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Research Article

Rapid and direct *in-vitro* shoot regeneration of black gram (*Vignamungo* L.) via cotyledonary explants; amend to transformation

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ABSTRACT

Rapid, short time and efficient protocol for simple and direct *in vitro* shoot initiation and plant regeneration was succeeded from cotyledonary explants of *Vignamungo*. All the explants were cultured on MS medium alone and MS+B5 vitamins supplemented with different concentrations and combinations of BA (0.5-2.0mg/L) and NAA (0.1-0.5mg/L). Young shoots were initiatedon MS+B5 vitamins medium fortified with 1.0mg/l BA under the incubation period in dark for three days in petri dishes. Green Shoots were aseptically sub-cultured in the same media for shoot elongation and 90% of shoot regeneration frequency was observed. The healthy and elongated shoots were transferred to half strength MS medium supplemented NAA (0.1-1.0mg/L)for root induction.Rooted plantlets were hardened under culture conditions and subsequently acclimatized green house facility. This short term protocol can be helpful for genetic improvement of black gramthrough *Agrobacterium* mediated transformation.

Keywords:Black gram, Direct shoot regeneration, B5 vitamins, Plant growth regulators and Transformation.

[I] INTRODUCTION

Plant biotechnological studies have been used legumes as a model plants for providing information in crop development.Legumes are adapted to tropical and sub-tropical condition, require low inputs, yields highly, and serves as a brilliant source of protein as seed or sprout. Because of its perfect combination of all nutrients (Karamany 2006), it is widely used overallin Asia especially in India and now also grown inother countries(Delic et al. 2009).The potentials of biotechnological tools can be explored by supplementing the conventional breeding approaches through insertion of gene of interest into genotypes of black gram. In general, Legumes are hard to tissue culture and are extremely genotype specific (Somer et al., 2003). Due to its recalcitrant nature, tissue culture approaches have met limited success in regenerating plants from mature explants (Tivarekar and Eapen 2001). Though few repots available on plant regeneration system via organogenesis from different explants including shoot apices (Goel et al., 1983), embryonic axes (Ignacimuthu and Franklin 1999) and cotyledons (Ignacimuthu et al., 1997; Sen and Guha-Mukherjee, 1998; Avinido and Hattori, 1999; Franklin and Ignacimuthu, 2000) in black gram, the shoot regeneration frequency was very low.However an efficient and high surveillance regeneration system is needed for genetic

transformation. In the present an attempt was take on to establish an effective and short time reproducible regeneration system using half dissect cotyledons with first two leaves for black gram.

[II]MATERIALS AND METHODS

2.1 Plant material and preparation of explants

All healthy and mature black gram seeds were surface sterilized with 70 % ethanol for 1 min followed by in 0.1 % mercuric chloride for 5 min. the surface sterilization process was continued by three rinses with sterile distilled water. Then seeds were dried on filter paper and placed on a solidified agar medium containing no growth regulators in sterile test tubes. Explants were primed from 3 day old seedlings by half excision of both cotyledons and first two leaves for direct shoot regeneration.

2.2 Culture media and culture conditions

All the prepared explants were germinated in petri dishes containing MS (Murashige and Skoog 1962) medium alone and MS with B5 (Gamborg et al. 1968) vitamins along with sucrose (30gm/L) and agar (8gm/L) as a gelling agent supplemented with various concentrations of BA (0.5, 1.0, 1.5 and 2.0mg/L)under dark conditions for three days (Figure 1A). All the media used in the study were adjusted to pH 5.8 before autoclaving. After three days of incubation period, responded explants with green colour were transferred to same media supplemented with various concentrations of BA for shoot elongation (Figure 1B). For rooting, healthy shoots (5-6 cm) were chopped and implanted to rooting medium consisting, of half strength MS medium supplemented with NAA(0.1-1.0mg/L).

The direct shoot regeneration frequency was estimated based on the percentage of explants respond to shoot initiation, similarly root regeneration frequency was estimated by percentage of explants forming roots.Visual observations of the cultures were helped to evaluate the importance of B5 vitamins and perfect combination of plant growth regulators. All the inoculated cultures were incubated in a culture room at 25 ± 2^0C with a relative humidity of 50-60% and around 16hrs photo period. With the intention of achieve acclimatization, plantlets with roots were separated from the medium and were shifted to polythene bags containing vermiculite, covered with polythene bags to maintain high humidity and kept under the culture room conditions for 2 weeks (Figure 1C). Plants with newly formed leaves were then transferred to the greenhouse.

[III] RESULTS AND DISCUSSION

The results of the half cotyledonary cultures on the development of direct shoots and root formation with different concentrations and combinations of plant growth regulators are shown in table 1. The cultured explants on different hormonal combinations showed varied results and became active with in weak after inoculation and new shoots became elongated by the second weak with leaves and internodes. Initially we started experiments with MS salts alone and we observed less shoot regeneration response with cotyledonary nodes and then we proceed with MS salts along with B5 vitamins. Similar encouraging results were noticed with B5 medium as per Gulati et al., (1994). The medium containing 1.0mg/L of BA and 0.2mg/L NAA induced maximum shoot initiation (3-4cm) within 10 dayswhere the BA concentration was highly influenced the shoot regeneration, as the concentration of BA was increased from 1.0mg/L to 2.0mg/L the shoot regeneration response was gradually decreased (table 1). The maximum number of days required for shoot induction was 22 days on MS medium without B5 vitamins containing 2.0mg/L BA and 0.2mg/L NAA, also observed a dose depended delay in the number of days required for shoot initiation. These results are in agreement with the investigation on black gram from Mony et al., 2010.

