the differentiated embryos to maturation medium containing 0.09 M sucrose and 10 μM abscisic acid (ABA) resulted in maturation of embryos. Mature embryos developed into plantlets on germination medium containing 0.09 M sucrose. Embryos developed from anthers on induction medium containing 200 μM spermidine showed maximum regeneration efficiency.

Zusammenfassung

Einfluss von Polyaminen auf die Androgenese von *Cucumis sativus* L.. Der Einfluss der Polyamine, Putreszin und Spermidin (5–1000 μM), wurde am Beispiel der Androgenese der *Cucumis sativus*-Sorten 'Calypso' und 'Green Long' untersucht. Die Zugabe von Putreszin oder Spermidin (5–200 μM) zum Embryo-induzierenden Medium [B5 (GAMBORG et al. 1968) mit 2.0 μM 2,4-Dichlorphenoxyessigsäure (2,4-D), 1.0 μM 6-Benzyladenin (BA) und 0.25 M Saccharose] verbesserte die Embryogeneserate, wobei Spermidin seine optimale Wirkung (90.66 bzw. 100.33 Embryonen pro 60 Antheren bei 'Calypso' bzw. 'Green Long') bei 200 μM entfaltete. Höhere Konzentrationen von Putreszin und Spermidin (500 oder 1000 μM) waren für die Embryogenese nicht förderlich. Die Differenzierung der Embryonen erfolgte auf B5-medium, dem 0.25 μM α-Naphthalensäure (NAA), 0.25 μM Kinetin (Kn) und 0.09 M Saccharose zugefügt wurden. Die Überführung der differenzierten Embryonen auf das Reifungsmedium mit 0.09 M Saccharose und 10 μM Abscisinsäure (ABA) war erfolgreich. Reife Embryonen entwickelten sich auf dem Keimmedium, das 0.09 M Saccharose enthielt. Die Embryonen aus Antheren, die auf dem Induktionsmedium mit 200 μM Spermidin kultiviert worden waren, wiesen die maximale Regenerationfähigkeit auf.

Key words. anther culture – cucumber – embryo induction – embryo differentiation – embryo maturation

Introduction

The improvement of cucumber is essential as the yield is greatly affected by fungal diseases, viruses and insect pests (MALEPSZY 1988). In various crop plants anther culture is an efficient method to generate homozygous lines for breeding (FERRIE et al. 1995). Yellow mosaic virus resistant lines in barley (FOROUGHI-WEHR and FRIEDT 1984) and *Fusarium oxysporum* resistant lines in *Solanum* spp. (RIZZA et al. 2002) have been produced through anther culture. Several factors influence androgenesis, including plant genotype, physiological state of the parent plant, microspore stage at the time of culture initiation, temperature pre-treatment of flower buds, incubation condition and culture medium (FERRIE et al. 1995). Culture medium composition is one of the most important factors that influence androgenesis. Generally, basal medium supplemented with growth regulators such as auxins, cytokinins, gibberellins and abscisic acid widely have been used for embryogenesis/organogenesis and plantlet regeneration from cultured anthers in many species. Polyamines have been regarded as a new class of plant growth regulators and greatly influence morphogenic events like organogenesis and embryogenesis (WALDEN et al. 1997; KAKKAR et al. 2000). Supplementation of polyamines to culture medium has triggered the process of somatic embryogenesis in various plants viz., rubber (EL-HADRAMI et al. 1989), soybean (NADOLSKA-ORCZYK and ORCZYK 1994), oat (KELLEY et al. 2002), ginseng (KEVERS et al. 2000; MONTEIRO et al. 2002), and oil palm (RAJESH et al. 2003). Polyamine supplementation to the culture medium also promoted androgenesis in potato (TIAINEN 1992) and gynogenesis in onion (MARTÍNEZ et al. 2000).

