An efficient protocol for rapid plant regeneration from deembryonated cotyledons of black gram [Vigna mungo (L.) Hepper]

R. Anandan, T. Deenathayalan, R. Bhuvaneswari, M. Merlin Monisha and M. Prakash*

Department of Genetics and Plant Breeding,
Faculty of Agriculture, Annamalai University, Annamalai Nagar-608 002, Tamil Nadu, India.
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ABSTRACT
Here an efficient protocol for micropropagation of black gram [Vigna mungo (L.) Hepper] cv. VBN 3 is reported. The deembryonated cotyledonary explants were cultured on MS medium containing different concentrations of plant growth regulators. The maximum frequency (72%) of direct shoot regeneration (devoid of callus phase), multiple shoot induction and shoot elongation was achieved from culturing the explants on MS medium containing 3.0 mg/l of 6-benzylaminopurine (BAP). Up to 65% of the regenerated shoots were rooted on MS medium containing 0.25 mg/l of α-naphthalene acetic acid (NAA) within 3 weeks after subculturing. The in vitro-raised plantlets were successfully hardened first under culture room conditions with 62% survival rate and then in greenhouse. The identified regeneration system could be efficiently used in various in vitro manipulation studies in black gram as well.

Key words: Black gram, Cotyledon, In vitro culture.

INTRODUCTION
Black gram [Vigna mungo (L.) Hepper] is a widely cultivated grain legume belongs to the family Fabaceae. It is an important nitrogen fixing, short-duration and tropical pulse crop grown in many parts of India. It is thought to have originated in the Indian subcontinent with maximum diversity in the Western Ghats. Black gram is also grown in the Southern United States, West Indies, Japan and other tropics and subtropics (Delic et al., 2009).

Black gram seeds contain high protein content (25–26%), carbohydrates (60%), fat (1.5%) and perfect combination of all nutrients, including, minerals, vitamins, basic amino acids (arginine, leucine, lysine, isoleucine, valine and phenylalanine), micronutrients (Calcium, magnesium, potassium, phosphorus and iron) and various phytochemicals (Karamany, 2006; Suneja et al., 2011; Prakash et al., 2018; Kamboj and Nanda, 2018). They also have appreciable quantity of all the essential amino acids excluding sulphur containing amino acids, which can be balanced to combine with cereals in daily intake.

The total production in India was estimated as 2.93 million tonnes from a cultivated area of 4.43 million ha (Anonymous, 2016). Madhya Pradesh stand first in area (19.40%), followed by U.P. (17.88%) and Andhra Pradesh (11.69%), whereas in production U.P. stands first (16.98%) followed by Andhra Pradesh (16.75%) and Madhya Pradesh (15.07%). The highest yield was recorded by the state of Bihar (898 kg/ha) followed by Sikkim (895 kg/ha) whereas the national average yield was 585 kg/ha (Anonymous, 2016).

The production constraints are susceptibility to several biotic (insect pests like gram pod borer and bruchids) and diseases such as yellow mosaic virus (VMYMCV), powdery mildew, and Cercospora leaf spot (Sahoo et al., 2002; Rao and Chand, 2006; Sarma and Borah, 2004) and abiotic stresses cause considerable damage and reduce the yield potential of this crop. Since not much progress could be made through classical breeding approaches because of narrow genetic base and sexual incompatibility with wild relatives, the development of pest and disease resistant in black gram cultivar still remains a challenge (Chopra and Saini, 2014; Adlinge et al., 2014).

In general, grain legume species are recognized as recalcitrant to in vitro regeneration methods and this has significantly slowed down advances in genetic manipulations (Chandra and Pental, 2003; Eapen, 2008). Genetic transformation is generally difficult in many of the crops and few reports on protocols for tissue culture were reported in sesame (Anandan et al., 2018) and green gram (Anandan et al., 2019). It shows that genetic transformation in this crop remains difficult due to the lack of an efficient plant regeneration protocols (Sainget al., 2005).

