Indian J. Plant Physiol., Vol. 8, No. 3 (N.S.) pp. 241-245 (July-Sept., 2003)

ENHANCED PLANT REGENERATION FROM COTYLEDONARY NODE EXPLANTS OF MUNGBEAN BY AMINO ACIDS

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Received on 24 Jan., 2002, Revised on 12 Sept., 2003

SUMMARY

In mungbean (*Vigna radiata* (L.) Wilczek), shoot buds were induced when cotyledonary node explants were supplemented with benzylaminopurine (10μM). When benzylaminopurine (BAP, 5 μM) and napthaleneacetic acid (NAA, 0.05 μM) were supplemented to the medium, 30-35 per cent of induced buds developed into micro shoots. Supplementation of amino acids like glutamine, proline and L-cysteine at the concentration of 40 and 80 mg dm^{-3} to the shoot bud development medium resulted in enhanced regeneration. The fresh mass increased with higher concentration of amino acids whereas number of micro shoots decreased. Ethylene and methane evolution was observed during the regeneration process. There was an increase in methane evolution and reduction in ethylene evolution with the addition of amino acids.

Key words : **Amino acids, benzylaminopurine, indole-3-acetic acid, mungbean, napthaleneacetic acid, regeneration.**

INTRODUCTION

Mungbean (*Vigna radiata* (L.) Wilczek) is an important grain legume with high protein content, which could adequately meet the protein requirements for a majority of the population in India. Genetic improvement of mungbean by conventional plant breeding methods is time consuming and labour intensive. In recent years, advances in plant genetic engineering have opened a new avenue for crop improvement. The success of genetic transformation is determined by several factors, among which an efficient regeneration protocol is a crucial one. Though a few reports on regeneration are available (Bajaj and Dhanju 1979, Mathews 1987, Gulati and Jaiwal 1990, Chandra and Pal 1995), the process of regeneration in these studies was slow and generally produced low frequencies of shoot regeneration. This was mainly due to the recalcitrant nature of this crop in *in vitro* culture.

Inclusion of certain amino acids in the culture medium has been shown to stimulate embryogenesis (Rao *et al.* 1995, Claparols *et al.* 1993). There is not much information on the effect of amino acids on *in vitro* organogenesis. In barley, supplementation of alanine, asparagine and glutamine increased the frequency of differentiation and percentage of plant regeneration (Zhu *et al.* 1990). Rajasubramaniam and Sarathi (1994) reported an enhanced frequency of adventitious shoot induction with addition of proline and glutamine.

Plant cells and tissues grown in culture have been known to produce ethylene (La Rue and Gamborg 1971) and hence likely to influence growth and differentiation in such system (Reid *et al.* 1985). Ethylene inhibited shoot regeneration in cotyledon explants of *Helianthus* (Chraibi *et al.* 1992), in callus culture of *Brassica* (Pua *et al.* 1999), and *Zea mays* (Songstad *et al.* 1988). Besides ethylene, role of the other gases including CO_2 in influencing shoot organogenesis and callus growth has been reported (Kumar *et al.* 1996). Methane is also released along with ethylene and plays some role in growth and differentiation of explants (Chandra *et al.* 1997).

The main objective of the present study was to enhance shoot bud development from induced buds in mungbean. For this purpose, the role of cysteine, proline and glutamine has been assessed on enhancing shoot bud development. Influence of these amino acids on evolution of ethylene has been studied on MS and B_5 media. Along with ethylene, the role of methane in growth and differentiation into buds, and development of the differentiated buds into micro shoots was assessed.

MATERIALS AND METHODS

Seeds of mungbean (*Vigna radiata* (L.) Wilczek) cv. Pusa-105 were obtained from the Division of Genetics, IARI, New Delhi-110012. Healthy seeds of uniform size were agitated in dilute solution of commercial liquid detergent, rinsed with tap water, surface sterilized with 0.1 per cent mercuric chloride solution for 5 min and washed 4-6 times with sterile double distilled water. About 3-5 sterilized seeds were germinated in culture tubes (60 cm^3) on sterilized cotton moistened with ¼ strength of either MS (Murashige and Skoog, 1962) or B_5 (Gamborg *et al.* 1968) media and plugged with cotton plugs. Liquid medium without agar was used for germination. The pH of the medium was adjusted to 5.5 before autoclaving.

