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DIRECT PLANT REGENERATION FROM COTYLEDONARY EXPLANTS OF BLACKGRAM (*VIGNA MUNGO* L.) HEPPER.

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ABSTRACT

The present study was undertaken to investigate the possibility for developing Efficient Plant regeneration protocol in *Vigno mungo* L. For this, the effect of Cytokinens on *in vitro* regeneration from Cotyledonary explants was studied in Black gram (*Vigno mungo* L.) Hepper Var Vamban-1. The cytokinins used were 6-benzylaminopurine (BAP), Thidiazuron (TDZ), and kinetin (Kn). The regenerated shoots elongate on the same medium. TDZ stimulated shoot regeneration and this effect was significantly enhanced. (4.0 mg/L) was proved to be best for induction of shoots from cotyledon explants The regenerated shoots elongated 5 cm for four weeks on the same medium. Micro shoots were rooted on MS medium fortified with IBA (3.0mg/L) followed by transfer to green house. We conclude that TDZ induces significant regeneration as well as shoot elongation from cotyledon in Black gram ver Vamban-1. For the first time our findings describe a direct protocol for *in vitro* shoot regeneration and elongation in Black gram with cytokinins.

KEY WORDS: - Black gram, grain legume, *In vitro* culture, Plant growth regulators, Plant Regeneration, and *Vigno mungo*

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INTRODUCTION

A significant part of human population relies on legumes as staple food for subsistence, particularly in combination with cereals. They are unique foods because of their rich nutrient content including starch, protein dietary fibre, oligosaccharides, phytochemicals and minerals[1] their nutritional contents contribute to many health benefits to humans[2,3] Most of the research on dry beans has been related to varietal selection. The criteria for selection have always been resistance to diseases or yields but nutritional quality[4].. The food legumes, particularly the grains or pulses are important food stuff in all tropical and subtropical countries. Pulses deal with those species of the plant which belongs to the family fabaceae. They constitute an integral part of human diet as mature dry seeds and may also be used as immature green seeds or as green pods with immature seed in it. They can be used for animals in the form of hay and straw. The pulses have high protein contents (average 20-26%). In addition to their value as food stuff, they are also important in cropping systems because of their ability to produce nitrogen through nitrogen fixing rhizobacteria resulting into an increase in the fertility of the soil and hence economical as these can partially replace the expensive nitrogenous fertilizers.

Black gram (*Vigna mungo* L.) Hepper is also one of the commonly grown pulse crops in many countries of the world. Its seed contains about 24% protein, 60% carbohydrates and 1.3% fats. Dry fodder (pod husk) is nutritive for milch animals. It is also referred to as cover crop. Being legume crop, it fixes nitrogen to an extent of 70-90 kg/ha and thus improves soil fertility. Efficient *in vitro* plant regeneration system is required for successful crop improvement programs through genetic engineering. In case of grain legumes, the crop improvement is mostly hampered due to the recalcitrant nature of leguminous tissues under *in vitro* condition Although on *in vitro* plant regeneration has been reported via organogenesis from various explants such as shoot apices[6] embryonic axes[7] and cotyledon[8,9,10,11] in black gram the number of shoots produced per explants was very low. Several attempts have been made to establish *in vitro* regeneration protocol for Black gram. There are some reports on the *in vitro* plant regeneration in Black gram using different explants[12, 13,14,15,1617,1819 and 20]. However, the *in vitro* regeneration protocols developed by the workers did not produce desired results using Black gram varieties from India. In this report we present a highly efficient protocol for rapid *in vitro* plant regeneration from cotyledons of black gram ver Vamban-1

MATERIALS AND METHODS

Seed germination

Seeds of Black gram (*Vigna mungo* L.) Hepper ver Vamban-1 procured from the Tamil Nadu Agricultural University, Coimbatore Tamil Nadu India. The seeds were soaked in distilled water for 24h, and surface sterilized using 0.1% $HgCl_2$ (Mercuric chloride)for 3 min, followed by four to five rinses with sterile distilled water. Seeds were germinated aseptically on[21] Murashige and Skoog (MS) (1962) medium containing 2% sucrose and solidified with 0.8% agar (Hi-media).

Regeneration and elongation medium

Cotyledon explants were excised from 15 and 25 day-old *in vitro* grown seedlings, respectively. Explants from cotyledons of 1 cm^2 were dissected and cultured in culture tubes in such a way that the abaxial surface was in close contact with the medium. When cotyledonary explants were cultured, the cotyledon tips and basal portions including the petiole were discarded. MS basal medium containing 2% sucrose was fortified with plant growth regulators TDZ (1.0-5.0mg/L), BAP (1.0-5.0mg/L), Kn (1.0-5.0mg/L).

