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Effect of Ethylene Inhibitors on *in vitro* Shoot Multiplication and their Impact on Ethylene Production in Cucumber (*Cucumis sativus* L.)

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Abstract: Effects of ethylene inhibitors like silver nitrate ($AgNO_3$), cobalt chloride ($CoCl_2$) and salicylic acid (SA) on multiple shoot induction and their impact on ethylene production using embryonal cotyledon cultures of *Cucumis sativus* L. were examined. The optimum concentration of $AgNO_3$ (40 μM), $CoCl_2$ (20 μM) and SA (20 μM), separately, induced maximum number of shoots in Murashige and Skoog's (MS) medium supplemented optimally with 4.44 μM BA and 0.25 μM NAA. Among the three ethylene inhibitors tested, $AgNO_3$ produced maximum number of shoots when compared to $CoCl_2$ and SA. Ethylene production was monitored in all the treatments with $AgNO_3$/$CoCl_2$/SA and it was observed that the treatment with $AgNO_3$ alone showed increase in ethylene production when compared to $CoCl_2$ and SA. Even though ethylene concentration was the highest in $AgNO_3$ treated explants, maximum number of shoots were observed.

Key words: Ethylene inhibitors, shoot regeneration, *Cucumis sativus* L.

Introduction

Cucumber (*Cucumis sativus* L.) is one of the major vegetable in the tropics, subtropics and milder areas of the temperate zones of both hemispheres. In recent years, cucumber tissue culture has been employed to produce cultivars with improved agronomic traits such as virus resistance (Chee and Slightom, 1991) and fungal resistance (Raharjo *et al*., 1996; Tabei *et al*., 1998). However, plant regeneration in cucumber still encounters many problems such as abnormal embryo development, poor differentiation of callus into shoots, poor survival of regenerated plants in soil and undesirable changes in ploidy level of regenerated plants (Ziv and Gadasi, 1986; Malepszy, 1988; Gambley and Dodd, 1990). Further, the frequency of regeneration in earlier studies was dependent on the source of explants, the cultivars, growth regulator combinations and physical conditions of culture.

In recent years, there has been accumulating evidences that growth and differentiation of plant cells and tissues *in vitro* can be affected considerably by ethylene. Ethylene is readily produced by plants and its production is associated with poor regeneration or recalcitrance behavior of culture materials (Chi *et al*., 1990). Many reports have demonstrated the positive effect of $AgNO_3$ on plant tissue culture (Mohiuddin *et al*., 1997; Mhatre *et al*., 1998; Saly *et al*., 2002). Ethylene inhibitors such as silver nitrate ($AgNO_3$), cobalt chloride ($CoCl_2$) and Salicylic Acid (SA) have been shown to be effective for shoot regeneration by inhibiting the ethylene production in cucumber (Roustan *et al*., 1992; Mohiuddin *et al*., 1997; Mhatre *et al*., 1998), muskmelon (Yadav *et al*., 1996), or its function by

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blocking certain steps in the pathway of ethylene synthesis in *Brassica campestris* (Pua *et al*., 1996) and *Chicorium intybus* (Bais *et al*., 1999). Addition of these compounds *in vitro* may be helpful for overcoming the recalcitrance or in enhancing regeneration. The present study was determined the effect of ethylene inhibitors on shoot regeneration efficiency from the embryonal explants of an important commercial cucumber cultivar Poinsett 76. In particular, we examined the interaction of ethylene and other plant growth regulator, SA. In addition, ethylene production from cultured explants and its impact on shoot induction frequency was investigated at the first time. Such studies are providing an update in regenerating large number of shoots from a single explant.

