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Effect of plant growth regulators on evolution of ethylene and methane by different explants of chickpea

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Abstract

Shoot tips, cotyledonary nodes and hypocotyls of chickpea (*Cicer arietinum* L.) were grown on 3 media: plant induction medium (PIM), callus induction medium (CIM), and shoot induction medium (SIM). Maximum growth and differentiation was seen in PIM, whereas minimum was observed in CIM. Shoot tips which differentiated to multiple shoots evolved negligible amounts of ethylene. Maximum ethylene evolution was recorded by hypocotyls in PIM. Ethylene appears to have stimulatory effect on shoot bud differentiation in cotyledonary nodes. But in hypocotyls, increased ethylene inhibited growth and differentiation. Calli on media containing only auxin (PIM) evolved significantly more ethylene, whereas those on media with cytokinin (SIM) evolved more methane. Callus forming explants like cotyledonary nodes and hypocotyls evolve more ethylene than shoot tips.

Additional key words: *Cicer arietinum*, differentiation, silver nitrate.

Introduction

Chickpea is an important pulse crop of India. When grown *in vitro* the explants tend to be recalcitrant. Because of this success in producing transgenic plant is limited (Fontana *et al.* 1993). One of the reasons for recalcitrance could be the evolution of ethylene and other gases which regulate germination (Gallardo and Matilla 1994), and growth, morphogenesis and differentiation (Jackson *et al.* 1991). The presence of ethylene in tissue culture is invariably the consequence of the method used for cultivation (medium composition, explant type, tightness of vessels). It was shown that ethylene accumulates in physiologically active levels (Riggetti *et al.* 1990). Using ethylene inhibitors,

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Abbreviations: CIM - callus induction medium; CN - cotyledonary nodes; DAI - days after inoculation; FM - fresh mass; H - hypocotyls; PGR - plant growth rate; PIM - plant induction medium; SIM - shoot induction medium; ST - shoot tips.

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enhanced shoot regeneration was obtained in *Raphanus sativum* (Pua *et al.* 1996), *Brassica campestris* genotype R500 (Palmer 1992), *Daucus carrota* (Roustan *et al.* 1990), and *Helianthus annuus* (Chraibi *et al.* 1991). Preliminary studies in our laboratory indicated that chickpea explants evolve significant amounts of ethylene and other gases (Patil *et al.* 1996). Besides direct effect of ethylene, its interaction with other gases like CO_2 and methane evolved during *in vitro* culture may also play a decisive role in growth and differentiation (Keevers 1992). So far, no attempt was made to relate evolution of ethylene and other gases to differentiation in this crop. The objective of the present study was to elucidate the role of ethylene and other gases in the regulation of growth, morphogenesis and differentiation of *in vitro* grown chickpea.

Materials and methods

Healthy seeds of chickpea (*Cicer arietinum* L. cv. BG-256) were washed thoroughly and sterilised with 0.1 % mercuric chloride solution for 3 min followed by repeated washings with sterile distilled water. Seeds were germinated aseptically in culture room with temperature 25 ± 2 °C. From 7-d-old seedlings, 3 explants of the size 5 mm *viz.* shoot tip (ST), cotyledonary node (CN) and hypocotyl (H) were cut and transferred under aseptic conditions to 3 different media: *1)* plant induction medium (PIM) - Murashige and Skoog basal medium (MS) supplemented with naphthaleneacetic acid (NAA) and isobutyric acid (IBA) (2 mg dm^{-3} each); *2)* callus induction medium (CIM) - MS supplemented with 2,4-D (2 mg dm^{-3}) and kinetin (0.25 mg dm^{-3}); *3)* shoot induction medium (SIM) - MS supplemented with benzylaminopurine (BAP; 2 mg dm^{-3}).

After transferring the explants, the culture tubes were sealed with rubber serum stoppers and maintained in a culture room with temperature 25 ± 2 °C and irradiance of 60 μmol m^{-2} s^{-1} for 12-h photoperiod. Growth as fresh mass (FM) accumulation was recorded at 1, 7, 15, 21 and 30 d after inoculation (DAI). For each treatment morphological changes occurring in each explant were also recorded as function of DAI. For each assay, 2 cm^3 of gas samples were withdrawn from each tube and were injected into gas chromatograph (*Sigma 2000, Perkin-Elmer,* USA) equipped with *Poropak N 80/100* mesh column and a flame ionization detector (FID). Carrier gas (nitrogen) flow was maintained at 20 cm^3 min^{-1}. Operational temperatures were set at 60 °C, and 110 °C for oven and detector, respectively. Standard ethylene and methane were injected in a similar way.

In order to ascertain whether ethylene affects growth and differentiation, ethylene inhibitor $AgNO_3$ (10 mM) was added to the media in which CN and H were grown. Growth, FM and ethylene evolution was recorded in these explants.

