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Efficient plant regeneration from cotyledons of black gram [*Vigna mungo* (L.) Hepper]

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An efficient and rapid regeneration protocol was developed from cotyledons of black gram [*Vigna mungo* (L.) Hepper] variety T-9. Murashige and Skoog's (MS) medium supplemented with Gamborg B_5 vitamins containing BAP @ 4.0 mg/L was most effective in producing regenerative calli. The cultures expressed maximum plant regeneration potential with 12 shoots per calli on regeneration media with no exogenous amino acid or plant growth regulator supplementation. Green and robust shoots thus developed were successfully rooted within 15 d on $^1/_3$ MS medium. Over 90% of rooted plants grew well and produced seeds normally when transferred to green house.

Keywords: black gram, grain legumes, *in vitro* culture, plant growth regulators, plant regeneration, *Vigna mungo*

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Black gram [*Vigna mungo* (L.) Hepper] is a widely cultivated pulse crop mainly for its protein rich seeds. It stands next to soybean in its dietary protein content. Although India is the main producer of black gram, but production is limited due to various biotic and abiotic stresses. The high susceptibility of the crop to yellow mosaic virus (VMYMV), fungal pathogens (powdery mildew, cercospora leaf spot), insects (bruchids)[1] and drought[2] result in significant yield losses. Attempts to enhance the tolerance of black gram against diseases and insect pests through classical breeding have met with limited success due to the absence of adequate and satisfactory level of genetic variability within the available germplasm. Though, genes conferring resistance to biotic and abiotic stresses have been found in many wild and related species, these are sexually incompatible with the cultivated ones. Some of such genes have been isolated, characterized and cloned from other plant species and microorganisms[3]. These genes can be transferred to cultivated crops, like black gram, in order to enhance the tolerance to stresses and, hence, stabilize yield. Success of such gene transfer approaches depends upon an efficient, reproducible and rapid *in vitro* regeneration system. In the past, tissue culture approaches to regenerate plants from juvenile and mature explants in black gram have met with limited success owing to the recalcitrant nature of this crop[4]. Although *in vitro* plant regeneration has been reported *via* organogenesis from various explants, such as shoot apices[5], embryonal axes[6] and cotyledons[7-10], in black gram, the number of shoots produced per explant was very low. For genetic transformation, however, a robust and high frequency regeneration system is needed. In this report, we present a highly efficient protocol for rapid *in vitro* plant regeneration from cotyledons of black gram.

Seeds of black gram genotype T-9 were obtained from Andhra Pradesh State Seed Development Corporation, Hyderabad. Surface sterilization of the seeds was done by rinsing them in 70% ethanol for 1 min, followed by 0.1% mercuric chloride for 5 min. The seeds were then rinsed in sterile distilled water 3-4 times and soaked in sterile water for 18 h in dark. The imbibed seeds were decoated and the two cotyledons were carefully separated. The cotyledon along with the embryonal axis was placed in such a way that the embryonal axis was in contact with the medium. The medium used was MS[11] containing B_5 vitamins[12], 3% (w/v) sucrose, 0.8% (w/v) agar and combinations of different plant growth regulators, *viz.* BAP, NAA and IAA. The plant growth regulator combinations tested were BAP (1, 2, 3, 4 mg/L) alone and in combination with 0.5 mg/L NAA or with 1 mg/A IAA. The cultures were maintained in dark at 25±1°C for 15 d with one subculture after 1 wk. They were subsequently transferred to regeneration media containing 3 mg/L BAP and maintained in light (85 μmol m^{-2} s^{-1}) for 20 d. Effect of inclusion of 575 mg/L proline, 760 mg/L glutamine, 3.0 mg/L BAP or 0.5 mg/L IBA was tested in ½ strength MS + B_5 regeneration medium for assessing their effect on shoot development.

The observations on morphogenetic response of the cultures such as callusing percentage and callus

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diameter were recorded 15 d after inoculation. Observations on regeneration frequencies, number of shoots and shoot length were recorded 20 d after transferring to regeneration media. Elongated shoots were rooted on $^1/_3$ MS medium. Plantlets transferred to plastic buckets (14 L capacity) containing farm soil were irrigated with water and/or half strength Hoagland solution alternatively. For histological studies 10-d-old calli were fixed in acetic acid: alcohol (3:1) for 24 h, stained with hematoxylin and viewed under Olympus CX31 light microscope.