Materials and Methods

Culture Method

Seeds of *Cucumis sativus* L. cv. Poinsett 76 (Indo-American hybrid seeds Ltd., Bangalore, India) were soaked in tap water for 15 min disinfested with 70% alcohol (v/v) for 1 min and 2.5% (v/v) commercial bleach 'Teepol' (5.25% sodium hypochlorite; Reckitt and Benckiser of India Ltd., Kolkatta, India) for 15 min followed by three rinses with sterile distilled water. Seeds were further disinfested by soaking in 0.1% mercuric chloride (w/v) for 8 min and germinated in darkness for 48 h in 25×150 mm test tubes (Borosil, India) containing sterile moist cotton. Embryonal cotyledon explants were isolated from one day-old germinating seeds under sterile conditions and were used as explants. To optimize the concentration of phytohormone for shoot regeneration, embryonal cotyledon explants were cultured on ten ml of shoot regeneration medium which was agar-solidified MS (Murashige and Skoog, 1962) medium supplemented with different concentrations and combinations of 6-benzyladenine (BA) (0-8.88 μM), kinetin (Kn) (0-9.2 μM) and α-naphthalene acetic acid (NAA) (0.05-1.0 μM). The effect of $AgNO_3$, $CoCl_2$ and SA were investigated by culturing the explants on MS medium containing 4.44 μM BA and 0.25 μM NAA with different concentrations of $AgNO_3$ (10-50 μM), $CoCl_2$ (10-40 μM) and SA (10-40 μM) individually. Silver nitrate and SA were filter sterilized through 0.22 μm Millipore filter (Sigma, USA) and added to the medium after autoclaving. After 4 weeks of culture, the adventitious shoots that formed on the explants were counted. Shoots developed from the explants were excised and transferred to rooting medium containing different concentrations of IBA (0.4, 2.4, 4.9 and 7.3 μM; Indole-3-butyric acid). The medium was adjusted to pH 5.8 before autoclaving at 121°C for 15 min. All cultures were incubated at 25°C under 16 h photoperiod of cool-white illumination (Philips, India) at 30 μmol m^{-2} sec^{-1}.

Acclimatization

Plants with profuse rooting were thoroughly washed in tap water to remove the agar and later transplanted to plastic pots containing a mixture of autoclaved sand, soil and vermiculite (1:1:1, v/v/v). Potted plants were grown in a growth chamber at 85% relative humidity for 4 weeks. Upon new leaf growth, the plants were kept in a shade house for 4 week before transferring to the field.

Statistical Analysis

Regeneration frequency (number of explants with shoots/total number of explants cultured) and shoot number per explant were assessed for 20 replicates. A complete randomized design was used in all experiments and a one-way analysis of variance (ANOVA) and comparisons between the mean values of treatments were carried out using Duncan's Multiple Range Test (DMRT). Significance was determined at the 5% level (Gomez and Gomez, 1976).

Ethylene Measurement

The embryonal cotyledon explant was cultured in a 9 mL glass tube (Borosil, India) (5 cm in length, 1 cm in diameter) containing 2.5 mL MS medium with or without different concentrations of

$AgNO_3$ (10-50 µM), $CoCl_2$ (10-50 µM) and SA (10-50 µM). The glass tube was sealed with an airtight seal septum until ethylene measurement. Each treatment consisted of five to six replicates with each glass tube containing 1 explant. Cultures were allowed to stand hood and at the end of 3 h, one milliliter of gas was sampled from each glass tube and measured using gas-chromatograph (Theologis, 1992). Ethylene produced by the cultured explant was examined at the interval of one week each during 4 week culture period.

Results and Discussion

Optimization of Culture Conditions for Shoot Regeneration

Among the different concentrations and combinations of BA, Kn and NAA tested, BA (4.44 µM) with NAA (0.25 µM) was found favourable for adventitious shoot induction and multiplication with a frequency of 62.6% for embryonal cotyledon explant (Table 1). In this medium, each explant regenerated 9.2 shoots. In the absence of plant growth regulators (control) in MS medium no shoot regeneration was observed. Similarly BA and NAA induced multiple shoots from various explants of cucumber viz., cotyledon (Selvaraj, 2002), shoot tip (Vasudevan *et al*., 2001; Selvaraj, 2002), hypocotyl (Wehner and Locy, 1981; Rajasekaran *et al*., 1983; Selvaraj, 2002) and shoot tip of *Momordica dioica* (Shiragave and Chavan, 2001). Hence, a combination of 4.44 µM BA and 0.25 µM NAA was chosen as a basal medium for investigating the effect of $AgNO_3$, $CoCl_2$ and SA on their role in enhancing shoot regeneration frequency and other growth parameters.

