BIOLOGIA PLANTARUM 44 (1): 13-18, 2001

Role of amino acids in evolution of ethylene and methane, and development of microshoots in *Cajanus cajan*

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Abstract

In pigeonpea (*Cajanus cajan* L.) shoot buds were induced when cotyledonary node explants were supplemented with benzylaminopurine (BAP; 2 mg dm^{-3}). When 0.1 mg dm^{-3} BAP and 0.01 mg dm^{-3} naphtahalene acetic acid were supplemented to the medium, the 34 - 35 % of induced buds developed to microshoots. By supplementing amino acids like proline, glutamine, asparagine and L-cysteine, shoot bud development to microshoots was enhanced at least by two fold. Amongst the amino acids proline gave maximum number of microshoots per explant. With increase in concentration of amino acids, fresh mass increased but microshoot number decreased. Also methane evolution was increased by addition of amino acids, and also in medium containing more of its nitrogen in the form of ammonia. Increased evolution of methane was accompanied by reduction in evolution of ethylene, and enhancement of efficiency of microshoot development.

Additional key words: asparagine, glutamine, L-cysteine, pigeonpea, proline.

Introduction

Pigeonpea (*Cajanus cajan* L.) is a major pulse crop of India. Though a few reports on regeneration are available (Eapen and George 1993, George and Eapen 1994, Prakash *et al.* 1994, Kunjumon *et al.* 1996), production of transgenics for resistance to pests and diseases could not be achieved frequently, due to recalcitrant nature of this crop in *in vitro* culture. In regeneration studies, inducing shoot buds by addition of BAP to the culture media is common practice (Gulati and Jaiwal 1994, George and Eapen 1994). However, induced buds do not always regenerate further to plants (Kamada and Harada 1979). The type and age of the explant (Cheng *et al.* 1980, Kumar *et al.* 1983), orientation of the explant in the culture medium (Eapen and George 1993), BAP concentration (Polisetty *et al.* 1997) and amino acids supplemented to the media (Zhu *et al.* 1990) might be important.

Though a number of studies demonstrated stimulation of embryogenesis by supplementing amino acids to the media (Rao *et al.* 1995, Claparols *et al.* 1993), there is very little information on the effect of amino acids on *in vitro* organogenesis (Santos *et al.* 1996). Rajasubramaniam and Sarathi (1994) reported an enhanced frequency of adventitious shoot induction with the addition of proline and glutamine. In melons role of proline in regulating benzyl adenine induced shoot organogenesis by increasing endogenous proline content was reported by Milazzo *et al.* (1998). In *Torenia fournieri*, in a medium containing BAP and NAA, alanine and aspargine stimulated formation of buds, whereas glutamic acid and aspartic acid enhanced bud formation when the media contained only NAA without BAP (Kamada and Harada 1979). In pigeonpea, high frequency plant regeneration was obtained with BAP in combination with IAA and aspartic acid (Eapen and George 1993).

Another aspect of regeneration is the role of evolved gases in *in vitro* cultures. Evolution and accumulation of ethylene in tissue culture is dependent on the method used (Rigghetti *et al.* 1990). Presence of ethylene was

Received 27 January 2000, *accepted* 25 May 2000.
Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; BAP - 6-benzylaminopurine; B5 medium - Gamborg medium; MS medium - Murashige and Skoog medium; NAA - naphthalene acetic acid.
Acknowledgement: The authors thank Mrs. Sangeeta Khetarpal and Dr. Anupam Raj for their help and co-operation during course of investigation. The authors thank the head, division of plant physiology for his help and cooperation. Authors PR and RC gratefully acknowledge the financial assistance given by ICAR-Cess fund. Authors GVSR thanks IARI for providing fellowship.
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found necessary for callus growth and for shoot regeneration (Perez-Bermudez *et al.* 1985, Kumar *et al.* 1996). However, ethylene inhibited shoot regeneration from callus cultures of *Brassica* (Pua *et al.* 1996), *Nicotiana* (Purnhauser *et al.* 1987), and *Zea mays* (Songstad *et al.* 1988). Non differentiating hypocotyl explants of chickpea were found to evolve significantly more ethylene than differentiating cotyledonary node explants (Chandra *et al.* 1997/98). In recalcitrant *Brassica* sps. addition of ethylene inhibitor to culture media enhanced shoot regeneration (Pua *et al.* 1996).

Besides ethylene role of other gases including CO_2, were reported to have influenced shoot organogenesis and callus growth (Kumar *et al.* 1996, Adkins *et al.* 1990). So far, little attention was paid to methane, which invariably is released along with ethylene. First reports on the role of methane on growth and differentiation of explants, and on development of induced embryos were reported by Chandra *et al.* (1997/98) and Guru *et al.* (1999).

