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CALLUS INDUCTION AND PLANTLET REGENERATION IN BLACKGRAM (*Vigna mungo* L. HEPPER)

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ABSTRACT

The present study was undertaken for callus induction and subsequent plantlet regeneration in blackgram. The study comprised of experiments for callus initiation, shoot regeneration and plantlet formation from cotyledon, hypocotyl, root tip and shoot tip explants. The effects of explants and different concentrations and combinations of BAP (0.0, 1.0, 2.5, 5.0 and 10 mg L^{-1}) and NAA (0.0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}) on callus induction were investigated first. Among the explants, hypocotyls showed the best performance in callus formation (92.33%) when cultured on MS medium supplemented with 2.5 mg L^{-1} BAP and 1.5 mg L^{-1} NAA followed by cotyledon, shoot tip and root tip explants, respectively. The height percentage of shoot regeneration from the calli derived from hypocotyls (56.33%) was achieved in MS medium supplemented with 3.0 mg L^{-1} BAP and 0.3 mg L^{-1} NAA and 0.5 mg L^{-1} GA_3. Calli from other explants had no shoot regeneration. The regenerated shoots were transferred to rooting medium supplemented with different concentrations of IBA and NAA. The high frequency (100 %) of rooting was observed with MS medium supplemented with 0.5 mg L^{-1} IBA. The rooted plants were transferred to pots for hardening.

Key words : Blackgram, Callus, Plantlet, Regeneration, *Vigna mungo* L. Hepper

INTRODUCTION

Blackgram (*Vigna mungo* L. Hepper, syn. *Phaseolus mungo* L.) is one of the most important pulse crops grown in Bangladesh. The cultivated blackgram belongs to the family Leguminosae and sub-family Papilionaceae. Blackgram is an annual food legume. It, also referred to as the urad, urd bean, urid, black lentil or white lentil, is grown Southern Asia like India, Pakistan, Bangladesh, Afghanistan, Myanmar. Like mungbean, it is a member of Asiatic *Vigna* crop group. It is mainly a day neutral warm season crop commonly grown in semi-arid to sub-humid low land tropics and sub-tropics. Blackgram grows on most soils, with a preference to loams with a pH of 5.5-7.5. The optimum temperature for growth ranges from 27-30^{o}C. Blackgram is more tolerant to waterlogging compared to mung bean (Lawn *et al.,* 1978).

Blackgram is very much popular in Bangladesh and ranks 3rd in terms of consumption and total area in which different varieties of this crop are cultivated (Gowda and

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Kaul, 1982). It ranks second in respect of yield and production of seed protein (Mian, 1976). It contains approximately 25-28% protein, 4.5-5.5% ash, 0.5-1.5% oil, 3.5-4.5% fibre and 62-65% carbohydrate on dry weight basis (Kaul, 1982). It has been reported that the average yield of blackgram is about 1000 kg ha^{-1} and the protein content is 25-26% (BINA, 2004). In spite of its various uses, its cultivation is decreasing day by day both in acreage and yield (BBS, 2006).

Plant biotechnology, a modern technique is mainly based on plant cell culture. Regeneration of plants from cells or tissues is an important and essential component of biotechnology, which is required for the genetic manipulation of plants. Plant cell culture has become an excellent method for plant cell differentiation as well as a supplementary technique for plant breeding programs through the uses of new and expanded genetic variability (Nakamura and Maeda, 1989). Production disease free healthy plant materials and genetic improvement through gene transfer technique it is very urgent and require a protocol for *in vitro* propagation in any crop plant. Regeneration and transformation procedures of pulses are not well developed in Asian countries compared to the success achieved in other grain legumes from Europe and North America.