Four day old cotyledonary node explants were used for the study. Explants consisting of cotyledonary node with intact cotyledons attached to embryonic axis (8 mm *i.e.* 4 mm each of epicotyl and hypocotyls) were excised and inoculated with 2 mm of hypocotyl end of embryonic axis in the medium. Inoculation of explant was done in 60 cm^3 culture tubes containing 20 cm^3 of either MS or B_5 media. Sucrose concentration was 3 per cent for MS and 2 per cent for B_5 and concentration of agar was 0.8 per cent for both media. Sucrose and agar (analytical grade) were obtained from Qualigens, India.

When the callus with buds were cultured continuously on induction medium (with 10 µM BAP) for more than 15 days, the induced buds reverted back to callus stage. Hence, the callus with buds were divided into 2-3 pieces and each of them was cultured individually on media (B_5 and MS) supplemented with 5 µM BAP along with 0.05 µM NAA. In this medium, buds developed into micro shoots and this was treated as control. As the bud development in this media was only 30-35 per cent, attempts were made to enhance efficiency of shoot bud development by supplementing the media with glutamine, proline and cysteine (40 and 80 mg dm^{-3}). These concentrations were selected based on the results of preliminary experiments.

Seed germination and maintenance of cultures were carried out in culture rooms where temperature was maintained at 25±2°C and photoperiod of 16 h light and 8 h dark cycle. 'Philips' white fluorescent tubes were used to obtain irradiance of 60 µmol $m^{-1}s^{-1}$. Measurement of rate of ethylene and methane production over 24 h periods, based on accumulation in sealed culture tubes 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 analysis. Column temperature was maintained at 60° 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 and regeneration to buds. For each experiment, there were 20 replicates. For measurement of ethylene and methane, the experiment was repeated thrice with three replicates each time. 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 *Micro software* of *CIMMYT*, Mexico was used.

Rooting and establishment: Well-developed shoots (3-4 cm) from regenerating explants were excised and rooted in MS medium supplemented with 1 µM IAA. Plantlets with well-developed roots were removed from culture tubes and after washing under running tap water, they were transferred to beaker containing Hoaglands solution for better survival rate. They were then transferred to pots containing sterile vermiculite and finally established on soil in the glasshouse.

RESULTS AND DISCUSSION

When the cotyledonary node explants were inoculated on B_5 medium supplemented with 10 µM BAP, green callus with numerous shoot buds were produced. If the callus and buds were continuously grown in B_5 medium containing 10 µM BAP, buds reverted back to callus stage thus becoming recalcitrant which is commonly observed in pulses grown *in vitro*. For enhancing regeneration, low concentration of NAA (0.05 µM), along with BAP (5 µM) was supplemented to the medium (Plate 1 & Plate 2). This medium is termed as shoot bud development medium, which served as control. The average number of shoot buds developed ranged from 4.3 in B_5 medium to 3.3 in MS medium (Table 1).

Table 1. Effect of amino acids supplemented to B_5 and MS shoot bud development medium (with BAP and NAA) on fresh mass (g explant^{-1}) and number of micro shoots [explant^{-1}]15 days after inoculation. Means followed by common letter within column are non-significant at P = 5%.

Amino acid	mg dm^{-3}	Fresh mass		Micro shoots	
		B_5	MS	B_5	MS
Control		0.614^{f}	0.593^{d}	4.3^{e}	3.3^{e}
L-glutamine	40	1.171^{d}	0.965^{bc}	6.7^{c}	5.7^{c}
	80	1.286^{c}	1.107^{a}	5.6^{d}	4.6^{d}
L-proline	40	1.081^{e}	0.896^{a}	8.3^{a}	6.3^{b}
	80	1.103^{de}	0.911^{bc}	7.7^{b}	6.0^{bc}
L-cysteine	40	1.623^{b}	1.023^{ab}	7.0^{bc}	7.2^{a}
	80	1.754^{a}	1.128^{a}	6.1^{cd}	6.0^{bc}
CD at 5%		0.08	0.029	0.35	0.18

Plate 1. Callus with shoot buds obtained from 15 day old cotylendonary node explants of mungbean variety Pusa 105. The explant was cultured on B_5 medium supplemented with 5 µM BAP and 0.05 µM NAA. Photograph taken 15 days after inoculation

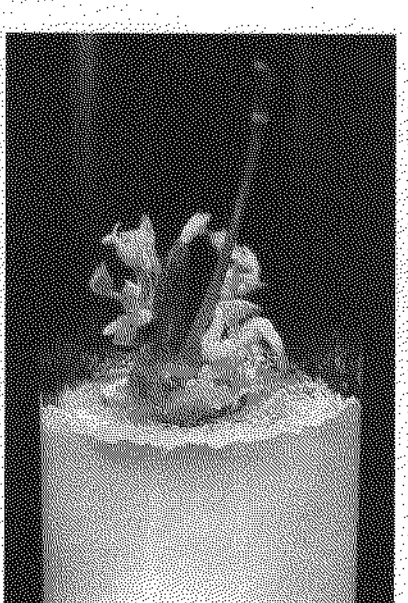

Plate 2. Callus with shoot buds obtained from 15 day old cotylendonary node explants of mungbean variety Pusa 105. The explant was cultured on MS medium supplemented with 5 µM BAP and 0.05 µM NAA . Photograph taken 15 days after inoculation