Despite the great success in induction of androgenesis in many species, it has been very difficult in cucumber (MALEPSZY 1988). In cucumber, plantlets were regenerated from anthers (LAZARTE and SASSER 1982), however, the ploidy of regenerants and the frequency

at which embryoids/plantlets were obtained was not given. Recently, we (ASHOK KUMAR et al. 2003) reported the androgenesis in cucumber and studied the role of auxins, cytokinins and temperature pretreatment of flower buds on embryogenesis from cultured anthers. The main objective of present study was to examine the influence of polyamines (putrescine and spermidine) on androgenesis of cucumber and report here positive role of putrescine and spermidine in embryo production and plant regeneration.

Materials and Methods

Plant material and surface sterilization of flower buds

The seeds of two cultivars of *Cucumis sativus* L., used in present experiments are 'Calypso' and 'Green Long'. Seeds of 'Calypso' and 'Green Long' were procured from Ken Agritech, Hubli, Karnataka, India and Mahyco vegetable seeds limited, Jalna, Maharastra, India, respectively. Plants were grown in the experimental plot, Department of Botany, Karnatak University, Dharwad, Karnataka, India using standard agronomic practices. The flower buds were collected when most of the microspores were at the uni-nucleate stage and stored at 4 °C for 2 days in dark (ASHOK KUMAR et al. 2003). Pretreated flower buds were washed in 1 % (v/v) Laboline (detergent) and 0.5 % Carbendazim (fungicide) for 10 min and surface sterilized with 5 % sodium hypochlorite solution for 20 min in orbital shaker at 100 rpm. The final step of sterilization was carried out in a horizontal laminar air flow chamber by rinsing the flower buds twice in sterile distilled water, followed by 0.1 % mercuric chloride solution for 5 min. Finally, the flower buds were rinsed five times in sterile distilled water. Anthers were isolated aseptically from flower buds and cultured onto an embryo induction medium. Auxins, cytokinins, abscisic acid, and polyamines were purchased from Sigma Chemical Company, USA, and membrane filters were purchased from Millipore Company, USA. Carbendazim was procured from Hyderabad Chemical Supplies Limited, Hyderabad, India.

Embryo induction, differentiation, maturation, and germination medium

B5 medium (GAMBORG et al. 1968) containing 0.25 M sucrose was used as basal medium. The embryo induction medium was B5 medium supplemented with 2.0 μM 2,4-D, 1.0 μM BA and 0.25 M sucrose (ASHOK KUMAR et al. 2003) [embryo induction medium (control); EI-0]. Putrescine and spermidine at 5, 10, 50, 100, 200, 500 and 1000 μM was added individually to B5 medium containing 0.25 M sucrose, 2.0 μM 2,4-D and 1.0 μM BA (EI-1 medium). For differentiation of embryos, the globular stage embryos/embryogenic calli with embryos were subcultured to differentiation medium – B5 medium supplemented with 0.25 μM NAA, 0.25 μM Kn and 0.09 M sucrose [Embryo differentiation medium; ED medium (ASHOK KUMAR et al. 2003)]. For maturation of embryos, the cotyledonary stage embryos were cultured onto maturation medium – B5 medium supplemented with 10 μM ABA (Embryo maturation medium; EM medium). Mature embryos were isolated aseptically and cultured onto germination medium – B5 medium containing 0.09 M sucrose. The pH of all the media was adjusted to 5.8 using 0.1N NaOH or HCl and was solidified with 0.8 % agar (Himedia, Mumbai, India). Media were sterilized by autoclaving at 120 °C for 20 min. Vitamins, polyamines and ABA were filtered using sterilized membrane filters (Millipore; 0.45 μm) and added to the autoclaved medium before aliquoting the medium into the culture tubes/Petri dishes or Magenta boxes.

Culture conditions and acclimatization of plantlets

The cultures were kept in the dark at 24±2 °C for two weeks and further incubation was at 24±2 °C and 16 h photoperiod of 40 μmol m^{-2} s^{-1} light provided by cool white fluorescent tubes for embryo induction. During embryo differentiation, maturation and germination, cultures were maintained at 24±2 °C and 16 h photoperiod of 40 μmol m^{-2} s^{-1} light provided by cool white fluorescent tubes. Well-developed plantlets were removed from cultures and washed in sterilized distilled water for not only to remove the traces of media but also to avoid infection. These plantlets were transplanted to plastic cups containing a mixture of autoclaved coco-peat, sand and garden soil (1:1:1). The transplanted plantlets were acclimatized in a plant growth chamber for 15 days under controlled humidity (80 %), temperature (24±2 °C), and light (40 μmol m^{-2} s^{-1}, 16 h photoperiod) before transferring to greenhouse for further growth.