ROOTING MEDIUM AND GREENHOUSE TRANSFER

After approximately one month's growth on regeneration medium the elongated shoots 3-4 cm in length were transferred for rooting onto MS medium supplemented with (2.0mg/L) IBA. The effect of plant growth regulators on rooting was investigated 3-4 weeks after culture on rooting medium. Rooted plants (4-5 weeks old) were weaned away from the tubes, the roots cleaned for agar by washing with sterile distilled water and the plantlets were transferred to pots Containing soil and compost (1:1) followed by transfer to greenhouse. A 65% relative humidity was maintained in the pots were covered with polythene bags until the plantlets were acclimated under greenhouse conditions. All media pH was adjusted to 5.7 with 1M KOH prior to addition of 0.8% agar, and the media were autoclaved at a pressure of 1.05 kg cm^{-2} for 15 min at 121°C. All cultures were incubated at 25 ± 2°C under white fluorescent light 40-60 µmol $m^{-2}s^{-1}$) with a 16 h photoperiod. All data were statistically analyzed by ANOVA followed by Duncan's multiple range tests for mean comparison. Data pertaining to shoot regeneration was obtained from 10 explants in each of two replicates for each treatment and the experiment was repeated twice.

RESULTS AND DISCUSSION

In the present study three kinds of cytokinins (TDZ, BAP and Kn were used either alone to investigate *in vitro* regeneration in Black gram cv. Vamban-1.

Effect of TDZ

TDZ showed efficient shoot bud induction and regeneration from cotyledon explants. Medium containing TDZ induced formation of shoot buds predominantly at the cut surface of the cultured explants within 1-2 weeks of culture (Fig.-a). Shoot buds were proliferated and developed into shoot primordia. Among the tested concentrations of TDZ (1.0-5.0mg/L), lower (1.0mg/L) and higher (5.0mg/L) concentrations showed least regenerative response with fewer shoots produced per explants compared to 1.0 and 5.0 mg/L of TDZ (Table-1) TDZ at (4.0mg/L) proved to be the optimal concentration producing a maximum number of shoots from cotyledon (4.3 ± 0.33) explants. TDZ at (1.0 mg/L) was less significant in terms of the number of shoots/explants produced as compared to (5.0 mg/L). At (5.0 mg/L) TDZ, the number of shoots produced from cotyledon (2.7 ± 0.32) explants were less than with (4.0mg/L) TDZ (Fig.-b) (Table -1).

Table–1

Effect of different concentrations of TDZ, BAP and Kn on regeneration of shoots from cotyledon explants of Black gram (Vigno mungo L.) Hepper Var Vamban-1.

Conc. of Cytokinins (mg/L)	No. of explants inoculated	% of Responsive explants	Average no of shoots / explants (S.E.)*	Average length of shoots after eight weeks (cm)
TDZ				
1.0	60	80	1.7 ± 0.32	4.2
2.0	60	90	1.8 ± 0.67	5.0
3.0	60	95	2.3 ± 0.33	5.5
4.0	60	70	4.3 ± 0.33	4.8
5.0	60	65	2.7 ± 0.32	4.4
BAP				
1.0	60	70	1.6 ± 0.75	3.9
2.0	60	60	2.0 ± 0.35	4.0
3.0	60	80	3.0 ± 0.33	4.6
4.0	60	85	2.0 ± 0.35	5.0
5.0	60	50	1.3 ± 0.32	4.8
Kn				
1.0	60	75	1.2 ± 0.35	4.2
2.0	60	80	1.4 ± 0.32	5.4
3.0	60	70	2.5 ± 0.34	5.0
4.0	60	60	3.2 ± 0.35	4.3
5.0	60	50	2.3 ± 0.45	4.0

S.E. Standard Error

EFFECT OF BAP AND KN

In the present study, we analyzed in parallel experiment the effect of BAP and Kn on Black gram regeneration. Medium with 1.0mg/L BAP and Kn induced compact callus and less number of shoots were observed from cotyledon explants. Shoot buds were formed directly on cotyledon explants at the concentration of BAP/Kn (1.0-5.0mg/L). Several of these shoot buds failed to regenerate into shoots. At (1.0-20mg/L) of BAP/Kn only a small number of albino shoots (1.6 ± 0.75 and 2.0±0.35/1.2±0.35 and 1.4± 0.32) regenerated from cotyledon explants (Table-1). BAP (3.0mg/L) (Fig-c,d) in the callus induction medium BAP was found to enhance regeneration frequency[22,] Similar results were also obtained in various grain legumes[24, 25,2618,27]. Culture of explants for a brief period on shoot initiation medium containing BAP followed by transfer to growth regulator free MS medium might be ideal for efficient shoot multiplication. Regenerated shoots become abnormal when cultured for long time on medium containing cytokinin. In other *Vigna* species namely *V aconitifolia* (18) and *V.unguiculete* (19), Kn /2,4-D were used to initiate organogenic calli from hypocotyl and cotyledon explants. At all tested concentrations, the presence of Kn in the medium induced callus formation and did not support shoot bud development from cotyledon explants in Black gram ver. 'Vamban-1'. From the above results we conclude that TDZ is the most potent cytokinin to induce *in vitro* regeneration from cultured cotyledon explants in Black gram cv. 'Vamban-1'. Among the tested cytokinins, BAP at a higher concentration proved to be second best to induce shoot regeneration; third best to induce shoot regeneration from explants cultured on Kn-supplemented medium. Thus *in vitro* regeneration in this genotype was highly influenced by the type of cytokinin used in the medium.