The study was, therefore, undertaken to find out suitable explants for regeneration and to develop a reproducible protocol for *in vitro* callus formation and plantlet regeneration

MATERIALS AND METHODS

The experiment was carried out at the Biotechnology Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh. The experimental materials (seeds) of *Vigna mungo* (2n=22) were collected from Bangladesh Institute of Nuclear Agriculture, for the establishment of culture. Root tips, cotyledons, hypocotyls and shoot tips were used as explants in the present study. Surface sterilization of mature seeds was carried out under Laminar Air Flow Cabinet. Seeds were washed by sterile distilled water for 3 to 5 minutes. Later, they were rinsed in 70% ethyl alcohol followed by washing with sterile distilled water for 3 times. Finally, surface disinfection was done with 0.1% $HgCl_2$ for five minutes with continuous agitation. The seeds were then washed 5 times with sterile distilled water to remove the sterilant. The mature seeds were placed on a solidified agar medium containing no growth regulators in sterile vials. Later the vials were wrapped with Parafilm. Seven to ten days after germination, root tips, cotyledons, hypocotyls and shoot tips of seedlings were dissected and cultured on MS medium supplemented with different combinations and concentrations of BAP (0, 1.0, 2.5, 5.0 and 10 mg/l) and NAA (0, 0.5, 1.0, 1.5 and 2.0 mg/l) for callus induction. Different concentrations of BAP (0.0, 3.0 and 5.0 mg/l) alone, different combinations of BAP (1.0, 2.0 and 3.0 mg/l) and NAA (0.1, 0.2 and 0.3 mg/l) with or without 0.5 mg/l GA_3 including the control were used for shoot induction from calli. For successful rooting, 3-5 cm usable shoots excised from multiple shoots were implanted to rooting medium consisting of MS medium supplemented with IBA (0.0, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) and NAA (0.20 and 0.50 mg/l).

MS (Murashige and Skoog, 1962) medium supplemented with different concentrations and combinations of phytohormones as per treatments was used for callus and shoot induction, shoot multiplication and rooting of shoots. Hormones were added separately to different media according to their requirements. pH of the media was adjusted to 5.8 and then agar @ 9.0 g was added to solidify the medium. All instruments, glass wares and culture media were sterilized by autoclaving at 1.16 kg/cm^2 pressure at 121^o C for 30 minutes. The temperature of the growth room was maintained at 25±2^{o}C by an air conditioner. A 16 hours light period was maintained with light intensity of 2000 lux. At least 16-20 explants were cultured in each experiment and all experiments were repeated thrice. One month old adequately rooted shoots when reached a height of 4-5 cm were brought out from culture room and kept in room temperature for 7 days. Plantlets were then taken out from vial and agar was washed out gently from roots. These plantlets were transplanted in small pots containing a mixture of compost, soil and sand (1:2:1) and were covered with transparent sheets. They were nurtured under room temperature in presence of sufficient light. In that conditions the plantlets were watered every alternate day.

The data for the parameters recorded in the present study were statistically analyzed by the programmed MSTATC and Microsoft Excel wherever applicable. The experiment was conducted in the culture room (Biotechnology Laboratory) following Completely Randomized Design (CRD). The analysis of variances for different parameters was performed and the means were compared by Duncan's Multiple Range Test (DMRT) at 5% level of probability for interpretation of results (Gomez and Gomez, 1984) using MSTATC Computer programme.

RESULTS AND DISCUSSION

The results had shown highly significant variation for percentage of callus induction (Table 1) from various explants used. The shoot tip explants showed the highest callus induction (52.01%) and the lowest callus induction (44.20%) was achieved from cotyledon explants. The cotyledon explants, however, showed callus induction earlier (14.77 days) than any other explants. The higher number of days was required in root tip (17.32 days) explants.

Table 1. Effect of explants on callus induction

Explants	Percentage of callus induction	Days required for callus induction	Weight of callus (g)
Cotyledon	44.20c	**14.77d**	0.90b
Hypocotyl	48.23b	16.59b	0.56c
Root tip	44.28c	17.32a	0.39d
Shoot tip	**52.01a**	15.17c	**0.99a**
CV (%)	9.51	3.31	9.26
$LSD_{0.05}$	1.445	0.1704	0.02037

Means followed by common letter(s) are statistically identical at 5% level of probability

Geetha *et al*. (1998) reported that callus development was best in shoot tip explants while hypocotyl was reported to be the best by Raman *et al*. (2004). We observed highest callus formation from shoot tip. This difference might be due to the different genotypes used by them and also in this study.