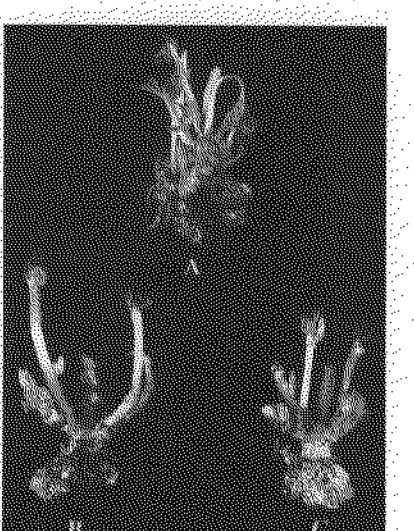

Plate 3. Effect of amino acids on micro-shoots development in mungbean variety Pusa 105. Amino acids were supplemented to B_5 shoot development medium [B_5 + BAP (5.0 µM) + NAA (0.05 µM)]. Photograph taken 15 days after inoculation.

A : Proline (40 mg dm^{-3})
B : Glutamine (40 mg dm^{-3})
C : Cysteine (40 mg dm^{-3})

Increased efficiency of shoot regeneration by supplementing different amino acids was reported by Eapen and George (1993) and Rajasubramaniam and Sarathi (1994). In the present study glutamine, proline and cysteine (40 and 80 mg dm^{-3}) supplemented to shoot bud development medium significantly increased fresh mass in all the treatments in B_5 and MS media over that of control (Table 1). Supplementing media with higher concentration of amino acids resulted in significantly more fresh mass than with lower concentration. All amino acid treatments significantly increased the number of micro shoots per explant over control at both the concentrations in MS and B_5 media (Table 1). Efficiency of regeneration was enhanced with amino acid treatment (Plate 3). Proline (40 mg dm^{-3}) and cysteine (40 mg dm^{-3}) led to maximum micro shoot development in B_5 medium and MS medium, respectively. Lower concentrations were more efficient than higher concentration in both B_5 and MS medium (Table 1).

Mungbean explants evolved ethylene and methane in both control and amino acid treatments (Table 2). With the addition of amino acids, evolution of ethylene was lower and that of methane was higher than that of control in both MS and B_5 media. All amino acids enhanced micro shoot development. Reduction in evolution of ethylene accompanied by increased methane evolution, thus established a relationship between ethylene, methane and the number of micro shoots. Amino acid treatments at higher concentration led to more methane and lesser ethylene production than that of lower concentrations in both MS and B_5 media. Methane evolution was significantly higher in MS medium containing more N in the form of NH_4^+ when compared to B_5 medium. Proline recorded maximum number of micro shoots in B_5 medium. Role of proline in inducing shoot regeneration was reported by 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 B_5 and MS media enhanced efficiency of development of shoot buds to micro shoots. At lower concentrations of amino acids, efficiency of bud development (as number of shoots developed to micro shoots) was more. On the other hand, increased fresh mass was observed at higher concentration of amino acids.

Table 2. Effect of amino acids supplemented to B_5 and MS shoot bud development medium (with BAP and NAA) on evolution of ethylene and methane (nmol g^{-1} fm s^{-1}). 15 days after inoculation. Means followed by common letter within column are non-significant at P = 5%.

		B_5		MS	
Amino acids	**mg dm^{-3}**	**ethylene**	**methane**	**ethylene**	**methane**
Control		0.053^{a}	0.020^{d}	0.057^{a}	0.038^{d}
L-glutamine	40	0.026^{ab}	0.024^{cd}	0.040^{b}	0.077^{c}
	80	0.015^{b}	0.031^{c}	0.034^{ab}	0.089^{c}
L-proline	40	0.012^{b}	0.051^{b}	0.035^{ab}	0.111^{b}
	80	0.009^{c}	0.022^{cd}	0.028^{c}	0.145^{a}
L-cysteine	40	0.021^{ab}	0.062^{a}	0.030^{c}	0.085^{c}
	80	0.016^{b}	0.081^{a}	0.022^{cd}	0.125^{b}
CD at 5%		0.005	0.012	0.003	0.014

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