Regeneration from both organogenesis and somatic embryogenesis has been recalcitrant in legume crops (Anand et al., 2001; Anwar et al., 2011). Even though, few reports are available on in vitro regeneration of black gram, the reproducibility of these results is very poor (Muruganantham et al., 2005; Mony et al., 2010). In the present investigation, deembryonated cotyledon explants of black gram were taken...
and an efficient shoot induction was achieved via direct organogenesis from in vitro grown seedling plants.

MATERIALS AND METHODS

Plant material and explant source: The mature seeds of black gram cv. VBN 3 were obtained from the Tamil Nadu Agricultural University, Coimbatore, India. Seeds were washed thoroughly in running tap water for 30 min. These seeds were surface sterilized with an aqueous solution of 0.1% (w/v) freshly prepared HgCl₂, for two min and finally rinsed five times with sterilized distilled water. The surface-sterilized seeds were germinated aseptically in half-strength MS (Murashige and Skoog, 1962) medium with 3% (w/v) sucrose (Qualigens, India) and 0.7% agar (bacteriological grade, Himedia, India) without exogenous supply of growth regulators.

Culture conditions: All the media were fortified with 30g/l sucrose and gelled with 0.7% agar (Himedia, India). The pH of the medium was adjusted to 5.8 by 1 N NaOH or 1 N HCl. The media were steam sterilized in an autoclave under 1.5 kg/cm² and 121°C for 15 min. All the cultures were grown at 25±2°C under a 16-h photoperiod supplied by two Philips TL 40W fluorescent tubes and 55±5% of relative humidity.

Adventitious shoot induction: Cotyledon explants were aseptically isolated from the sterilized mature seeds. These cotyledon explants were dissected from the seeds in such a way that the embryonic axis was removed completely, thus having a cut at the proximal portion of the cotyledon (also known as deembryonated cotyledon). The deembryonated cotyledon explants were cultured on MS medium with various concentrations of 6-benzylaminopurine (BAP). After 4-6 weeks of culturing, the frequency of explants producing shoot directly without callus phase and the average number of shoots per explant was recorded. All the cultures were transferred to fresh medium after every 15 days. MS basal medium without exogenous supply of growth regulators was used as control. The cultures were maintained in Tissue Culture Laboratory, Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University.

Table 1: Effect of different concentrations of cytokinin with full strength MS on black gram regeneration.

| BAP (mg/l) | Regeneration response (%) | Mean number of shoots per explant (mean ± SE) | Nature of plantlets |
|-----------|---------------------------|---------------------------------------------|---------------------|
| 0.0       | 0.00 ± 0.00               | 0.00                                        | -                   |
| 0.5       | 15.0 ± 1.52a              | 1.0 ± 0.5a                                  | +                   |
| 1.0       | 18.0 ± 0.70ef             | 1.0 ± 0.7a                                  | +                   |
| 2.0       | 37.2 ± 0.83f              | 2.0 ± 0.4c                                  | +                   |
| 3.0       | 72.6 ± 1.34g              | 6.0 ± 0.3e                                  | +++                 |
| 4.0       | 55.8 ± 1.58de             | 3.0 ± 0.5d                                  | ++                  |
| 5.0       | 45.8 ± 0.83d              | 2.0 ± 0.3b                                  | ++                  |
| 6.0       | 34.2 ± 0.83c              | 2.0 ± 0.5b                                  | +                   |
| 7.0       | 22.2 ± 0.83b              | 1.0 ± 0.4a                                  | -                   |
| 8.0       | 8.4 ± 1.14b               | 1.0 ± 0.5a                                  | -                   |
| 10.0      | 6.0 ± 1.58a               | 1.0 ± 0.2a                                  | -                   |

Values represent means ± SE of 20 replicates per treatment in three repeated experiments. Values followed by the same letter are not significantly different at P<0.05 according to Duncan’s multiple range tests.

