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Differential response of *in vitro* derived explants of Mungbean under varied hormonal regimes

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Abstract
Green gram is an important legume crop grown widely in different parts of the Asian countries. The need for food grains increases due to the growing world's population. The recalcitrance nature of green gram for *in vitro* regeneration and differential response of explants, cultivars hampers the genetic improvement of the crop. Therefore *in vitro* regeneration protocol is essential for improvement of each cultivar/variety. For the present study, explants of cotyledon, hypocotyls and leaf of Vamban 2 variety derived from five and seven day old *in vitro* grown seedlings were used. The excised explants were inoculated in different combinations of BAP and NAA in MS basal medium. The response of regeneration varies among the explants and also the age of the seedlings used. The inclusion of hormone NAA in two different concentrations and in combination with BAP produced hard calli with roots. Cotyledon and leaf explants produced multiple shoots in the shoot induction media supplemented with BAP (0.5 mg/L) and the elongated shoots were rooted in ½ MS media with 1 mg/L IBA. The protocol established can be used for improvement of cultivar through genetic engineering or genome editing for important traits.

Keywords: Mungbean, *in vitro* regeneration, differential response, hormones

1. Introduction
Mungbean (*Vigna radiata* (L.) Wilczek) is one of the important grain legume crops grown in the rice-based farming systems in south Asia and it is also gaining popularity as cash crop in other countries. It is grown mainly under rainfed condition during kharif season with and contributes about 15% of total pulse production (about 24 million tonnes in 2020-21, Mahendra *et al*. 2020) [16]. The cultivation of green gram was increasing due its early maturity, nutritional value and easy digestability. Mungbean is a potential source of dietary protein for human nutrition and it is also used as feed for livestock. The sprouted Mungbean seeds used as a vegetable/salad which is succulent, and it is rich in protein, minerals and vitamins (Poehlman 1991) [19]. They are also rich in Sulphur containing amino acids *viz*., methionine, cystine and cysteine. Even though mung bean is used widely in Indian households and serves as an important pulse crop, its production and productivity remain low in the Indian subcontinent due to biotic and abiotic constraints (Tiyagi and Alam 1992) [28].
The introgression of novel genes for enhancing pest/disease resistance and yield from wild species through conventional breeding or using biotechnological tools will facilitate in genetic improvement in green gram. The introgression of desirable genes for yield and YMV resistance led to the development of new improved cultivars *viz*., IPM-99-125, IPM-02-03 and IPM-02-14 (Singh *et al*., 2010) [26]. The introgression of resistant genes from wild species through interspecific or Intergeneric crosses often leads to fertilization problems or growth of fertilized embryos. To overcome the constraints, the immense potential of biotechnological tools can be used to supplement the breeding Programme by introducing novel genes into elite cultivars. For introducing new genes from other source or manipulating the target genes through CRISPR/Cas9 approach, efficient regeneration protocol is essential for generation of transgenic plants possessing desirable trait. The legume crops are recalcitrant in nature for *in vitro* culture as well as for its genotype specificity. Regeneration of plants through somatic embryogenesis is limited due to less conversion rate of somatic embryos into plantlets (Devi *et al.* 2004; Girija *et al.* 2000) [6, 9]. The successful regeneration procedure for generation of plants under selection agent is rather limited in green gram compared to other crops. Direct *in vitro* organogenesis would be a better and rapid multiplication method for accessing the expression of transgene. Yadav *et al.* (2009) [29] developed multiple shoots using double cotyledonary node explants in green gram variety ML267 on MS based media with 2 mg/L BAP and very low concentrations of NAA, IBA.

The multiple shoot induction was induced using 6.63µM (Raveendar and Ignachimuthu, 2010) [20], 1 mg/L BAP (Sagare and Mohanty, 2014) [21]. The response of explants on culture media varies based on genotype of cultivars. So, the present study was taken up to optimize the efficient regeneration protocol for the variety Vamban 2 using different combinations of BAP and NAA on MS based media.

2. Materials and Methods

Mung bean [*Vigna radiata* (L.) cv. Vamban 2] seeds were used for the present study. The seeds were surface sterilized with Tween 20 for 5 min followed by 70% ethanol for 5 min. Then the seeds were rinsed with 5% sodium hypochlorite for 5 min and washed repeatedly for 5 times with sterile water. The sterilized seeds were blot dried and placed on ½ strength MS medium (Murashige and Skoog (1962) [27] salts with B5 (Gamborg *et al.* 1968) [8] vitamins solidified with 0.8% agar, pH 5.8) for 7 d. They were maintained at 25 ± 2 ºC, 60% relative humidity and 16-h photoperiod. The five and seven-days old explants were used for the inoculation into shoot initiation media supplemented with different hormonal regimes.