Table 1: Effect of BA, Kn and NAA on multiple shoot induction from cotyledon explants of cucumber cv. Poinsett 76

Concentration (µM)		Percentage of explants with shoots	Mean No. of shoots		Shoot length (cm)	No. of nodes
			Initial culture	After 2nd transfer		
0		9.4±0.16qr	1.6±0.16j	2.4±0.16n	1.7±0.16lm	1.1±0.08lm
		(F = 6291.25)***	(F = 6.410)***	(F = 15.83)***	(F = 58.40)***	(F = 21.87)***
BA						
0.44		12.6±0.11p	1.8±0.16hi	3.0±0.34l	2.2±0.11k	1.4±0.11kl
0.88		18.4±0.11mn	1.9±0.32h	3.6±0.11jk	2.4±0.23k	1.6±0.11k
1.76		23.6±0.11k	2.1±0.16gh	4.2±0.11i	3.6±0.11h	2.4±0.11i
2.22		32.2±0.11hi	2.5±0.40e	4.8±0.46g	3.8±0.11gh	2.6±0.11h
4.44		43.4±0.23e	2.8±0.16d	5.4±0.23ef	4.6±0.11e	3.4±0.11ef
8.88		15.8±0.20no	1.8±0.16hi	3.4±0.23k	2.6±0.11jk	1.8±0.11jk
		(F = 1238.32)***	(F = 31.645)***	(F = 13.52)**	(F = 31.66)***	(F = 15.92)**
Kn						
0.92		9.2±0.23r	0.9±0.12l	2.0±0.34o	1.2±0.11n	0.8±0.08n
1.84		12.4±0.23q	1.6±0.11j	2.8±0.11lm	2.0±0.34kl	1.2±0.11ln
2.76		17.2±0.11n	1.8±0.11hi	3.2±0.11kl	2.6±0.23jk	2.0±0.34j
3.68		22.4±0.11l	2.0±0.11gh	3.8±0.46j	3.0±0.11ij	2.2±0.30ij
4.64		30.8±0.46i	2.4±0.11ef	4.6±0.23gh	3.8±0.11gh	2.8±0.11g
9.28		10.8±0.23q	1.2±0.11k	2.4±0.11n	1.8±0.11l	1.2±0.23l
		(F = 5507.31)***	(F = 16.954)***	(F = 56.89)***	(F = 187.12)***	(F = 75.28)***
BA	NAA					
4.44	0.05	46.6±0.11d	2.8±0.11d	5.6±0.11e	4.4±0.11ef	3.6±0.11e
	0.10	58.4±0.23b	3.6±0.34b	7.4±0.23c	6.0±0.23c	5.0±0.34bc
	0.25	62.6±0.11a	4.8±0.46a	9.2±0.23a	7.2±0.11a	5.8±0.23a
	0.50	38.4±0.23fg	2.4±0.11ef	4.8±0.46g	3.6±0.11h	2.6±0.11gh
	0.75	32.8±0.11q	2.1±0.08g	4.0±0.23ij	2.8±0.11j	2.0±0.11j
		(F = 1942.95)***	(F = 91.393)***	(F = 24.54)***	(F = 40.80)***	(F = 58.07)***
Kn	NAA					
4.64	0.05	27.4±0.23j	3.6±0.11b	5.2±0.30f	4.0±0.40g	2.8±0.11g
	0.10	38.6±0.40f	3.4±0.11c	6.4±0.23d	5.2±0.11d	4.0±0.23d
	0.25	49.8±0.34c	3.5±0.08bc	7.8±0.50b	6.4±0.23b	5.2±0.11b
	0.50	22.6±0.11kl	2.1±0.08g	4.2±0.41i	3.2±0.30i	2.4±0.23i
	0.75	19.4±0.23m	1.6±0.11j	3.4±0.23k	2.2±0.11k	1.4±0.23kl

