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Multiple Shoot Regeneration from Detached Embryonic Axis in Greengram (*Vigna radiata*) cv.SML 668

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ABSTRACT

Background: Under prevailing regime of climate change and dynamic pest-crop interactions, greengram genetic improvement requires a speedier and precise tool such as genetic transformation to develop improved lines. In this context, an *in vitro* multiple shoot regeneration system *via* organogenesis was demonstrated in mungbean cv. SML668, a parent cultivar used in crop improvement programs, using double cotyledonary node (DCN) and detached embryonic axis (EA) explants with 1.0 mg l^{-1} BAP.

Methods: While both the explants responded to *in vitro* regeneration, number of shoots regenerated was higher with EA (4.02±0.19) than with DCN (3.1±0.08). 6-benzyl aminopurine (BAP) was found to be most effective for inducing and regenerating multiple shoots, in comparison to all other phytohormones (NAA, IAA and TDZ) and supplements (amino acids) tested. Sub culturing twice on BAP supplemented media followed by two subcultures on basal media was optimal for multiple shoot regeneration. Rhizogenesis was obtained on basal media devoid of any phytohormones in EA explants and in 1.0 mg l^{-1} IAA for DCN explants. The *in vitro* regenerated plantlets were successfully hardened in a mixture of soil, sand and vermiculite that flowered, produced pods and viable seeds on maturity.

Result: The study revealed that detached embryonic axis was a potential explant for *in vitro* regeneration in SML668, a cultivar not tested for its *in vitro* regeneration ability before.

Key words: Double cotyledonary node, Embryonic axis, Green gram, *In vitro,* Multiple shoot regeneration.

INTRODUCTION

A short duration crop with low input requirements and outstanding nutritional value of easily digestible protein (Itoh *et al.* 2006; Gan *et al.* 2017), the grain legume mungbean (*Vigna radiata* L. Wildzek) belonging to the family Leguminoseae (Fabaceae), is grown globally in an area of about 7.3 million ha with a global production of 5.3 million tons (2015-17) (https://avrdc.org/intl-mungbean-network). India and Myanmar account for 30%, China for 16% and Indonesia for 5% of the total global produce of mungbean. Yet, the average seed yield of the crop in major growing countries is low (aprox. 0.5 to 1.5 t ha^{-1}) (Nair and Schreinemachers, 2020) due to loss in yields from both biotic and abiotic stress(es).

Amongst viral diseases, Yellow Mosaic Disease (YMD) is a major constraint not only in India but also globally (Kulkarni *et al.* 2019; Mishra *et al.* 2020). Conventional integrated pest management strategies desperately need an update especially in the prevailing regime of changing climate. Improved mungbean bred by conventional means tend to lose their resistance to disease and face problems of narrow genetic base in pulses. Further genetic improvement in mungbean now requires implementation of modern biotechnological tools like translational genomics, genome editing and transgenic (s) (Kang *et al.* 2014, 2015; Mishra *et al.* 2020). In this context, it is essential to have an efficient *in vitro* regeneration system for mungbean in desired genotype for further research on genetic transformation or genome editing. Earlier reports on *Agrobacterium* mediated transformation have shown low frequencies of regeneration and recovery of transformants despite several optimizations in protocols (Gulati and Jaiwal, 1990; Khatun *et al.* 2008; Sahoo *et al.* 2016). Recalcitrance of mungbean towards regeneration and transformation (Atif *et al.* 2013; Chandra and Pental, 2003; Chaudhary *et al.* 2010) still exists and is also genotype and explant dependent (Anandan *et al.* 2019).

With limited reports on popular Indian mungbean cultivars in this context, this study was initiated with the sole objective to evaluate the impact of different plant growth regulators on *in vitro* shoot organogenesis from double cotyledonary node and embryonic axis explants to establish an optimal regeneration system.

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MATERIALS AND METHODS

Plant material

The cultivar SML668, having a high demand in Indian market (Mehandi *et al.* 2019) and derived through selection from NM 94 was selected for this study. Being YMD sensitive (Suman *et al.* 2015) this cultivar has huge scope of improvement through *in vitro* studies. Pure seeds of Breeder's grade were obtained from ICAR-IIPR, Kanpur. The entire experiment was conducted in Division of Plant Biotechnology, ICAR-Indian Institute of Pulses Research, Kanpur during the years 2019-2020.

Seed sterilization

Handpicked healthy and uniform seeds were surface cleaned with Tween 20 for 15 mins and rinsed under running tap water. Thereafter seeds were treated with the 0.2 % $HgCl_2$ solution for 3 mins, 70% ethanol for 1 min and rinsed thrice with sterile distilled water. Sterilized seeds were placed on sterilized blotting sheets for further use.