In the present study, we also observed the shoot regeneration percentage, the results showed that the highest percentage (92.7%) of shoot regenerated on MS medium supplemented with 1.0mg/L BA and 0.2mg/L NAA along with B5

vitamins and the lowest percentage (41.9%) was observed in MS medium alone supplemented with 2.0mg/L BA and 0.2mg/L NAA. The percentage of shoot regeneration was also decreased gradually with the increase of BAP concentration in the medium (table 1). All the cultures arerespond highly in medium contained B5 vitamins, this is because of MS salts were inferior to B5 salts for shoot regeneration (table 1).

Table 1: The effect of different concentrations and combinations of BA and NAA on days required and shoot regeneration from cotyledonary node explants.

Media type	BA (in mg/L)	NAA (in mg/L)	Percentage of direct shoot re generation	Days required for shoot initiation
MS+	0.5	0.2	43.1	21
MS+	1.0	0.2	62.8	18
MS+	1.5	0.2	56.2	19
MS+	2.0	0.2	41.9	22
MS+B5 vitamins	0.5	0.2	65.3	16
MS+ B5 vitamins	1.0	0.2	92.7	10
MS+ B5 vitamins	1.5	0.2	81.3	13
MS+ B5 vitamins	2.0	0.2	74.1	15

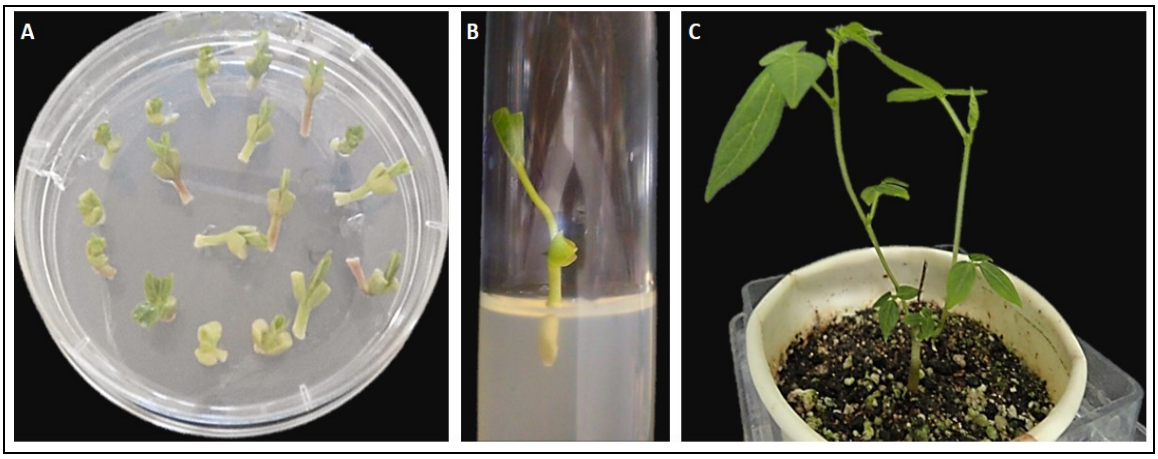

Fig:1. Regeneration of black gram: A. Direct shoot regeneration of black gram, **B.** Shoot elongation, **C.** Hardened plants maintaining in culture room.

Consequently, addition of B5 vitamins to MS salts, resulted in enhancement of the shoot regeneration percentage buds as compared to MS salts alone. The obtained results also suggested by Das et al., 2002where the formation of number of shoot buds were enhanced in B5 salts. Hence, the overall effect of B5 vitamins was better than MS salts alone for shoot regeneration.The efficiency of BA on the induction of multiple shoot formation has been reported in *Vigna radiate* L. (Himabinduet al., 2014), BA was also found to enhance the regeneration frequency as reported by Gulati and Jaiwal (1930) and Chandra and Pal (1995). Young and green shoots (4-5cm) regenerated within 10-15 days were transferred to rooting media of half MS medium supplemented with different concentrations of NAA(0.1-1.0 mg/L), without B5 vitamins half strength MS media with 0.5mg/L NAA, worked out efficiently for initiation of rooting. Healthy plant lets with well root systems, were transferred to sterile soil and maintained in controlled conditions in the cultureroom. Later plantlets were transferred to green house.

[IV] CONCLUSION

In conclusion, though a handful of reports describe multiple shoot formation from cotyledonary node most of them were time required efforts. All these aimed us to attempt to establish a rapid and short time in vitro direct shoot regeneration protocol for black gram

through which elongated shoots can produced within less than two weeks. In additionall the cultures were exposed to different treatments such as dark co-cultivation so on so which are amend to *Agrobacterium* mediated transformation. Thus, the proposed protocol in the present investigation would be the best regeneration practice with cotyledonary node as explant by the growth hormone BA and this can be helpful with *Agrobacterium tumeficienes* for development of transgenics.

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