Results and discussion

Morphogenesis: Plant growth regulators (PGRs) in the media influenced the morphogenesis, as a result, the same explant differed in morphogenesis in different media. ST differentiated to multiple shoots in PIM, callus in CIM and callus with shoot

buds in SIM. CN firstly differentiated into callus in all the three media, but further it differentiated to plantlet in PIM and shoot buds in SIM. In contrast to the above explants, H differentiated only into callus in all the three media (Chandra *et al.* 1993). Thus in media with auxins (PIM) or cytokinin (SIM) differentiation was seen but in CIM (auxin + cytokinin), no differentiation was observed. 2,4-D in CIM strongly inhibited shoot regeneration and produced only non-morphogenetic callus (Purnahauser *et al.* 1987).

Growth: Fresh mass (FM) accumulation was maximum in PIM and was minimum in CIM (Fig. 1). Maximum FM was seen in CN (which differentiated to plantlets) followed by ST (which differentiated to multiple shoots) when they were grown in PIM. H recorded minimum growth in all the three media tested, lowest FM was in CIM, where undifferentiated H recorded even reduction in FM. In PIM at 30 DAI, FM was 25 times more over initial FM in ST, 8 times more in CN and only 5 times more in H (Fig.1). Differentiation of chickpea H tissue into callus was earlier reported by Singh *et al.* (1982). Similarly in *Brassica campestris* ssp. *pekinensis* (Chi *et al.* 1991), in *Brassica oleracea* var. *botrytis* (Sethi *et al.* 1990) and in *Helianthus annuus* (Chraibi *et al.* 1991, 1992) H was less regenerative than CN. Thus our study indicates a positive relation between increased FM and differentiation of the tissue.

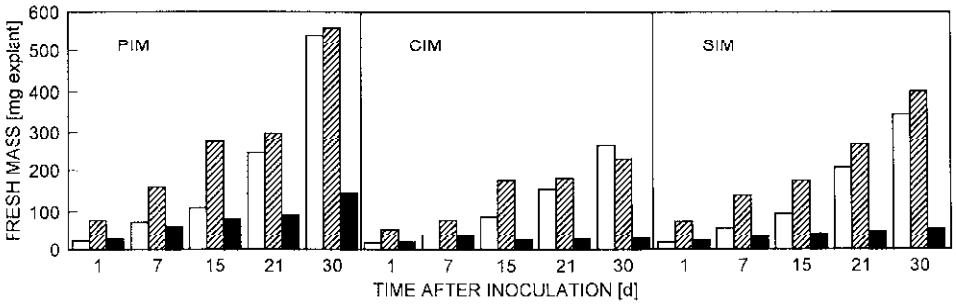

Fig. 1. Fresh mass (FM) accumulation in different chickpea explants [shoot tips (ST) - *white columns*, cotyledonary nodes (CN) - *grey columns*, and hypocotyls (H) - *black columns*] grown for 30 d on different media (PIM, CIM, and SIM - for composition see Materials and methods). The values are means of 4 replicates each from 5 independent experiments. For each experiment 40 explants of each ST, CN, and H were inoculated.

Ethylene evolution: Growth and differentiation was influenced by evolution of ethylene. It varied with the type of the explant. Maximum ethylene evolution was seen at 7 DAI and it gradually reduced to half at 15 DAI in all the three media and in all the three explants studied, after which ethylene production decreased sharply at 21 DAI (Fig. 2). ST which formed shoots in PIM, callus in CIM and buds in SIM, evolved no ethylene in PIM and very little ethylene in CIM and SIM. Low ethylene evolution in ST might have resulted in differentiation of buds and increased FM. When shoot tips were cultured in media containing 1 cm^3 dm^3 ethrel, the cut end at the base bulges but did not affect the shoot tip differentiation. But at higher concentration (10 cm^3 dm^{-3}) bulging of tissue is accompanied by non-differentiation. In PIM and SIM, maximum ethylene evolution was seen in H at 7 DAI and this level was maintained upto 15 DAI.

In CN, ethylene evolution was recorded in all the 3 media, maximum being in CIM at 7 to 15 DAI (Fig. 2). In this media CN recorded minimum growth when compared to that in PIM and SIM and even more ethylene evolution than H, which generally recorded higher evolution (Fig. 2).

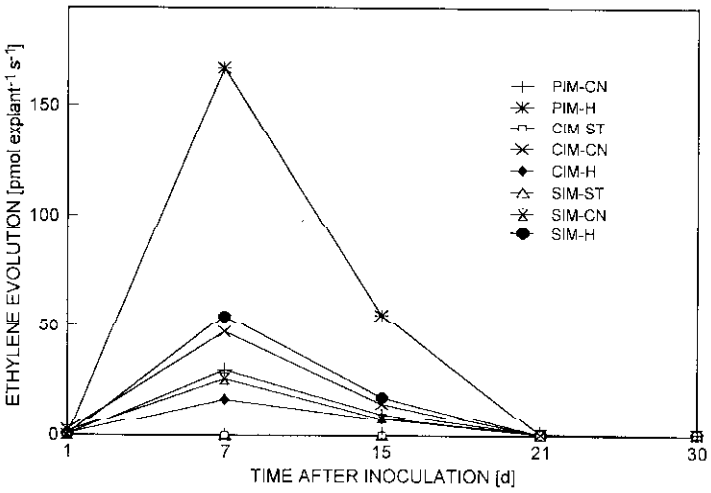

Fig. 2. Evolution of ethylene in chickpea explants of cotyledonary node (CN), hypocotyl (H), and shoot tip (ST) grown on different media (PIM, CIM, and SIM).