Explant preparation

Surface sterilized seeds were aseptically germinated on MS (Murashige and Skoog, 1962) media with B_5 vitamins (Gamborg *et al.* 1968) (MSB_5) devoid of any phytohormones, 3% (w/v) sucrose solidified with 0.8% (w/v) agar and with pH 5.8.

Double cotyledonary nodes were excised from 4 old seedlings by removing the seed coat and cut on both the sides covering both hypocotyl and epicotyl along with the apical portion of cotyledons.

Embryonic axis excised from sterilized and soaked seeds in sterile distilled water for 24 h in dark were split open to detach both the cotyledons exposing 3-5 mm embryonic axis.

Inoculation and shoot multiplication

Aseptically prepared explants were inoculated in shoot bud induction media *i.e.* MSB_5 fortified with different concentration of phytohormones *viz.* BAP alone (0.5 mgl^{-1}, 1 mgl^{-1} and 2 mgl^{-1}) or in combination with other phytohormones (NAA, IAA, TDZ). Amino acid (glutamine, proline, and cysteine) supplementation in basal media was also tried in effort to enhance shoot proliferation. On emergence of primary shoots, they were excised and transferred to MSB_5 media devoid of phytohormones for further elongation. Sub culturing was done every fortnight. With emergence of new shoots, they were excised and placed on fresh media for elongation. Callus appearing at base of explant was intermittently removed.

In each experiment, MS medium was used for culture and pH of the medium was maintained at 5.8 ± 0.02. Cultures were maintained at 25±2°C under a 16:8 h (light, dark) photoperiod with light intensity 25 µmol m^{-2} s^{-1} provided by cool white fluorescent tube lights.

Rhizogenesis and hardening of plantlets

In vitro regenerated shoots with a length more than 2 -3 cm were individually transferred onto root initiation media: MSB_5 supplemented with 1.0 mg l^{-1} of IAA or IBA or NAA for DCN explant. EA derived shoots were rooted in MSB_5 media without any phytohormone. Shoots with a developed rooting system were then gently removed from media, washed with tap water to remove agar entangled in roots, initially hardened in small pots with soilrite and covered with poly bags having pores for 2 weeks in culture room. The bags were then removed and established plants were transferred to a mixture of soil, sand and vermiculite kept in greenhouse for further development. Prevailing temperature recorded was 27±2°C with relative humidity of ca. 30-35%.

Data recorded

Each treatment had 15 explants in triplicates. Visual observations of increase in shoot number, length and callus were taken every week and data for shoot multiplication were recorded as frequency of shoot differentiation, numbers of shoots regenerated per explant and shoot length, 4 weeks post inoculation. The root length, percentage of shoots with rooting and days to rooting were recorded 2-3 weeks post inoculation in rooting media.

RESULTS AND DISCUSSION

In vitro shoot regeneration

DCN as explant

Basal culture medium *viz*. MSB_5, devoid of any phytohormones, did not regenerate multiple shoots. Assessment of number of shoots regenerated *in vitro* using different phytohormones individually and in combination along with amino acids was hence done (Table 1). Cytokinins are known to be a prerequisite for legume *in vitro* regeneration, as in ground nut (Venkatachalam and Jayabalan, 1997); lentil (Fratini and Ruiz, 2002) and urdbean (Adlinge *et al.* 2014) and amongst many cytokinins, BAP has been the most responsive, especially with *Vigna* species (Chandra and Pal, 1995). Hence, BAP was chosen as the cytokinin for this study. In comparison to basal media alone, higher number of shoots regenerated in presence of BAP with a maximum of 3.1±0.08 shoots per explant in presence of 1.0 mgl^{-1} BAP (Fig 1).

Though per cent regeneration was not statistically different in media supplemented with BAP at 0.5 mgl^{-1} and 1.0 mgl^{-1}, number of shoots regenerated were significantly higher at 1.0 mgl^{-1} BAP. Reports indicate that combination of BAP and auxin aid in axillary shoot induction and elongation (Rasool R *et al.* 2009; Yadav *et al.* 2010, Singh V *et al.* 2014) across different crops; hence influence of BAP in combination with auxin on shoot regeneration was also tested. It was observed that shoot regeneration in media supplemented with BAP (1.0 mgl^{-1}) in combination with NAA (0.1 mgl^{-1}) was at par with BAP 1.0 mgl^{-1} alone but there was a significant decrease in per cent regeneration. Mahalaxmi *et al.* 2003 had reported a positive effect of amino acid supplements on *in vitro* regeneration of cotyledonary node explant. On a similar line, basal media supplemented with BAP and NAA and amino acid (s), (L-Cysteine, Proline and