Nature of plantlets was evaluated qualitatively as -: no, +: abnormal shoots ++: normal shoots with leaves, +++: multiple shoots.

RESULTS AND DISCUSSION

An efficient plant regeneration system that could be applicable to a wide group of genotypes for a given species is inconsistent among the cultivated varieties of black gram. This indicates that a genotype independent regeneration protocol for black gram has not been reported. Several scientists reported that black gram is a recalcitrant species for in vitro regeneration and genetic transformation (Chandra and Pental, 2003; Anand et al., 2001; Anwar et al., 2011). An efficient, reliable and reproducible tissue culture protocol is an essential prerequisite for genetic improvement as well as in vitro induction of mutation in plants.

The deembryonated cotyledon explants excised from mature seeds of cv. VBN 3 were evaluated for their ability to induce adventitious shoots on MS media supplemented with different concentrations of BAP (Fig 1a, b & c; Table 1). In the present study, Shoot organogenesis occurred directly from the proximal end of explants without the formation of a distinct intermediate callus and well-defined shoot buds were visible within 4 weeks of culture.
Table 2: Effect of different levels of auxins and MS medium strengths on rooting of adventitious shoots of black gram cv VBN 3.

| Culture medium         | Rooting response\(^a\) % (mean ± SE) | Mean number of roots/shoot(mean ± SE) | Plant conversion rate\(^b\) (%) |
|------------------------|---------------------------------------|--------------------------------------|---------------------------------|
| Half MS                | 26 ± 9.8c                             | 2.00 ± 0.1b                          | 32.0 ± 5.4c                     |
| Full MS                | 40 ± 8.4c                             | 2.04 ± 0.08b                         | 35.0 ± 5.4c                     |
| MS+NAA (0.5 mg/l)      | 65 ± 12.5a                            | 3.02 ± 0.04a                         | 62.0 ± 5.4 a                    |
| MS+NAA (1.0 mg/l)      | 47 ± 13.2c                            | 2.00 ± 0.06b                         | 42.0 ± 5.4 b                    |
| MS+IBA (0.5 mg/l)      | 55 ± 12.5b                            | 4.50 ± 0.08a                         | 45.0 ± 5.4 a                    |
| MS+IBA (1.0 mg/l)      | 34 ± 15.4c                            | 1.02 ± 0.04c                         | 37.0 ± 5.4 b                    |

\(^a\)10 adventitious shoots/replicate, three replicate/treatment and experiment repeated thrice, data taken after 4 weeks.
\(^b\)10 plant conversion indicates the plantlets with well-developed shoot and root.

Values followed by the same letter are not significantly different at \(P<0.05\) according to Duncan’s multiple range tests.

Fig 1: Adventitious shoots regeneration from deembryonated cotyledon of black gram cv. VBN-3. a. Surface sterilized seeds of black gram. b. Germination of seeds on MS basal medium. c. Deembryonated cotyledon dissected from in vitro grown seedlings. d. Development of multiple shoots from deembryonated cotyledon on MS medium containing BAP (3 mg/l). e. Elongated shoots with normal leaves on MS medium + BAP (3 mg/l). f. Rooting on MS medium containing NAA (0.25 mg/l). g. Plantlets acclimatized in sand, clay and soil mixture (1:1:1). h. Mature plants grown in greenhouse. Scale bars: 2 mm (a, b, c, d, e, f, g and h).
(Fig 1d & e). Regeneration of shoots from cotyledon explants has been previously reported in black gram genotypes with low regeneration frequencies using immature cotyledonary nodes (Igriculta et al., 2005), mature cotyledons (Gill et al., 1987) as explants. Plant micropropagation through direct shoot regeneration allows large-scale multiplication of genetically uniform plantlets compared with plantlets derived through callus phase, which often leads to somaclonal variation (Rani et al., 2000).