Histological and cytological studies

Cultured anthers were collected at different intervals of time (7 days) and embryos at different developmental stages were fixed in FAA (Formalin/glacial acetic acid/70 % ethanol, 10:5:85) for 12 h at room temperature, dehydrated through a graded ethanol-butyl alcohol series and embedded in paraffin wax (FOWKE and RENNIE 1996). The tissues sectioned at a thickness of 7 μm were stained with 0.05 % toluidine-blue solution and examined under compound microscope. To determine the ploidy level of anther derived plants, the root tips of randomly selected 20 regenerants of each cultivar were harvested and treated with 2 mM aqueous solution of 8-hydroxyquinoline at room temperature for 4 h, followed by an addition of equal volume of cold 0.1 M colchicine and cooled for 12 h at 2 °C as 8-hydroxyquinoline and colchicine facilitate arresting of cell division and metaphase accumulation in root tip cells (ARMSTRONG 1996). The pre-treated roots were fixed in ethanol: glacial acetic acid (3:1) for 24 h, subsequently they were washed in water and hydrolysed in 1 N HCl at 60 °C for 10 min. Hydrolysed root tips were stained in Feulgen stain for 1 h and squashed in 45 % acetic acid (ARMSTRONG 1996).

Data analysis

The experiments were arranged in a complete randomised design with 12 replicates in each experiment and each replicate consisting of 4–6 anthers. Each experiment was repeated three times. The cultures were observed periodically and morphological changes were

recorded at weekly intervals. The number of responding anthers, embryos and plantlets produced in each treatment were counted and the results expressed as percentage of responding anthers per treatment, total number of embryos per 60 anthers per treatment and plantlets per anther per treatment. The number of embryos (mean number of embryos per 60 anthers) induced and plantlets (mean number of plantlets per anther) regenerated per experiment was statistically analysed. Results were subjected to analysis of variance (ANOVA) and mean values were separated according to Duncan's multiple range test (DMRT).

Results and Discussion

Embryo induction

Anthers of both cultivars swell on induction medium in two weeks (Fig. 1 A). In 'Calypso', embryos were induced directly, but callus-mediated embryogenesis was noticed occasionally. 'Green Long' exhibited callus mediated embryogenesis only. Microspores enlarged and began to divide after few days of inoculation (Fig. 1B) and subsequently developed into embryo (Fig. 1C) or embryogenic callus. In 'Calypso', during direct embryogenesis, globular embryos were induced directly from the swollen anthers in four weeks. Embryogenic callus was induced from swollen anthers of 'Calypso' and 'Green Long' in two weeks (Fig. 1D) and subsequently embryos developed from embryogenic calli in another two weeks (Fig. 1E).

Embryo induction was improved with the addition of 10, 50, 100 or 200 μM putrescine to the embryo induction medium compared to control (Table 1). Whereas supplementation of higher concentrations of putrescine (500 or 1000 μM) was not beneficial. Among different levels of putrescine, optimum of 82.33 and 94.33 embryos were developed from 60 cultured anthers of 'Calypso' and 'Green Long', respectively on embryo induction medium supplemented with 200 μM putrescine. Putrescine is an important modulator of biological processes such as cell division, growth, and differentiation (WALDEN et al. 1997), and embryogenesis was improved with the addition of putrescine to embryo induction medium (KELLEY et al. 2002).

Addition of spermidine to embryo induction medium was shown to increase the embryogenic process in potato (TIAINEN 1992), and ginseng (MONTEIRO 2002). In the present study, supplementation of spermidine at concentrations 10, 50, 100 or 200 μM improved the embryo production (Table 1). Maximum of 90.66 and 100.33 embryos were produced from 60 anthers in 'Calypso' and 'Green Long' respectively on embryo induction medium supplemented with 200 μM spermidine.