Values represents the treatment means of 20 replicates, Values with the same letter(s) within the column are not significantly different according to Duncan's Multiple range Test (DMRT) at $p \leq 0.05$ level ***, Highly significant ($p<0.001$); **, Highly significant ($p<0.01$)

Table 2: Effect of ethylene inhibitors in multiple shoot induction on MS medium containing BA (4.44 μM) and NAA (1.59 μM)

Compound (μM)	Percentage of explants with shoots	No. of shoots		Mean shoot length (cm)	No. of nodes
		Initial culture	After 2nd transfer		
BA + NAA	62.6d	4.8gh	9.2j	7.2b	5.8b
$AgNO_3$					
10	48.6± 0.12g	7.2 ± 0.12d	13.4 ± 0.17g	4.6 ± 0.12g	3.2 ± 0.16fg
20	62.4 ±0.17de	8.4 ± 0.17cd	16.2 ± 0.11ef	5.4 ± 0.18e	4.6 ± 0.18d
30	71.6 ± 0.11b	10.6 ± 0.12b	21.6 ± 0.24c	6.2 ± 0.12c	5.0 ± 0.21c
40	84.2 ± 0.16a	13.8 ± 0.24a	37.4 ± 0.17a	7.6 ± 0.14a	6.2 ± 0.24a
50	34.6 ± 0.30k	6.0 ± 0.17f	9.6 ± 0.12i	3.4 ± 0.12ij	2.6 ± 0.21gh
$CoCl_2$					
10	30.8 ± 0.11l	2.8 ± 0.14k	6.6 ± 0.12l	2.2 ± 0.12m	1.4 ± 0.12ij
20	36.4 ± 0.11j	3.6 ± 0.11ij	8.2 ± 0.14k	2.8 ± 0.14k	1.6 ± 0.12hi
30	68.6 ± 0.34c	8.8 ± 0.24c	27.2 ± 0.24b	5.6 ± 0.21de	4.4 ± 0.24de
40	52.4 ± 0.17f	7.2 ± 0.12de	16.4 ± 0.17e	4.8 ± 0.21fg	3.6 ± 0.18f
50	44.6 ± 0.22i	4.8 ± 0.14g	11.4 ± 0.14h	3.4 ± 0.18i	2.0 ± 0.14h
Salicylic acid					
10	21.8 ± 0.18n	1.8 ± 0.17m	3.0 ± 0.12no	1.6 ± 0.12h	1.0 ± 0.08j
20	26.4 ± 0.20m	2.4 ± 0.21kl	3.8 ± 0.14n	2.6 ± 0.16kl	1.6 ± 0.12i
30	62.4 ± 0.34e	5.8 ± 0.14fg	20.6 ± 0.17cd	5.8 ± 0.24d	4.8 ± 0.20cd
40	47.6 ± 0.28gh	4.4 ± 0.24h	9.6 ± 0.12ij	5.0 ± 0.18f	4.2 ± 0.18e
50	34.8 ± 0.24jk	3.6 ±0.17i	5.4 ± 0.12m	3.8 ± 0.16gh	3.0 ± 0.21g

Values represents the treatment means of 20 replicates, Values with the same letter(s) within the column are not significantly different according to Duncan's Multiple range Test (DMRT) at $p \leq 0.05$ level