An efficient method for plant regeneration via callus formation was reported in pigeonpea by Abhijeeta and Rajesh (2018) using two varieties as BDN-2 and GT-101. Three different explants viz. leaf, hypocotyl and root excised from 15-20 days old in vitro raised seedlings were cultured on basal MS medium and more shoots were observed in MS + 1.0 mg/l NAA + 4.0 mg/l Adenine and roots on Elongated shoots in MS + 1.0 mg/l IAA media. BAP and Kinetin @ mg/L was found more effective in inducing shoot organogenesis and TDZ is also found to be the best suited for inducing multiple shoot in black gram (Thallapathy, 2017). Adventitious shoot formation was severely affected when the explants were cultured on media containing either lower (0.5 mg/l) or higher (10.0 mg/l) concentrations of BAP (Table 1). After incubation over a period of 4 weeks, the highest rate of adventitious shoot formation (72%) was achieved on MS medium fortified with BAP (3.0 mg/l). An increase in the number of primary shoot induction was observed when the concentration of BAP was increased from 0.5 to 3.0 mg/l. The lowest percentage of shoot induction was observed with 8.0 and 10.0 mg/l of BAP. BAP is considered as the most effective cytokinin for stimulating efficient shoot induction in black gram (Muthukumar et al., 1996; Chakraborti et al., 2006; Das et al., 2004).

The highest regeneration efficiency (72%) observed in the present study may be possibly due to the use of the decumbentated cotyledon explants derived mature seeds. Our results are in contrast to an earlier report where shoot regeneration response from cotyledon explants obtained from seedlings drastically declined (Igriculta et al., 1997; Gill et al., 1987). The results of the present study indicate that the induction of multiple shoots and also elongation of shoots were observed on the same media composition (MS media supplemented with 3.0 mg/l of BAP). Black gram has been found to be recalcitrant to in vitro regeneration and also multiple shoot formation and the efficiency of regeneration has been found to be dependent on various parameters, viz. explant size, age, type and genotype, and media composition (Saini et al., 2002). They reported that the multiple shoot induction (6 shoot buds/explant) was obtained from axillary shoots on medium having 1.0 mg/l BAP. Mony et al. (2010) reported that the lowering of BAP concentration (1.0 mg/l) produced maximum number of shoots (9.33) in case of black gram. They had also stated that the increase the BAP concentration in medium resulted in the formation of callus. Variation in regeneration response may be due to genotype specific differences or different culture medium, as it has been mentioned in previous regeneration studies on black gram (Saini et al., 2002).

Induction of rooting was found to be an extremely difficult process in black gram. Initiation and development of roots at the base of in vitro grown shoots is an essential step to establish tissue culture-derived plantlets in the soil. Adventitious shoots at 2 to 3 cm in height (Fig. 1d & e) were transferred to hormone-free half-strength or full-strength MS or MS+ NAA (0.5 and 1.0 mg/l) or MS+ IBA (0.5 and 1.0 mg/l) for in vitro induction of roots (Table 2) (Fig 1e). The maximum percentage of root induction (65 %) was found with that of MS+NAA (0.5 mg/l), followed by 52 % with 0.5 mg/l IBA. This advantageous effect of NAA or IBA on in vitro induction of roots is well documented in previous tissue culture studies in black gram (Adlinge et al., 2014). Mony et al. (2010) found that percentage of rooting was higher in IBA but number of roots per plant was higher in NAA. The rooted plantlets were acclimatized in sand, clay and soil mixture (Fig 1g) and mature plants grown in greenhouse (Fig 1h) with survival rate of 62 %.

CONCLUSION
It is concluded that the tissue culture system reported here using deembryonated explants is a rapid and direct shoot regeneration method for Vigna mungo cv. VBN 3. The protocol for plant regeneration from black gram cotyledons through adventitious shoot formation can now be used for commercial multiplication, germplasm conservation and genetic transformation studies.

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