The explants were excised and inoculated into shoot initiation media with different combinations of NAA and BAP (Table 1). The shoot initiation media was supplemented with 30 g/L sucrose, 0.8% agar. The pH of the media was adjusted to 5.8 and autoclaved after adding agar. The response in different hormonal regimes was recorded and the shoot initiated was maintained in same media for shoot elongation. The produced multiple shoots were separated and transferred into rooting media (1/2 MS+1 mg/L IBA+30 g/L sucrose+0.8% agar). The rooted plantlets were transferred to soil mixture (soil: vermicompost) in 2:1 ratio. The transferred plantlets were maintained in culture room for 3 days and further transferred to green house facility.

The induction of shoot was assessed after three subcultures for all the explants. The shoot induction percentage is calculated as, no of explants showing shoot initiation to total number of explants and expressed in percentage. The rooting percentage of elongated shoots was calculated from number of shoots rooted to total number of shoots transferred to rooting media. The observation on shoot induction and rooting under *in vitro* condition was based completely randomized design with three replications.

Table 1: Different hormonal combinations used for regeneration of mung bean

Media	Media composition
M1	MS + 0.5mg/L NAA
M2	MS + 0.5 mg/L BAP
M3	0.5 mg/L NAA+0.5 mg/L BAP
M4	MS + 1.0 mg/L NAA
M5	MS + 1.0 mg/L BAP
M6	MS + 1.0 mg/L NAA + 1.0 mg/L BAP

3. Results and Discussion

In mung bean, the regeneration of plants under *in vitro* condition is limited compared to other legume species. Earlier regeneration of plantlets through somatic embryogenesis was attempted by several workers using various hormonal combinations was not successful (Bose *et al*. 1992, Sarker and Siddiqua, 2004) [4, 25]. Further reports on *in vitro* regeneration of mung bean from cotyledon, hypocotyl and immature leaflet derived calli were reported by Amudha *et al*. (2003) [1] and Mendoza *et al*. (1992) [18]. The regeneration of plants through indirect organogenesis takes longer time duration which leads to Somoclonal variation affecting normal agronomical features of variety/cultivar (Fontana *et al*. 1993) [7]. Several authors emphasized on *in vitro* regeneration through direct organogenesis to overcome the difficulties incurred during indirect organogenesis (Hoque *et al*. 2007; Khatun *et al*. 2008; Yadav *et al*. 2010) [13, 15, 29].

The present study was undertaken to develop regeneration protocol through direct organogenesis in local variety Vamban 2. The surface sterilized healthy seeds were inoculated into ½ MS medium and the explants were excised from five-and seven-day old *in vitro* germinated seedlings. The cotyledon, hypocotyls and leaf explants were inoculated in MS media with different concentration of NAA and BAP (Table 2 and Fig 1).

The explants showed differential response in media containing different hormonal combinations. The media containing NAA in two different combinations showed induction of roots in all the explants excised from both 5 day and 7 day old seedlings. Cotyledon and leaf explants produced dull brown hard calli along with roots while hypocotyl explants showed Profused rooting. NAA in combination with BAP showed varied response among different explants. In media containing hormonal combinations of 0.5 mg/L NAA+0.5 mg/L BAP it produced hard green/white calli while hypocotyls explants produced dull brown calli. Leaf explants showed only increase in size of the explants and initiation of calli was not observed. NAA in combination with BAP produced callus with formation of roots (Gulati and Jaiwal, 1992; Yadav *et al*. 2010) [12, 29]. The organogenic potential of explants depends on the type of the explants, concentration of hormones used and also the hormonal combinations (Ignachimuthu *et al*. 1997) [14].

In the media containing BAP (0.5 mg/Land 1.0 mg/L), cotyledonary and leaf explants showed shoot initiation while hypocotyl explants produced hard white and green calli. More number of multiple shoots was produced by cotyledon and leaf explants in media containing 0.5 mg/L BAP (Fig 2), whereas cotyledonary explants produced hard green calli in media with increased concentration of BAP (1 mg/L BAP). In the same media, hypocotyl explants showed shoot initiation after 35 days. BAP is an effective cytokinin which is used widely for *in vitro* regeneration of shoots in grain legumes (Sahoo *et al*. 2002; Saini and Jaiwal, 2002) [23, 24]. Bhajan *et al*. (2019) [3] reported multiple shoot initiation in MS media containing 4.0 µM BAP using Mungbean variety BARI Mung-6. In another *Vigna* sp. shoot bud primordia initiation was reported after 2-3 weeks of inoculation in media supplemented with 8.88 µM BAP with coconut water and 75 mg/L adenine sulphate (Malabadi. 2005) [17].