Effect of Ethylene Inhibitors on Shoot Regeneration

In the present study the addition of $AgNO_3$ (10-50 μM) was beneficial to shoot regeneration (Table 2). The high frequency of shoot regeneration for embryonal cotyledon (84.2%) with maximum number of shoots (37.4/explant) as well as increase in the length of shoots (7.6 cm) were achieved on the medium containing 40 μM $AgNO_3$; shoot number was four times more than that was obtained on the medium without $AgNO_3$. $AgNO_3$ concentration below 40 μM also induced multiple shoots but to a lower extent (Table 2). Silver nitrate treatment at 50 μM inhibited shoot multiplication in terms of both in shoot number (9.6/explant) and length of shoots (3.4 cm). Roustan *et al.* (1992), in melon and Mohiuddin *et al.* (1997) in cucumber reported the efficiency of $AgNO_3$ on multiple shoot induction in cotyledon and hypocotyl explants respectively. However, they were able to obtain only lower number of shoots per explant (about 25 shoots). But in the present experiment, we could get more number of shoots (37.4 shoots/explant) at the end of second subculture. As Ag^+ ions can prevent a wide variety of ethylene-induced plant responses, including growth inhibition and senescence, the effect was assumed to be mediated *via* the inhibition of the physiological action of ethylene (Beyer *et al*., 1984), a potential inhibitor of many plant regeneration systems. In other words, the addition of $AgNO_3$ or Ag_2SO_4 causes precipitation in culture medium, enhanced shoot regeneration on these media suggests that free Ag^+ ions are still available. In a similar way, $AgNO_3$ enhanced shoot regeneration in *Nicotiana plumbaginifolia* and *Triticum aestivum* (Purnhauser *et al*., 1987), *Zea mays* (Songstad *et al*., 1991), *Brassica campestris* sp. Oleifera (Burnett *et al*., 1994), sp. Pereinensis (Chi *et al*., 1990) and *Raphanus sativus* (Pua *et al*., 1996).

Treatment with $CoCl_2$ was also effective for improving shoot regeneration frequency (Table 2). In the present study, a high frequency of shoot regeneration was achieved by $CoCl_2$ (30 μM) with embryonal cotyledon explant (68.6%). The number of shoots were 27.2/explant with a mean shoot length of 5.6 cm. Roustan *et al.* (1992), Mhatre *et al.* (1998) reported that $CoCl_2$ induced multiple shoots but a lower frequency of 11 shoots/explant in melon and cucumber respectively. In some cases, $CoCl_2$ was equally effective as $AgNO_3$ in eliciting morphogenesis (Chraibi *et al*., 1991).

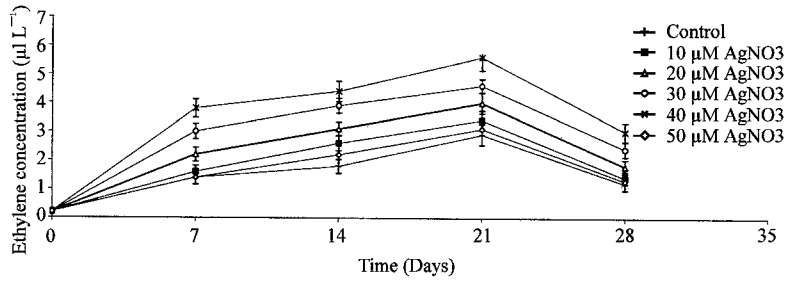

Fig. 1: Effect of various concentrations of $AgNO_3$ (10-50 µM) on ethylene production in cucumber cv. Poinsett 76

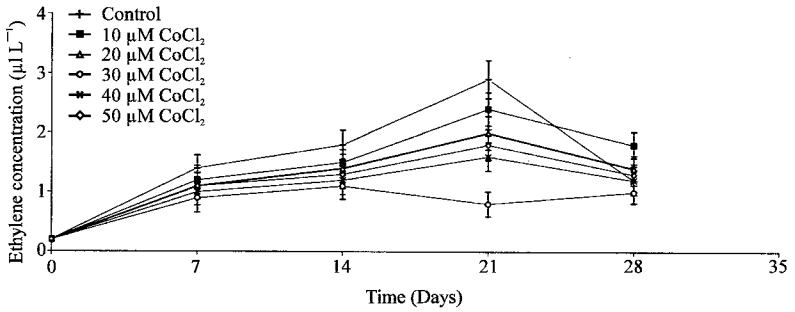

Fig. 2: Effect of various concentrations of $CoCl_2$ (10-50 µM) on ethylene production in cucumber cv. Poinsett 76

Among the different concentrations of SA tested, 30 µM concentration induced maximum shoot proliferation (Table 2) but lees than $AgNO_3$ and $CoCl_2$ treatments with 20.6 shoots/explant. Present study was in conformity with Mhatre *et al.* (1998) who reported that acetyl SA induced multiple shoots at 20 µM concentration in cucumber (about 9 shoots/explant). Experiments with apple suspension culture SA reduced ethylene production over a short period of time but less effective over longer periods (Leslie and Romani, 1988). Increased concentration of 10 to 30 µM, enhances the shoot growth and development, suggesting that there is a correlation between a decrease in ethylene level and an increase in SA level.