The combination of BAP and NAA exhibited significant influence on the percentage of callus induction (Table 2). The highest percentage (86.42%) of callus induction was observed in medium supplemented with 2.5 mg L^{-1} BAP and 1.5 mg L^{-1} NAA and the lowest percentage was found with 10 mg L^{-1} BAP and 1.5 mg L^{-1}NAA. The medium without BAP and NAA had no callus formation. Geetha *et al*. (1998) reported that callus development was best in shoot tip explants with 3.0 mg L^{-1} BAP and 1.5 mg L^{-1} NAA. Mathur and Prakash (1997) reported that MS medium supplemented with 0.5 mg L^{-1} kinetin and 2.0 mg L^{-1} 2,4-D gave the maximum callus induction in *Vigna mungo*.

Table 2. Effect of hormone concentrations on callus induction

Hormone concentrations (mg L^{-1})		Days required for callus induction	Percentage of callus induction	Weight of callus (g)
BAP	NAA			
0.0	0.0	0.00l	0.00k	0.00n
1.0	0.5	20.83de	42.83h	0.60j
	1.0	19.75g	49.25fg	0.78i
	1.5	18.83h	58.92e	1.04f
	2.0	20.25f	59.33e	0.85h
2.5	0.5	18.50h	69.17d	0.96g
	1.0	17.17j	78.42bc	1.28cd
	1.5	**14.75k**	**86.42a**	**1.66a**
	2.0	17.25j	75.00c	1.20e
5.0	0.5	18.58h	68.92d	1.01fg
	1.0	17.92i	77.67bc	1.31c
	1.5	19.92j	79.08b	1.50b
	2.0	17.75i	67.25d	1.23de
10.0	0.5	23.33c	52.42f	0.55k
	1.0	20.58ef	60.67e	0.73i
	1.5	19.50g	37.92e	0.88h
	2.0	21.17d	51.17f	0.65j
CV%		3.31	9.51	9.26
$LSD_{(0.05)}$		0.4260	3.613	0.05091

Means followed by common letter(s) are statistically identical at 5% level of probability

Highly significant variation was observed in combined effect of explants and hormone concentrations on the percentage of callus induction. The highest percentage (92.33%) of callus induction was found in hypocotyl explants with 2.5 mgL^{-1} BAP and 1.5 mgL^{-1} NAA followed by cotyledon (91.00%) and shoot tip (87.33%) explants in the same concentration of treatment and the lowest percentage (37.33%) was observed in cotyledon explants at 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA (Table 3).

Table 3. Combined effect of explants and hormone concentrations on callus induction

Hormone concentrations (mg L^{-1})		Percentage of callus induction			
BAP	NAA	Cotyledon	Hypocotyl	Root tip	Shoot tip
0.0	0.0	0.00\	0.00\	0.00\	0.00\
1.0	0.5	37.33yz	38.33yz	41.67xy	54.00p-v
	1.0	52.33s-w	38.33yz	48.00u-x	58.33m-s
	1.5	61.67l-q	53.33q-v	58.33m-s	62.33l-p
	2.0	57.33n-t	61.67l-q	61.67l-q	56.67o-u
2.5	0.5	68.33h-l	68.33h-l	76.67c-h	63.67k-o
	1.0	69.67g-l	83.33bc	78.33c-g	82.33bc
	1.5	**91.00a**	**92.33a**	75.00c-i	87.33ab
	2.0	82.67bc	80.67b-d	54.00p-v	82.67bc
5.0	0.5	72.33e-j	68.33h-l	71.67f-k	63.33k-o
	1.0	76.67c-h	80.67b-e	76.67c-h	76.67c-h
	1.5	79.00b-f	82.33bc	73.33d-j	81.67b-d
	2.0	66.67i-m	76.67c-h	49.00t-x	76.67c-h
10.0	0.5	42.33xy	62.33l-p	44.w-y	61.00l-r
	1.0	52.33s-w	65.67j-n	52.33s-w	72.33e-j
	1.5	33.33z[	68.33h-l	50.00s-x	80.00b-f
	2.0	31.67z[	46.67v-x	58.33m-s	68.00h-i
CV%			9.51		
$LSD_{(0.05)}$			7.227		