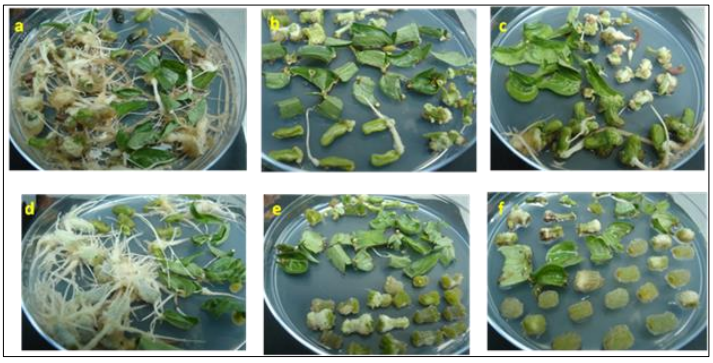
Based on the response of explants on different concentrations of growth hormones, further experiments were carried out with 0.5 mg/L BAP using cotyledon, hypocotyls and leaf explants with three replications. True leaf excised from 7 day old seedlings produced maximum number of shoots. Most of the explants showing shoot initiation after 21 days produced multiple shoots after 2 subcultures. The shoot induction percentage of leaf explant ranged from 22 to 25%. The shoots were separated and transferred to rooting medium (MS+1mg/L IBA+3% sucrose). Root initiation was observed after 15 days in all the shoots transferred to rooting. Sagare

and Mohanty, (2015) [21] observed multiple shoots in cotyledonary explants on shoot induction medium with 0.5 mg/L BAP using the green gram variety Sujatha. They reported poor response in other explants *viz.*, hypocotyls, true leaves indicating the *in vitro* response depends on the variety used and vary among the cultivars. Even the response of different explants and cultivars was different, BAP enhances the shoot induction irrespective of other factors involved in shoot regeneration and it is the most widely used and effective cytokinin reported for *Vigna* species (Chandra and Pal, 1995; Gulati and Jaiwal, 1990, Sahoo *et al*. 2002; Saini *et al*. 2002; Yadav *et al.* 2010) [5, 11, 23, 24, 29]. Amutha *et al*. (2006) [2] reported multiple shoot induction using cotyledonary node and hypocotyl explants derived from 3 day old seedlings with 0.9 µM of TDZ supplemented MS medium.

The findings in the present investigation revealed that, the *in vitro* response of mung bean vary among different varieties, different types of explants used and also the days of the seedlings raised for excision of explants. The observed findings can be further used for establishment of the efficient transformation protocol for the variety Vamban 2, so that the cultivar can be further improved for commercially important traits.

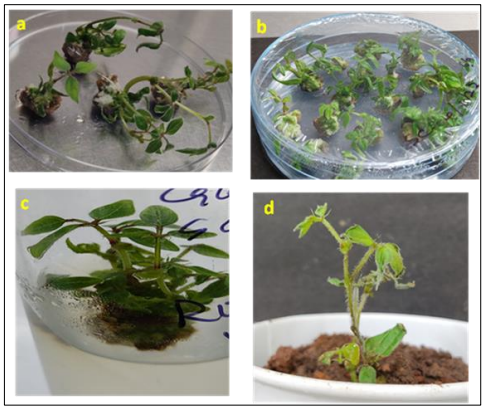
Table 2: Response of *in vitro* cultured explants in different hormonal regimes

Media composition	Cotyledon		Hypocotyl		True leaf	
	5th DAI	7th DAI	5th DAI	7th DAI	5th DAI	7th DAI
0.5 mg/L NAA	Formation of roots	Formation of roots	Formation of roots	Formation of roots	Formation of roots	Formation of roots
0.5 mg/L BAP	Shoot initiation	Hard green calli	Hard white and green calli	Hard white and green calli	Shoot initiation	Hard white and green calli
0.5 mg/L NAA+ 0.5 mg/L BAP	Hard green calli with roots	Hard white calli initiation	Hard white calli	Dull brown calli initiation	Increase in explant size	Increase in explant size
1.0 mg/L NAA	Root formation	Root formation	Root formation	Root formation	Root formation	Root formation
1.0 mg/L BAP	Hard green calli	Hard green calli	Hard white and green calli	Shoot initiation	Hard white and green calli	Hard white and green calli
1.0 mg/L NAA+1.0 mg/L BAP	Hard white calli with roots	Hard white and green calli	Hard white calli and formation of roots	Greenish watery calli	Increase in explant size with brownish calli	Increase in explant size



a. Explants showing root formation with basal callusing (0.5 mg/L NAA)
b. Explants showing shoot initiation (0.5 mg/L BAP)
c. Hard white calli and root formation in 0.5 mg/L NAA+ 0.5 mg/L BAP)
d. Root formation in MS+1.0 mg/L NAA
e. Hard green calli in MS+1.0 mg/L BAP
f. Dull green calli initiation in MS+1.0 mg/L NAA+ 1.0 mg/L BAP

Fig 1: Response of different explants on media containing combinations of NAA and BAP



a) & b) Explants with single and multiple shoots
c) Elongated multiple shoots d) Hardened plant

Fig 2: Regeneration of mung bean under *in vitro* conditions

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