In our previous studies (ASHOK KUMAR et al. 2003), maximum of 70.8 and 81.66 embryos per 60 cultured anthers of 'Calypso' and 'Green Long', respectively were developed on B5 medium supplemented with 0.25 μM 2,4-D and 1.0 μM BA.

Embryo differentiation, maturation, and germination

Embryos or embryogenic calli with putative embryos induced on different induction media were differentiated into cotyledonary stage embryos (Fig. 1F) through torpedo stage in 4 weeks on differentiation medium (ED medium) supplemented with 0.25 μM NAA, 0.25 μM Kn and 0.09 M sucrose. Accumulation of storage products is the important process during embryo maturation in androgenesis as well as somatic embryogenesis. ABA enhances this process by eliciting the transcription of genes for protein and lipid biosynthe-

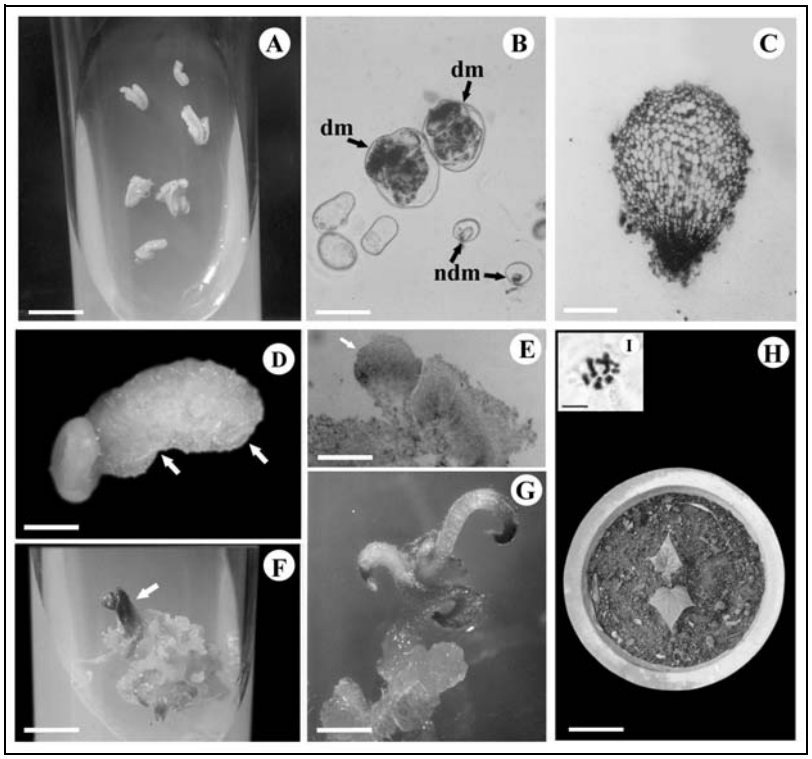

Fig. 1. Embryogenesis and plantlet regeneration from anther cultures of *Cucumis sativus* L. (cvs. 'Calypso' and 'Green Long'). **A**: Swollen anthers of 'Calypso' (bar = 0.71 cm). **B**: Squash preparation of swollen anther showing dividing (dm) and non-dividing microspores (ndm) (bar = 0.01 mm). **C**: Longitudinal section of globular embryo (bar = 0.5 mm). **D**: Embryogenic callus induction (arrows) from anthers of 'Calypso' (bar = 3 mm). **E**: Section through a portion of embryogenic callus showing embryo (arrow) (bar = 1.2 mm). **F**: Differentiation of cotyledonary stage embryo (arrow) (bar = 0.62 cm). **G**: Developing plantlet on germination medium (bar = 3 mm). **H**: A potted anther derived plantlet (bar = 7 cm). **I**: Cytology of root tip cell of 'Calypso' showing haploid number of chromosomes (n = 7) (bar = 0.007 mm).

Table 1. The influence of polyamines on embryogenesis and plantlet regeneration from cultured anthers of *Cucumis sativus* L. cvs. 'Calypso' and 'Green Long'.