The main objective of the present study was to enhance shoot bud development from induced buds in pigeonpea. For this purpose, the role of cysteine, proline, asparagine and glutamine has been assessed on enhancing shoot bud development. Influence of these amino acids when supplemented to the both MS and B5 media, on evolution of ethylene has been studied. Along with ethylene the role of methane in growth and differentiation to buds, and development of the differentiated buds to microshoots has been assessed which was not done so far.

Materials and methods

Seeds of pigeonpea (*Cajanus cajan* L.) cv. P-855 were obtained from Division of Genetics, IARI, New Delhi-110012. Healthy seeds of uniform size were agitated in dilute solution of commercial liquid detergent, rinsed under tap water, surface sterilized with 0.1 % mercuric chloride solution for 5 min, and washed for 4 - 6 times with sterilized double distilled water. In 150 cm^3 conical flask about 4 - 5 sterilized seeds were germinated on sterilized cotton, moistened with 1/4 strength of either MS (Murashige and Skoog 1962) or B5 (Gamborg *et al.* 1968) media, supplemented with 2 mg dm^{-3} BAP (*Sigma*). Liquid medium without agar was used for germination. The pH of the medium was adjusted to 5.5 before autoclaving.

Four-day-old seedlings were used for the study. Explant consisted of cotyledonary node along with intact cotyledons but from which both radicle and plumule were excised. When the explant was inoculated with plumule side portion of cotyledonary node in contact with the medium, numerous shoot buds along with callus were induced.

Inoculation of the explant was done in 60 cm^3 culture tubes containing 20 cm^3 of either MS or B5 media. Sucrose concentration was 3 % for MS and 2 % for B5; agar quantity remained 0.8 % for both the media. Sucrose and agar are of analytical grade obtained from *Qualigence Company*, India.

If the callus with buds were cultured continuously in induction medium (with BAP 2 mg dm^{-3}) for more than 15 d, the induced buds reverted back to callus stage. Hence the callus with buds was divided into 2 - 3 pieces and each was cultured individually in media (B5 and MS) supplemented with 0.1 mg dm^3 BAP along with 0.01 mg dm^{-3} NAA. In this media buds developed to microshoots and this was treated as control. As the bud development in this media was only 34 - 35 %, attempts were made to enhance efficiency of shoot bud development, by supplementing the media with proline, glutamine, asparagine and L-cysteine at two concentrations (50 or 100 mg dm^{-3}). These concentrations were selected based on the results of preliminary experiments. All the amino acids are from *Hi-media Laboratories*, Mumbai, India.

Seed germination and maintenance of cultures were carried out in culture rooms where the temperature was maintained at 25 ± 2 °C and photoperiod 16 h. *Philips* white fluorescent tubes were used to obtain irradiance of 60 µmol m^{-1} s^{-1}.

Measurement of rate of ethylene and methane production, based on accumulation in sealed culture tubes over 24 h periods were made using the method of Wilson *et al.* (1994). Culture tubes containing the explant were sealed with *Suba-seal* rubber stoppers, 24 h prior to taking gas samples. *Perkin Elmer* gas chromatograph fitted with FID detector was used for this purpose. Column temperature was maintained at 60 °C and that of injector and detector at 200 °C. Exactly 1 cm^3 of the gas sample was injected for each treatment. For calibration standard ethylene and methane were obtained from *EDJ Research Company*, London, UK.

All experiments were repeated thrice for measurement of fresh mass, moisture content and regeneration to buds. For each experiment, there were 20 replicates. For measurement of ethylene and methane the replicates were 3 each time and the experiment was repeated thrice. Completely randomized design (for single factor) and factorial completely randomized design (for more than one factor) were used. Means were evaluated at $P = 0.05$ using Duncan's New Multiple Range Test (DMRT). For statistical analysis standard methods and *Microsoftware* of *CIMMYT*, Mexico was used.

Results and discussion

When the cotyledonary node explant (with both plumule and radicle exiced), was inoculated with its plumule side touching the medium and with its radicle side up, green callus with numerous shoot buds were produced. Buds developed in the plumule side touching the medium, whereas callus developed in radicle portion, away from the medium. Induction of buds in this orientation was recorded in both B5 and MS media. Similarly high frequency of plant regeneration was obtained when adaxial surface of cotyledon was in contact with the medium in *Tamarindus indica* (Jaiwal and Gulati 1991). Eapen and George (1993) also reported influence of orientation of explant on shoot regeneration in peanut and pigeonpea.