The type, combination and concentration of plant growth regulators strongly influenced the organogenic potential of the cotyledonary explants of variety T-9 of black gram. Initial culturing of the explants on MS medium gave rise to calli, which were creamish green on media containing BAP alone and white when BAP was used in combination with NAA and IAA (Table 1; Fig. 1a). The callusing frequency ranged from 60 to 90% in response to various plant growth regulators. The frequency of callusing seemed to depend more on concentration of BAP. Hence, when NAA and IAA were used, the callusing percentage increased at increasing BAP concentrations. The callus diameter was higher on BAP in combination with NAA and IAA as compared to BAP alone (Table 1). Histological analysis of 12-d-old calli revealed initiation of meristemoids in the form of meristematic zones from the peripheral region of the callus (Fig. 1f). Patel *et al*[13] obtained organogenic calli in mung bean (*V. radiata*) using NAA/2,4-D along with BAP. Benzyl adenine (BAP) is the most widely used and effective cytokinin for various legumes including *Vigna* spp[1,14,15].

Inclusion of BAP in callus induction medium facilitated callusing as well as subsequent shoot regeneration. Shoot initials developed in the cultures soon after transfer to regeneration medium (Fig. 1b). Maximum shoot regeneration frequency was observed on 4.0 mg/L BAP medium, while the number of shoots per callus was highest (4.3) at BAP (3 mg/L) in the callus induction medium. BAP was found to enhance regeneration frequency[16,17]. Inclusion of NAA and IAA in the callus induction medium, on the other hand, hampered the regeneration potential of the calli (Table 1). In other *Vigna* species, namely *V. aconitifolia*[18] and *V. unguiculata*[19], Kn/2,4-D were used to initiate organogenic calli from hypocotyl and cotyledon explants.

On the regeneration medium, the maximum number of shoots per explant (12) and the maximum average shoot length (4.7 cm) were obtained in the treatment without any exogenous addition of plant growth regulator or amino acid (Table 2). Inclusion of BAP or IBA in the regeneration medium resulted in reduction of shoots and only about 4 or 7 shoots per

Table 1—Effect of various concentrations of BAP, NAA and IAA in callus induction medium on callusing and shoot regeneration from cotyledonary explants in black gram (*V. mungo*)

Growth regulators			Responding explants	Callus diameter	Regeneration frequency	Number of shoots per explant
BAP	NAA (mg/L)	IAA	(%)	(mm)	(%)	
0	0	0	n.d	n.d	n.d.	n.d.
1	0	0	90 ± 5.77	5.0 ± 0.58	50.0 ± 5.77	1.7 ± 0.67
2	0	0	70 ± 5.77	5.3 ± 0.33	63.3 ± 3.33	2.3 ± 0.33
3	0	0	80 ± 6.77	6.3 ± 0.67	76.7 ± 3.33	4.3 ± 0.33
4	0	0	80 ± 6.77	6.7 ± 1.33	83.3 ± 3.33	2.7 ± 0.33
1	0.5	0	60 ± 8.82	9.0 ± 1.15	n.d.	n.d.
2	0.5	0	70 ± 3.33	7.7 ± 0.88	n.d.	n.d.
3	0.5	0	80 ± 3.33	7.3 ± 0.33	n.d.	n.d.
4	0.5	0	90 ± 3.33	8.0 ± 0.58	n.d.	n.d.
1	0	1.0	60±11.55	7.3 ± 0.33	n.d.	n.d.
2	0	1.0	70 ± 8.82	7.7 ± 0.33	16.7 ± 3.33	1.3 ± 0.33
3	0	1.0	80 ± 6.67	6.7 ± 0.33	23.3 ± 3.33	1.7 ± 0.33
4	0	1.0	90 ± 5.77	7.3 ± 0.33	n.d.	n.d.

Regeneration was tested for assessing regeneration potential, calli were cultured on media containing 3mg/L BAP in light (85 μmol m^{-2} s^{-1}) for 20 d
100 explants were taken for observation:
Values represent mean ± SE of three replicates in three individual experiments
n.d.: not detected

Table 2—Effect of various plant growth regulators and amino acids on shoot regeneration from cotyledon-derived calli of black gram (*V. mungo*)