Ethylene Production from Cultured Explants

The addition of $AgNO_3$ to basal shoot regeneration medium enhanced the level of ethylene produced by the cultured explants (Fig. 1). Increasing $AgNO_3$ concentrations coincided with an increase in ethylene production in embryonal culture. In the untreated cucumber explants the maximal ethylene level was 2.9 µL L^{-1} after 3 week in the culture. Then the capacity of explant to produce ethylene declined gradually after a week (1.4 µL L^{-1}). In the presence of $AgNO_3$ at 40 µM the ethylene production was dramatically increased after 3 week over the control (Fig. 1). On the 28th day, ethylene production by $AgNO_3$ treated or non treated tissue had decreased. Although the mechanism in which ethylene production is stimulated in response to $AgNO_3$ is unknown, ethylene over production as a result of Ag^{2+} ions treatment has been reported in tomato fruits (Penarrubia *et al.*, 1987). Theologis (1992) reported that ethylene production by the stimulation of $AgNO_3$ can be explained by receptor interference by Ag^{2+} ions that triggers cells to overproduce ethylene.

When embryonal cotyledon explants were cultured with $CoCl_2$, ethylene production was strongly inhibited over the control treatment. On the 21st day its effectiveness as an inhibitor of ethylene production was concentration-dependent (Fig. 2). Co^{2+} inhibits ethylene production by blocking the

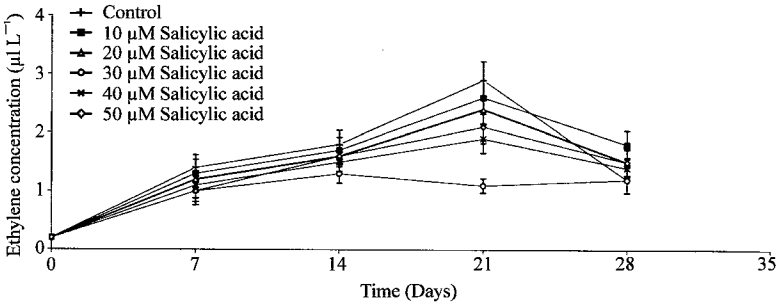

Fig. 3: Effect of various concentrations of Salicylic acid (10-50 µM) on ethylene production in cucumber cv. Poinsett 76

conversion of 1-aminocyclo-propane 1-carboxylic acid to ethylene (Yang and Hoffman, 1984). In the presence of this inhibitor, shoot regeneration increased with the level of inhibition of ethylene production.

In the present study, the amount of ethylene production in SA treated explants strongly inhibited. In the presence of SA at 30 µM, the ethylene level was strongly inhibited after 3 week (Fig. 3). Supporting the previous assumption that SA inhibits ethylene production by blocking the action of ethylene forming enzyme (EFE) (Leslie and Romani, 1988). In the present study, like $CoCl_2$ treatment SA induced multiple shoots by inhibiting ethylene production. Salicylic acid may have an inhibitory effect on ethylene biosynthesis on signaling (Jirage *et al.*, 2001), on the other hand, ethylene can also inhibit SA accumulation.

The present study concluded that ethylene plays an important role in the growth of cell cultures and it is also possible to improve the frequencies of shoot regeneration in cucumber by supplementing the regeneration medium with ethylene inhibitors. Inhibitors of ethylene biosynthesis on receptor binding that confer enhancement of shoot induction and provide efficient way of shoot propagation. As a result, we obtained more number of shoots from embryonal explants of cucumber. Further the ethylene production from embryonal cotyledon explants was analyzed for the first time in cucumber.

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