Means having common letter(s) are statistically identical at 5% level of probability

Days required for callusing was recorded by careful observation of the explants every day. Number of days required for callus initiation varied depending on the explants and hormonal treatments. The cultured explants showed significant variation on days required for callus induction. Cotyledon explants responded earlier towards callusing and required least number of days (14.77) for callus initiation followed closely by shoot tip (15.17), hypocotyl (16.59) and root tip (17.32) explants (Table 1).

Days required for callus induction was significantly influenced by the concentrations of BAP and NAA. The least number of days required for callus induction was 14.75 at 2.5 mg L^{-1} BAP and 1.5 mg L^{-1} NAA. Maximum numbers of days (25.83) was required for callus formation in 10.0 mg L^{-1} BAP and 1.5 mg L^{-1} NAA (Table 2).

In order to induce shoot regeneration, calli derived from cotyledons, hypocotyls, root tips and shoot tips were cultured on shoot induction media containing different concentrations of BAP and/or NAA and combination of BAP, NAA and GA_3. Only hypocotyl explants developed shoots (Fig. 1B and 1C). The calli of cotyledon, root tip and shoot tip explants failed to differentiate into any shoots. The failure might be due to the absence of appropriate concentration and combination of hormone or lack of any additives essential for regeneration. However, Geetha *et al.* (1997) reported that that cotyledon explants developed shoots.

Days required for shoot induction from hypocotyl explants were significantly influenced by different hormone concentrations (Table 4). The least number of days required for shoot induction was 19.33 at 3.0 mg L^{-1} BAP, 0.3 mg L^{-1} NAA and 0.5 mg L^{-1} GA_3 followed by 3.0 mg L^{-1} BAP and 0.3 mg L^{-1} NAA (22.23 days). The maximum number of days required for shoot induction was 30.00 at 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA.

Table 4. Effect of hormone concentrations on shoot induction from hypocotyl explants

Hormone concentrations (mg L^{-1})			Days required for shoot initiation	Percentage of shoot initiation	No. of shoots calli^{-1}	Length of shoots (cm)	
						At 35 DAI	At 45 DAI
BAP	NAA	GA_3					
0.0	0.0	0.0	0.00g	0.00h	0.00f	0.00g	0.00h
3.0	0.0	0.0	0.00g	0.00h	0.00f	0.00g	0.00h
5.0	0.0	0.0	26.67b	15.67g	1.33e	0.51f	0.62g
1.0	0.1	0.0	30.00a	25.00f	1.67de	0.93e	1.08f
2.0	0.2	0.0	26.00c	38.67d	2.00d	1.50d	2.23e
3.0	0.3	0.0	22.33e	46.00b	2.67c	1.63c	2.47d
1.0	0.1	0.5	26.33bc	32.33e	1.33e	2.13b	2.97b
3.0	0.3	0.5	**19.33f**	**56.33a**	**4.33a**	**2.63a**	**3.60a**
5.0	0.5	0.5	23.67d	24.33c	3.33b	2.17b	2.73c
$LSD_{0.05}$			0.1745	0.7137	0.1926	0.0446	0.0446

Means followed by common letter(s) are statistically identical at 5% level of probability

The results showed that the highest percentage (56.33) of shoots regenerated in medium supplemented with 3.0 mg L^{-1} BAP, 0.3 mg L^{-1} NAA and 0.5 mg L^{-1} GA_3 followed by 46.00% in 3.0 mg L^{-1} BAP and 0.3 mg L^{-1} NAA. The lowest percentage (15.67%) was observed in 5.0 mg L^{-1} BAP (Table 4). The best response towards shoot regeneration from primary leaf callus of blackgram was reported on MS medium supplemented with 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA (Meijer and Broughton, 1981), and also on medium supplemented with 3.0 mg L^{-1} BAP and 1.5 mg L^{-1} NAA (Geetha *et al.*, 1998).