Glutamine 50 mgl^{-1} each) was tested for multiple shoot regeneration in mungbean to find that the combination regenerated lesser number of shoots in comparison to media supplemented with NAA alone or BAP alone. Among all the treatments tested, media with 1.0 mgl^{-1} BAP + 0.1 mgl^{-1} NAA responded better for number of shoots per explant (2.86±0.19) followed by the media 1.0 mgl^{-1} BAP + 0.1 mgl^{-1} NAA + 50 mgl^{-1} Glutamine (2.34±0.19) (Fig 2). A decrease in per cent regeneration was also documented.

Role of TDZ as a shoot multiplication hormone (Kumar *et al.* 2003; Amutha *et al.* 2006) for mungbean cultivars was tested but failed to reproduce the results. TDZ induced multiple shoot node formation rapidly and effectively in comparison to BAP, but these formations remain stunted and converted into callus. Even in combination with BAP, TDZ regenerated single shoot with callusing that ultimately died due to tissue necrosis.

Although there are number of published optimized *in vitro* regeneration protocols for mungbean reporting regeneration of more than 104 shoots per explant using TDZ (Amutha *et al.* 2006); 27 shoots per explant using combination of BAP and NAA (Yadav *et al.* 2010) however, they were not reproducible in the cultivar SML 668. The phytohormone BAP (1.0 mgl^{-1}) was found most efficient for *in vitro* multiple shoot induction in mungbean cultivar SML668. The maximum average shoot length recorded was 4.15±0.75 with 1.0 mgl^{-1} BAP (Table 2). This study highlighted that SML 668 is not much responsive towards tissue culture while using DCN as choice explant.

Embryonic axis as explant

Cotyledonary node segments produce shoots from nodal sections and remain the first choice for shoot differentiation *via* organogenesis amongst legumes (Chandra and Pental,

Table 1: Effect of different concentrations of phytohormones on multiple shoot proliferation from DCN explant after 30 days of inoculation.

MSB_5 + growth regulators (mgl^{-1})							%	No. of shoots /
BAP	TDZ	NAA	IAA	L-Cysteine (C)	Proline (P)	Glutamine (G)	regeneration* (Mean±SE)	sxplant (Mean±SE)
0.5	-	-	-	-	-	-	98.0±1.6	2.18±0.07
1	-	-	-	-	-	-	99.1±0.28	3.1±0.08
2	-	-	-	-	-	-	90.5±0.54	2.52±0.07
1	-	0.1	-	-	-	-	82.7±2.74	2.86±0.19
1	-	-	0.1	-	-	-	77.8±3.21	2.31±0.14
1	-	0.1	-	50	-	-	62.9 ±1.99	2.30±0.13
1	-	0.1	-	-	50	-	57.1±0.79	1.78±0.13
1	-	0.1	-	-		50	70.2±0.21	2.34±0.19
1	-	0.1	-	50	50	50	56.2±1.57	2.08±0.21
-	0.01	-	-	-	-	-	59.7±2.22	1.71±0.13
-	0.05	-	-	-	-	-	51.9±3.31	1.47±0.11
-	0.10	-	-	-	-	-	35.4±2.3	1.26±0.07
1	0.01	-	-	-	-	-	68.1±1.49	1.84±0.10

* % regeneration: number of explants responding to *in vitro* regeneration.

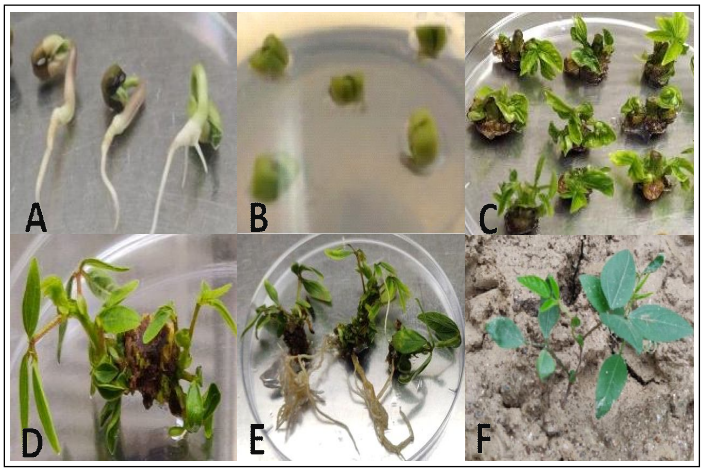

Fig 1: Stages of regeneration from double cotyledonary node (DCN) on MS with 1.0 mgl^{-1}BAP. A- Aseptically geminated seedlings in MS media. B- DCN explant from 4 day old seedlings. C-after 20 days of inoculation MSB_5+BAP (1.0 mgl^{-1}). D- Shoot proliferation after 30 days in MSA. E- Root initiation in MSB_5+ IAA 1.0 mgl^{-1}. F- Establishment of plantlets in soil after primary hardening.