Fresh mass and moisture content of the explant were higher in B5 than in MS medium (Table I). After 15 d of culture, green callus with numerous morphologically similar buds were induced in both the media. Induction of shoot buds were seen in almost all explants, it ranged from 100 % in B5 medium to 96 % in MS medium (Table 1). Similarly more shoot buds were observed in B5 medium (Table 1).

In the present study, attempts were made to relate evolution of ethylene and methane to growth and differentiation. In pigeonpea cotyledon explants evolved both ethylene and methane in both MS and B5 media (Table 1). Ethylene evolution was twice in B5 media to that in MS medium. Methane evolution, on the other hand was two and half times more in MS medium over that of B5 medium (Table 1), indicating that media play a role in evolution of the gases. As, explant, growth regulators and other culture conditions are similar, the observed differences in evolution of these gases may be attributed only to the differences in media components such as concentration and form of nitrogen, and to that of sucrose and other micro and macro nutrients. Based on the detailed studies on the role of form and concentration of nitrogen and sucrose in chickpea (Guru 1997), it was inferred that increased methane evolution in MS medium and increased ethylene evolution in B5 media are due to increased concentration of NH_4^+ in MS medium (NO_3^- : NH_4^+ is 2:1 in MS medium and 12.5:1 in B5 medium) and to a total nitrogen concentration (60 mM in MS and 27 mM in B5).

The ethylene to methane ratio ranged from about 1:0.92 in B5 to 1:6 in MS at 15 d after inoculation when buds with callus were observed (Table 1).

Table 1. Effect of medium on fresh mass [g explants^{-1}], moisture content [%], percentage of pigeonpea cotyledonary node explants regenerating to callus with buds, number of shoot buds per callus, percentage of shoot buds developed, and ethylene and methane evolution [pmol g^{-1}(f.m.) s^{-1}] at 15 d after inoculation in media supplemented with 2 mg dm^{-3} BAP.

Medium	Fresh mass	Moisture content	Callus regeneration	Shoot buds per callus	Shoot buds developed	Ethylene	Methane
B5	1.115 ± 0.58	90.80 ± 1.4	100	42.8 ± 3.3	35.98	1.79 ± 0.14	1.64 ± 0.54
MS	0.955 ± 0.89	88.23 ± 1.9	96	39.8 ± 3.8	34.17	0.76 ± 0.17	4.21 ± 0.96

If the callus and the buds were continuously grown in B5 medium containing 2 mg dm^{-3} BAP, buds reverted back to callus stage thus becoming recalcitrant which is commonly observed with pulses grown *in vitro*. Hence, after 15 d the callus with buds were devided into 2 - 3 pieces and subcultured in the respective media (B5 and MS) supplemented with BAP (0.1 mg dm^{-3}) along with NAA (0.01 mg dm^{-3}). For enhancing regeneration, supplementing low concentration of NAA, along with BAP was reported by White and Voisey (1994). This medium is termed as shoot bud development media which served as control. The number of shoot buds developed ranged from 7.7 in B5 medium to 6.3 in MS medium (Table 2). Though recalcitrance was prevented by this medium, the percentage of buds developed to shoots to that of total shoot buds induced remained 34 - 35 %. This percentage is higher than 26.6 % reported by George and Eapen (1994) with cotyledonary node explants.

Increased efficiency of shoot regeneration by supplementing different amino acids was reported by Milazzo *et al.* (1998), Eapen and George (1993), and Rajasubramaniam and Sarathi (1994). In the present study four amino acids glutamine, asparagine, proline, and L-cysteine at two concentrations supplemented to bud development medium significantly increased fresh mass in all the treatments in B5 and MS media over that of control except for proline at lower concentration (50 mg dm^{-3}) in B5 medium. Maximum fresh mass was seen with glutamine in B5 medium and with asparagine in MS medium (Table 2). Supplementing the media with higher concentrations of amino acids resulted in significantly more fresh mass than with lower concentrations in MS media (Table 2).

All the amino acid treatments significantly increased number of microshoots per explant over control, at both the concentrations and in both the media (Table 2). Thus efficiency of microshoot development was enhanced with amino acid treatment. Proline (50 mg dm^{-3}) recorded maximum microshoot development followed by glutamine, asparagine and cysteine. Lower concentrations were more efficient than at higher concentrations in both B5 and MS medium (Table 2).

Table 2. Effect of various amino acids supplemented to B5 and MS shoot bud development medium (with BAP and NAA) on fresh mass [g $explants^{-1}$] and number of microshoots [$explant^{-1}$]. Data was recorded 15 d after inoculation. Means followed by common letter within the column are non-significantly different at $P = 5$ %.