Amino acid/ Growth regulator	Responding explants (%)	No. of shoots per explant	Average shoot length (cm)
½ MS + B5	74.7 ± 2.6	12.3 ± 0.3	4.7 ± 0.2
½ MS + B5 + 3.0 mg/L BAP	76.7 ± 3.3	4.3 ± 0.3	3.5 ± 0.3
½ MS + B5 + 575 mg/L proline	45.3 ± 0.9	5.3 ± 0.3	1.6 ± 0.1
½ MS + B5 + 730 mg/L glutamine	51.0 ± 2.1	6.3 ± 0.3	2.3 ± 0.2
½ MS + B5 + 0.5 mg/L IBA	58.3 ± 2.0	7.0 ± 0.6	3.7 ± 0.3

100 explants were taken for observation
Values represent mean ± SE of three replicates in three individual experiments

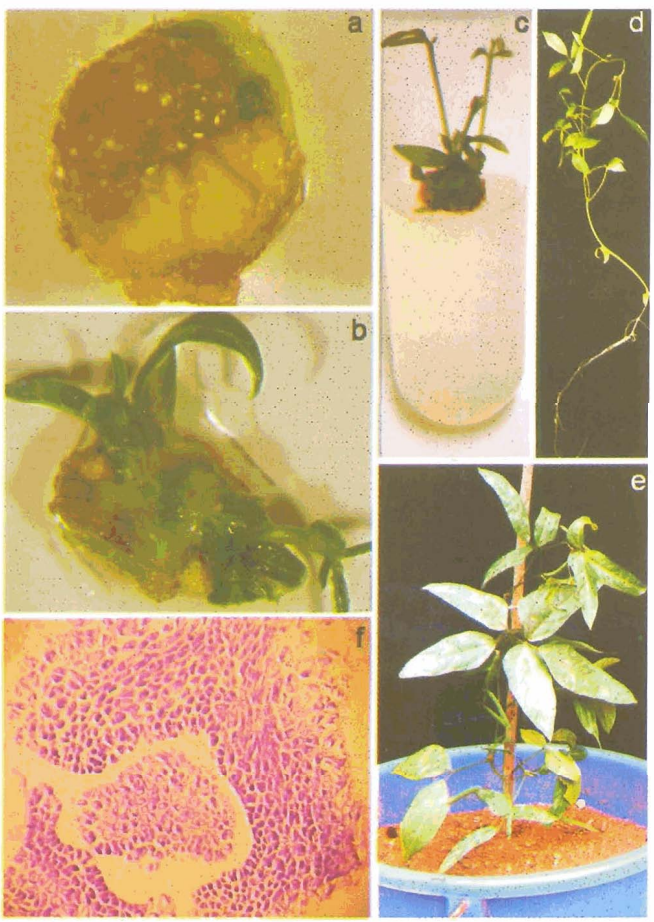

Fig. 1—Regeneration of plantlets from cotyledon derived callus in black gram (*V. mungo*): (a) callusing from cotyledon explants 12 d after inoculation; (b) shoot formation from cotyledon derived callus; (c) elongated shoots from cotyledon derived callus; (d) *in vitro* rooting; (e) regenerated plants growing in pots; & (f) histological section of cotyledon derived callus showing meristematic zones at the periphery.

explant, respectively were obtained. The average shoot lengths of these plants were also reduced to 3.5 and 3.7 cm, respectively with BAP and IBA. Addition of proline and glutamine in the regeneration medium resulted in further reduction in shoot number as well as average shoot length (Table 2). It is interesting to note that maximum shoot regeneration potential was expressed with no exogenous supplementation of plant growth regulator in regeneration medium. This could be related to a probable reorientation of balance amongst the endogenous levels of various plant growth regulators in particular with those of auxins and cytokinins in the cultures. This hypothesis is supported by reports where the organogenic potential of the explant was strongly influenced by the type of growth regulator[2,7,19]. Further, cultures on basal medium without any plant growth regulators might have been induced with 'withdrawal shock', forcing them to regenerate.

Green healthy shoots regenerated within 20 d (Fig. 1c) on regeneration medium developed healthy roots within 15 d on MS medium containing $^1/_3$ inorganic and organic addendum without any supplementation of plant growth regulator (Fig. 1d). About 90% of the rooted plantlets efficiently hardened in pots containing soil and farm yard manure and produced seeds normally (Fig. 1e).

In summary, the present work reports an efficient and rapid plant regeneration protocol for *V. mungo* even in the absence of any plant growth regulator supplementation in the regeneration media. This kind of an efficient regeneration system from cotyledonary explants would be useful for development of transgenics of black gram *via* microprojectile bombardment or *Agrobacterium* mediated transformation.

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