Inhibitory effects of ethylene on differentiation was reported by Zobel and Roberts (1978). According to Perrata *et al.* (1986), prevention of aerobic respiration due to accumulation of ethanol and ethylene in cultures treated with 2,4-D was responsible for callus formation.

Evolution of methane: Significant amount of methane was released in culture tubes along with ethylene and its quantitative production was influenced by PGRs and type of the explant.

Influence of PGRs on evolution of ethylene and methane: In PIM (medium with auxin) and CIM (medium with high auxin concentration) significantly more ethylene was evolved than in SIM (Fig. 2). In SIM (medium with cytokinin only) significantly more methane was evolved when compared to medium with only auxins (PIM) (data not given). To assess the role of methane evolved in cytokinin media, intact cotyledons, in which radicle was removed were grown in MS media containing 2 mg dm^{-3} BAP. This resulted in multiple shoot formation (Fig. 3). In these cultures, evolution of methane was 90 times more than that of ethylene (data not given). When cotyledonary explants were treated with CIM, callus without buds developed. Thus our study implies that evolution of methane in cytokinin media is related to the induction of shoot bud differentiation. Similarly when methyl methane was supplemented in the media in which CN explants of cucumber were grown, more shoot buds were induced which was related to evolution of methane (Mustafa *et al.* 1991). Not only ethylene and methane

but their interaction with other gases might play a key role in growth and differentiation (Keevers *et al.* 1992).

Fig. 3. Multiple shoots regenerated from 4-d-old intact cotyledon explant of chickpea. The explant was cultured on MS medium supplemented with 2 mg dm^{-3} BAP. Photograph taken at 25 DAI.

Ethylene was shown to be involved in plant morphogenesis *in vitro* by enhancement of growth and differentiation of cultured cells and tissues of several recalcitrant plant species (Adkins *et al.* 1990, Pua *et al.* 1996). In recalcitrant *Brassica campestris* significantly more ethylene evolution was observed in which enhanced shoot bud regeneration was achieved by using silver nitrate in the media (Palmer 1992). The only report on chickpea on evolution of ethylene was by Gallardo and Matilla (1994) who reported that ethylene evolution influences germination.

Effect of $AgNO_3$ on growth of explants: $AgNO_3$ (inhibitor of ethylene evolution; Patil *et al.* 1996) was added to the media and in both CN and H, FM increased (Table 1). The increase in FM with this treatment was most apparent in H in CIM. In control (without $AgNO_3$) H recorded even reduced FM from 7 to 15 DAI (Fig. 1). This result confirms the role of ethylene in preventing cell proliferation and growth in H. In CN the increase in FM with $AgNO_3$ treatment was less. This provides the evidence that the proliferation of the cell in CN was to certain extent stimulated by evolution of ethylene as observed in case of *Brassica* (Sethi *et al.* 1990) and in *Petunia* (Theriou *et al.* 1993). As ST evolved no ethylene, no attempt was made to treat it with $AgNO_3$.

Use of $AgNO_3$ not only enhanced FM but induced shoot bud differentiation also in CN and H. In H, a callus forming tissue, shoot buds appeared after 25 DAI. When $AgNO_3$ was used in the medium, enhanced shoot bud regeneration was reported in *Brassica compestris* (Palmer 1992), *Helianthus annuus* (Chraibi *et al.* 1992), and

Table 1. Influence of $AgNO_3$ on fresh mass and ethylene evolution in 7- and 15-d-old *in vitro* grown explants (CN and H) of chickpea on different media (PIM, CIM, and SIM).

Medium	Explant	Fresh mass [mg explant^{-1}]				Ethylene [pmol explant^{-1} s^{-1}]			
		7 d		15 d		7 d		15 d	
		control	$AgNO_3$	control	$AgNO_3$	control	$AgNO_3$	control	$AgNO_3$
PIM	CN	162	180	272	290	108	53	36	14
	H	66	93	83	148	600	140	197	82
CIM	CN	80	97	178	182	169	83	53	28
	H	38	72	25	89	61	26	127	14
SIM	CN	142	152	176	201	93	33	29	14
	H	38	81	47	109	195	84	64	30
SE at 5 %		2.85				2.08			
CD at 5 %		12.20				9.96			

Daucus carrota (Roustan *et al.* 1990). In chickpea enhanced growth and differentiation in H which is otherwise non differentiating tissue was not so far reported. Our study suggests why application of $AgNO_3$ in the media does not always induce shoot bud regeneration. $AgNO_3$ application may be useful only in tissues evolving excess ethylene (like H), or the media (PIM and CIM) which contribute to the maximum ethylene evolution. Our data suggests the possible role of ethylene and methane on differentiation. Further studies are needed to understand the precise regulation of differentiation by various gases.

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