2003), but embryonic axis as an explant can also be utilized for tissue culture due to presence of fully exposed and broad preexisting meristems that have predetermined fate of regeneration. Embryonic axis (EA) has been explored for establishing efficient regeneration and transformation systems in other crops such as *Cicer* (Krishnamurthy *et al.* 2000; Aasim *et al.* 2011, Shukla *et al.* 2015); *Glycine* spp. (McCabe *et al.* 1988), *Phaseolus vulgaris* (Gatica Aria *et al.* 2010) and several *Vigna* sps. (Bhargava and Smigocki,1994; Acharjee S *et al.* 2012; Ivo *et al.* 2008), but no such report was retrieved for *Vigna radiata.*

In this study, percent regeneration and number of shoots regenerated per explant were assessed in basal media supplemented with different concentrations of BAP alone.

Table 2: Average shoot length of *in vitro* regenerated shoots on MSB5 medium supplemented with BAP 30 days after inoculation.

BAP (mgl^{-1})	Shoot length (cm) (Mean±SE)
0.5	3.07±0.09
1	4.15±0.75
2	2.10±0.09

Maximum shoot proliferation (4.02±0.19) was recorded in MSB_5 supplemented with 1.0 mgl^{-1} BAP (as with DCN (Table 3, Fig 3). This is the first ever report of EA as an explant in cv. SML668 for direct organogenesis in mungbean along with comparative analysis with DCN.

A single shoot regenerated in media without any phyto hormone using EA as explant and was similar to that observed while using DCN as explant. Embryonic axis was more responsive than DCN and that too for all doses of BAP, as evident by the per cent regeneration response as given in Table 3. Maximum number of shoots regenerated per explant (4.02±0.19) was found in media supplemented with 1.0 mgl^{-1} BAP and was significantly higher than other doses of BAP. It was also significantly higher to the number of shoots regenerated (3.1±0.08) in same dose of BAP using DCN as explant.

Effect of duration of exposure of explant to BAP on shoot regeneration

Significant increase in shoot multiplication was observed in explants (both DCN and EA) exposed to BAP for 20 days and then kept in basal media devoid of any hormone (Table 4). Exposure to phytohormones BAP for more than 20 days reduced the number of shoots produced, and

Fig 2: Effect of DCN explant in growth medium A-Shoot differentiation in 1.0 mgl^{-1} BAP + 0.1 mgl^{-1} NAA, B- Shoot differentiation in 1.0 mgl^{-1} BAP + 0.1 mgl^{-1} NAA + 50 mgl^{-1} Glutamine.

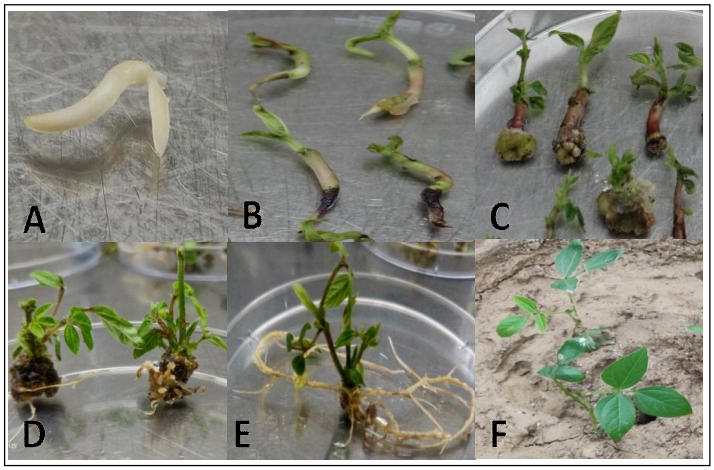

Fig 3: Stages of regeneration from embryonic axis (EA) explant on MS with 1.0 mgl^{-1} BAP. A- Excised embryonic axis. B-After 10 days of inoculation in MSB_5 + BAP (1.0 mgl^{-1}) C- After 20 days in same media. D- Shoot proliferation after 30 days in MSA. E- Root initiation in MSB_5+ IAA 1.0 mgl^{-1}. F- Establishment of plantlets in soil after primary hardening.