SHOOT ELONGATION

The major problem encountered during tissue culture studies in Black gram is the formation of rosettes or leaf-like structures that do not develop further into shoots. In addition, the regenerated shoots do not elongate on the same medium and retain the albino phenotype as was also observed during present investigation when using TDZ. Interestingly, during our investigation shoot elongation occurred on medium supplemented with TDZ (4.0mg/L). Fully elongated micro shoots developed on the same medium, and thus transferring the stunted shoots for further elongation onto a fresh medium was avoided. Thus the described protocol is essentially a direct regenerated for protocol for plant regeneration in Black gram cv. 'Vamban-1'.

ROOTING OF MICRO SHOOTS

Prominent shoots (5.0-5.4 cm) were developed from cut ends of explants three weeks after culture on regeneration medium. Upon additional 7 days of culture on the same medium, fully elongated shoots of 4-5 cm length were excised and transferred onto rooting medium supplemented with (1.0-5.0 mg/L) IBA. It was observed that IBA induced rooting from cut ends of micro shoots within 3-4 weeks of culture on rooting medium. Maximum number of roots per rooted micro shoot was (4.1 ± 0.17) at (3.0mg/L) IBA that attained an average length of (3.2 ± 0.13) (Table-2) (Fig-e) Rooted micro shoots were successfully transferred to the greenhouse with 80% survival rate. The rooting of shoots was significantly affected by the auxin concentration (Table-2). 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.[30] reported that MS medium containing 0.25 mg l-1 IBA performed best and required four weeks for rooting.[28] 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 (Fig-e). [29]reported that efficient rooting (100%) of the shoots on medium containing half MS salts, full MS vitamins and

Figure 1

Direct Plant regeneration from Cotyledon explants of Black gram (Vigno mungo L.) Hepper (a) Formation of multiple shoots on MS+TDZ (3.0) mg/L after 3weeks (b) Proliferation of multiple shoots on MS+TDZ (3.0mg/L) after 6 weeks (c) Development of multiple shoots on MS+BAP (3.0mg/L) after 6 weeks. d) Formation of shoots on MS+ Kn (3.0mg/L) after six weeks e) Rooting of individual micro shoots on MS+IBA (3.0mg/L) (f) hardening of plantlet IBA (2.5 µM). [27] reported that medium containing 0.25 mg l-1 IBA obtained only 25% roots and [28] reported that medium containing 3 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.[28] reported that medium containing 3.0 mg l-1 IBA produced 14.5 roots/plants. It was clear from the above discussion that 2.0 mg/L IBA was better for root formation than any other treatments[30, 28.] reported that IBA was effective for rooting of black gram, while[31] 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. (Fig-e,f)

TABLE 2

Rooting ability of regenerated micro shoots from Cotyledonary explants culture of Black gram var Vamban-1 cultured on MS medium supplemented with IBA.

Conc. of Growth Hormone (mg/L)	No. of explants inoculated	% of Responsive explants	Average no of roots (S.E)*	Average length of roots (S.E)*
IBA				
1.0	60	80	2.7 ± 0.32	1.7 ± 0.32
2.0	60	90	3.8 ± 0.67	2.6 ± 0.57
3.0	60	95	4.1 ± 0.17	3.2 ± 0.23
4.0	60	70	2.6 ± 0.33	2.3 ± 0.33
5.0	60	65	2.0 ± 0.32	2.7 ± 0.32

**** Mean ± Standard Error***

In conclusion, our findings describe an efficient *in vitro* regeneration protocol from cotyledon explants of Black gram cv. 'Vamban-1'. The optimized regeneration method is based on a direct regenerated protocol that does not need any additional culture steps to achieve shoot regeneration and elongation. Shoot bud initiation, proliferation, and shoot elongation occur on a common TDZ/BAP/Kn medium. Thus the regeneration protocol is simplified to a large extent and can be completed in a short duration. The present protocol facilitates genetic manipulation studies for Black gram improvement.

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