Number of shoots from hypocotyl explants was significantly influenced by different hormonal concentrations (Table 4). The results showed that the highest number of shoots (4.33) was proliferated on MS medium supplemented with 3.0 mg L^{-1} BAP, 0.3 mg L^{-1} NAA and 0.5 mg L^{-1} GA_3 and the lowest (1.33) with 5.0 mg L^{-1} BAP and also with 1.0 mgL^{-1} BAP, 0.1 mgL^{-1} NAA and 0.5 mg L^{-1} GA_3.

Different concentrations and combinations of BAP and/or NAA and BAP, NAA and GA_3 had significant influence on shoot length (Table 4). The results showed that the highest shoot length (2.63 cm) at 35 days after inoculation and (3.60 cm) at 45 days after inoculation was obtained in MS medium supplemented with 3.0 mg L^{-1} BAP, 0.3 mg L^{-1} NAA and 0.5 mg L^{-1} GA_3.

The rooting of shoots was significantly affected by the auxine concentration (Table 5). MS medium supplemented with 0.25 mg L^{-1} IBA and 0.2 mg L^{-1} NAA performed better and required least number of days (8.33) for rooting. Khawar *et al*. (2002) reported that MS medium containing 0.25 mg L^{-1} IBA performed best and required four weeks for rooting. Geetha *et al*. (1998) reported that roots emerged within 15 days. A higher percentage of rooting (100%) was found with 0.5 mg L^{-1} IBA in the present study. Raman *et al*. (2004) reported that efficient rooting (100%) of the shoots on medium containing half MS salts, full MS vitamins and IBA (2.5 µM). Khawar *et al*. (2002) reported that medium containing 0.25 mg L^{-1} IBA obtained only 25% roots and Geetha *et al*. (1998) reported that medium containing 3.0 mg L^{-1} IBA showed 78.3% of rooting. The maximum number of roots (14.33) per shoot was recorded in medium containing 0.5 mg L^{-1} NAA. Geetha *et al*. (1998) reported that medium containing 3.0 mg L^{-1} IBA produced 14.5 roots/plants. It was clear from the above discussion that 0.5 mg L^{-1} IBA was better for root formation than any other treatments. Das *et al*. (2002) and Geetha *et al*. (1998) reported that IBA was effective for rooting of blackgram, while Roy *et al*. (2007) reported that NAA was effective for rooting. In our study we found higher percentage of rooting using IBA while number of roots/plant was higher using NAA.

Table 5. Effect of IBA and NAA on root induction

Hormone concentrations (mg L^{-1})		Days required for root initiation	Percentage of shoots showing roots	Number of roots $shoot^{-1}$	Length of roots (cm) at 15 DAI
MS medium		13.33b	76.67b	6.67d	3.13a
IBA	0.25	**8.33f**	96.67a	9.67c	**3.22a**
	0.50	9.33e	**100.0a**	11.33b	2.68b
	1.00	10.33d	90.00a	9.67c	2.42b
	2.00	10.67d	73.33b	7.67d	2.42b
	3.00	11.67c	73.33b	7.67d	2.67b
	4.00	12.33c	70.00b	6.67d	1.78c
	5.00	14.33a	66.67b	6.67d	1.75c
NAA	0.20	9.33e	73.33b	**14.33a**	0.92d
	0.50	10.67d	53.33c	9.33c	0.93d
CV%		4.96	7.59	6.75	8.58
$LSD_{(0.05)}$		0.9396	10.07	1.039	0.3209

Means followed by common letter(s) are statistically identical at 5% level of probability

Healthy plantlets of 7-10 cm in height were planted in a mixture of garden soil, sand and cowdung at the ratio of 2:1:1. Immediately after transplantation, the plants along with pots were kept in diffused sunlight in the controlled environment of the growth room. Survival rate of the transplanted plantlets was 80-85% (Fig. 1E and 1F).