Polyamine	µM	No. of anthers cultured	Embryogenesis [a]				Plantlet regeneration [a] (Mean no. of plantlets per anther)	
			'Calypso'		'Green Long'			
			Responding anthers (%)	Mean no. of embryos per 60 anthers	Responding anthers (%)	Mean no. of embryos per 60 anthers	'Calypso'	'Green Long'
Control	0	60	46.11	70.33 f	52.21	81.33 gh	0.67 i	0.80 i
Putrescine	5	60	46.11	70.66 f	52.21	81.66 gh	0.67 i	0.80 i
	10	60	46.66	72.66 de	52.76	83.00 g	0.69 gh	0.84 g
	50	60	47.21	74.33 d	53.33	85.66 f	0.75 f	0.88 e
	100	60	47.76	77.66 c	54.43	89.33 d	0.78 e	0.92 d
	200	60	50.55	82.33 b	56.10	94.33 b	0.89 c	0.94 c
	500	60	46.11	71.00 ef	52.76	82.33 gh	0.70 g	0.82 h
	1000	60	45.55	69.66 f	51.66	80.66 h	0.65 j	0.78 j
Spermidine	5	60	46.10	71.00 ef	52.76	82.00 gh	0.70 g	0.83 gh
	10	60	46.66	73.33 d	53.33	84.66 f	0.75 f	0.87 ef
	50	60	47.76	77.33 c	53.88	87.66 e	0.84 d	0.93 c
	100	60	49.43	83.33 b	55.00	92.66 c	0.98 b	1.07 b
	200	60	51.10	90.66 a	57.21	100.33 a	1.23 a	1.35 a
	500	60	46.66	72.66 de	52.21	81.66 gh	0.74 f	0.87 ef
	1000	60	46.10	70.66 f	51.66	80.66 h	0.68 i	0.80 i

[a] In each column, mean values followed by same letters are not significantly different according to DMRT at P=0.05.
Control: B5 medium supplemented with 2,4-D (2.0 µM), BAP (1.0 µM) and 0.25 M sucrose.

sis (MERKLE et al. 1995; PALMER and KELLER 1997). ABA treatment was necessary for maturation of embryos and subsequent development into normal plantlets in cucumber (ASHOK KUMAR et al. 2003). Similarly, in this study, embryos were matured in 2 weeks on maturation medium (EM medium) containing 10.0 µM ABA. Mature embryos developed into plantlets (Fig. 1G) in another 2 weeks on germination medium containing 0.09 M sucrose. However, the frequency of plantlet development depends on the composition of media used for the induction of embryos or embryogenic calli (Table 1). Polyamines, especially spermidine and putrescine significantly increased not only embryo yield but also the conversion of embryos into normal plantlets in *Solanum tuberosum* (TIAINEN 1992). Similarly, in this study, the embryos that originated from polyamine-containing medium especially spermidine at 200 µM showed highest frequency of plantlet regeneration (Mean number of 1.23 and 1.35 plantlets per anther of 'Calypso' and 'Green Long' respectively) on germination medium (Table 1).

Transplantation and cytological analysis

The regenerated plantlets were taken out of the culture vessels and washed thoroughly with sterile water to avoid infection and transplanted into plastic cups containing mixture of coco-peat, sand, and garden soil (1:1:1). Plantlets were hardened in a growth chamber under controlled environmental conditions, which was necessary for the survival of the plantlets. In each cultivar, 68 plantlets were transferred to greenhouse, of which 29 plantlets of 'Calypso' and 34 plantlets of 'Green Long' survived after 20-days (Fig. 1H). The cytology of root tip cells of randomly selected 20 plantlets in each cultivars revealed that, 16 and 12 plantlets were haploids in 'Calypso' (Fig. 1I) and 'Green Long' respectively and remaining plantlets were diploids.

From the present investigation it can be concluded that the efficiency of embryogenesis and plantlet regeneration from cultured anthers of cucumber was increased with the addition of polyamines to the induction medium and spermidine found to be most effective.

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