Table 3: Response of EA explants cultured on MSB5 medium containing different concentrations of BAP, 30 days post-inoculation.

BAP (mgl^{-1})	EA		
	% Cultures regenerating shoots (Mean±SE)	Shoot number (Mean±SE)	Shoot length (cm) (Mean±SE)
0.5	99.00±0.36	3.05±0.17	4.11±0.19
1	99.60±0.31	4.02±0.19	5.01±0.16
2	99.13±0.28	2.72±0.11	3.04±0.22

Table 4: Effect of duration of BAP (1.0 mgl^{-1}) treatment on shoot multiplication from DCN and EA explant.

Culture duration in days	Shoot number/explant (Mean±SE)	
	DCN	EA
10	2.11±0.08	2.16±0.08
20	3.41±0.12	4.55±0.18
30	2.98±0.10	4.22±0.16
40	2.63±0.12	3.16±0.10

Table 5: Effect of different concentrations of auxins on root induction from DCN derived shoots.

MSB_5+ auxins (1 mgl^{-1})	Root length (cm) (Mean±SE)	Days to rooting (Days)	% of rooting (Mean±SE)
IAA	5.74±0.29	12-14	93.30±1.50
IBA	4.78±0.25	17-19	77.70±1.05
NAA	2.73±0.21	13-15	61.70±0.94

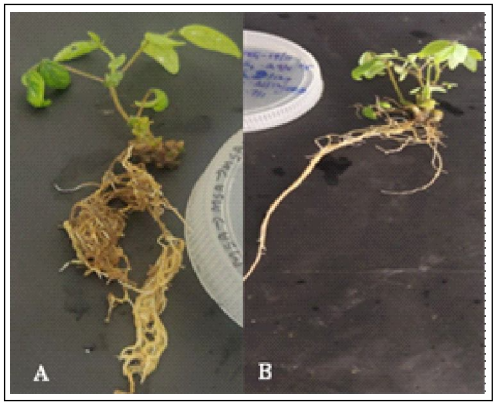

Fig 4: Rooted Plantlets A: DCN in MSB_5 + IBA 1 mgl^{-1}, B: EA in MSB_5.

gradually caused necrosis and shoot deterioration. Further elongation in shoots was obtained on basal medium itself.

Rhizogenesis

Root formation was successfully obtained in 1.0 mgl^{-1} IAA within 2 weeks from DCN explant. Root initiation frequency at same concentration of IBA (77.70%) and NAA (61.70%) revealed growth of an improper and less natural root system. The roots developed were shorter and thicker on use of NAA and response on use of IBA was delayed (by ca. 5-10 days) in comparison to IAA (Table 5). This result is in accordance with those of (Gulati and Jaiwal, 1994) and (Mahalakshmi *et al.* 2003) who reported that IAA was best for inducing (Khatun *et al.* 2008) reported NAA and (Yadav *et al.* 2010) and (Patra *et al.* 2018) reported IBA as best rooting hormone for mungbean.

EA regenerated shoots successfully rooted (rooting frequency ca. 96.30%) in MSB_5 medium without any exogenous growth regulator giving an average root length of 8.70±0.31cm (Fig 4). This self-induction might be the results of pluripotent cells present at hypocotyl region that aid in endogenous induction of rooting. The *in vitro* multiplied plantlets were hardened and acclimatized in a mixture of sand, soil and vermiculite with a survival rate of 85-90%. The hardened shoots were transferred to the greenhouse where they developed pods and set viable seeds.

Conclusion

Efficient *in vitro* regeneration system in *Vigna radiata* using BAP as choice phytohormone has been reported by several researchers, however, when tested in *cv.* SML 668, the number of shoots regenerated per explant was found to be low. Understanding that response of genotype is a crucial parameter to develop an efficient and reproducible *in vitro* regeneration system, equally important is the choice of explant and its response. In this context, efforts were made to study the effect of phytohormones and explant on mungbean genotype SML668. Embryonic axis was a better responding explant that gave higher shoot number per explant in comparison to the cotyledonary node with 1.0 mg l^{-1} BAP. Thus, we conclude that EA carries equal potential for direct multiple shoot regeneration as the explant DCN and this is the first report in mungbean SML668 where embryonic axis could be successfully used for *in vitro* regeneration. Further studies with use of supplements and different phytohormones and explants may pave way for efficient regeneration in mungbean in this cultivar and many others.

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Conflict of interest: None.

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