In conclusion, the callus formation and regeneration ability of the explants have the potential for future biotechnological studies for the improvement of blackgram. However, further study is needed to standardize the protocol of regeneration via callus from different explants other than hypocotyls.

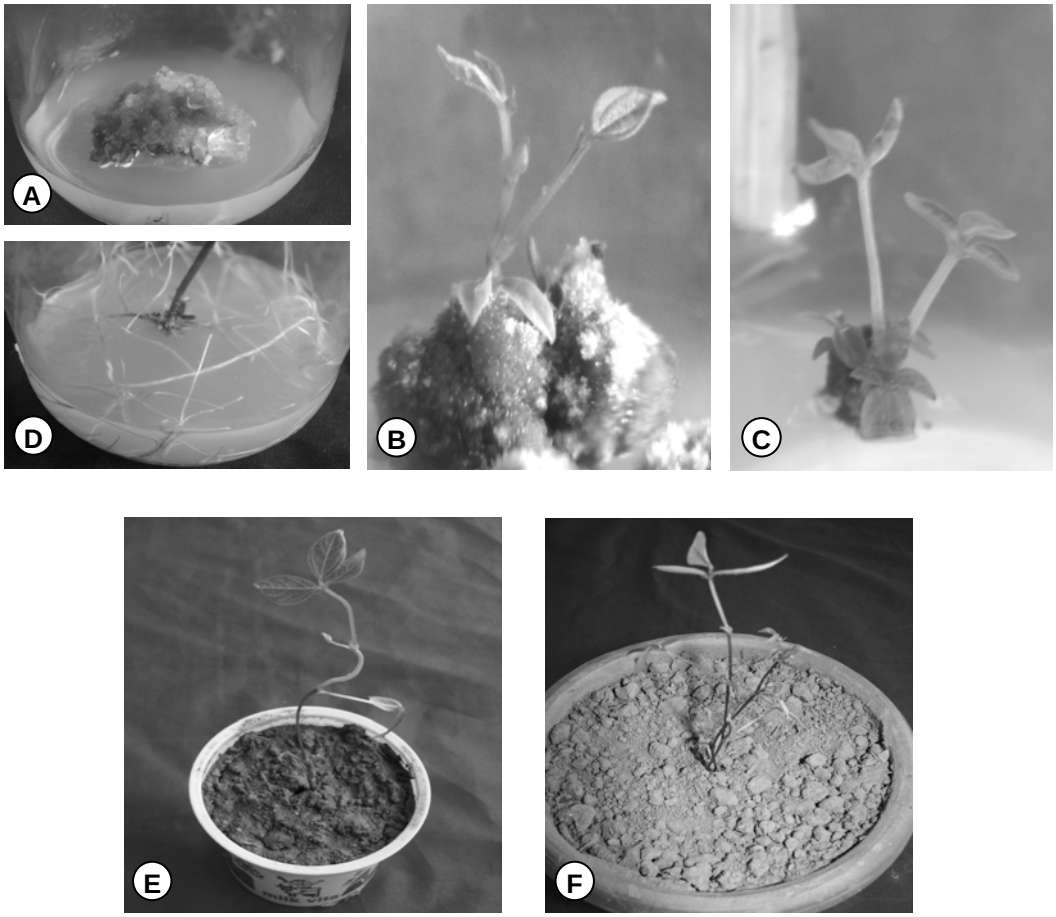

Fig. 1. A) Callus proliferation from hypocotyl explants on MS medium containing 2.5 mg/l BAP and 1.5 mg/l NAA at 26 days after inoculation (DAI). B) and C) Shoot regeneration from hypocotyl callus on MS medium containing 3.0 mg/l BAP and 0.3 mg/l NAA and MS medium containing 3.0 mg/l BAP, 0.3 mg/l NAA and 0.5 mg/l GA_3, respectively. D) Root formation from regenerated shoots on MS medium supplemented with 0.5 mg/l IBA. E) *In vitro* raised plant of blackgram transfer to plastic pot. F) Established blackgram plant.

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