Amino acid	[mg dm^{-3}]	Fresh mass B5	MS	Microshoots B5	MS
Control		0.897^{d}	0.577^{d}	7.7^{f}	6.3^{f}
L-asparagine	50	1.207^{c}	0.675^{ab}	14.6^{c}	13.6^{cd}
	100	1.382^{bc}	0.719^{a}	11.4^{e}	10.7^{e}
Glutamine	50	1.592^{a}	0.659^{abc}	16.9^{b}	14.3^{bc}
	100	0.784^{a}	0.702^{a}	$12.3d^{e}$	9.6^{e}
L-cysteine	50	1.221^{c}	0.581^{c}	16.8^{b}	12.7^{d}
	100	1.430^{b}	0.608^{c}	13.3^{cd}	10.2^{e}
L-proline	50	0.897^{d}	0.590^{cd}	19.4^{a}	17.9^{a}
CD at 5 %		0.193	0.080	1.36	1.32

Table 3. Effect of various amino acids supplemented to B5 and MS shoot bud development medium on evolution of ethylene and methane [pmol g^{-1}(f.m) s^{-1}]. Data was recorded 15 d after inoculation. Means followed by common letter within the column are non-significantly different at $P = 5$ %.

Amino acid	[mg dm^{-3}]	B5 ethylene	methane	MS ethylene	methane
Control		1.51^{a}	0.47^{f}	2.34^{a}	1.84^{f}
L-asparagine	50	0.65^{d}	0.90^{e}	1.26^{b}	2.5^{e}
	100	0.43^{f}	1.51^{c}	0.54^{d}	3.30^{d}
Glutamine	50	1.16^{b}	0.76^{c}	1.37^{b}	4.5^{c}
	100	0.76^{c}	1.15^{d}	0.72^{c}	7.81^{ab}
L-cysteine	50	0.79^{c}	1.70^{c}	0.61^{cd}	2.95^{d}
	100	0.43^{f}	2.60^{b}	0.22^{e}	7.42^{b}
L-proline	50	0.54^{e}	2.50^{b}	0.50^{d}	3.06^{d}
	100	0.32^{g}	0.39^{a}	0.25^{e}	8.06^{a}
CD at 5 %		0.11	0.22	0.18	0.25

Pigeonpea explants evolved ethylene and also methane in both control and amino acid treatments (Table 3). With the addition of amino acids evolution of ethylene was lower and that of methane was higher than that of control in both the media (Table 3). Highest ethylene to methane ratio was recorded with cysteine (1:35) and proline (1:32) at higher concentrations.

All amino acids enhanced microshoot development. Thus reduction in evolution of ethylene accompanied by increased methane evolution resulted to increased microshoots, thus establishing a relationship between ethylene, methane and the number of microshoots, especially at lower concentrations of the amino acids (Table 2).

Amino acids at higher concentrations recorded more methane and lesser ethylene than at lower concentration in both B5 and MS media (Table 3). Maximum methane was recorded in proline treatment in both the media followed by glutamine and cysteine (Table 3).

Methane evolution was significantly higher in MS medium containing more N in form of NH_4^+ when compared to B5 medium and addition of amino acids enhanced methane evolution. Thus a clear relationship between NH_4^+ in media to that of methane evolution was established which was not reported earlier. Similarly in habituated organogenic calli of sugar beet, activation of C-1 pathway (which include methyl and formyl groups) was related to organogenesis in these lines (Hagege *et al.* 1994). On the contrary, positive influence of amino acids on inducing organogenesis was seen in *Torenia fournieri*, when media contained KNO_3 as a sole source of N but not NH_4NO_3 (Kamada and Harada 1979).

Proline treatment recorded maximum microshoots in both MS and B5 media. Role of proline in regulating benzyl adenine induced shoot regeneration was reported by (Milazzo *et al.* 1998). When endogenous levels of proline was increased by the addition of proline precursors or by proline itself, stimulation of the purine and aromatic metabolism resulted in enhancement of organogenesis in melons (Milazzo *et al.* 1998).

With the addition of proline, ethylene evolution decreased whereas methane evolution increased. In maize callus cultures, supplementing 1-aminocyclopropane-1-carboxylic acid (ACC), a ethylene precursor, increased evolution of ethylene which was accompanied by reduction in free proline content and also a reduction in regeneration rates (Songstad *et al.* 1988).

From the present study, it can be concluded that addition of amino acids to both B5 and MS media enhanced efficiency of development of shoot buds to microshoots by 1 to 8 fold. At lower concentrations of amino acids efficiency of bud development as number of shoots developed to microshoots was more. On the other hand, at higher concentrations fresh mass was more increased.

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