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Investigating the *in vitro* regeneration potential of mungbean cultivar Samrat

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ABSTRACT

***In vitro* regeneration of mungbean, being genotype dependent, needs to be standardized with release of new cultivars to be able to use them for further genetic improvement, especially in the regime of genome editing. Samrat is a mungbean cultivar that has reigned mungbean production in past years and is a stable resistant source against Yellow mosaic disease (YMD). The regeneration ability this cultivar, using double cotyledonary node (DCN) and embryonic axis (EA) explants was investigated in Murashige Skoog (MS) medium supplemented with different concentrations of BAP (6-Benzyl Amino Purine) for varied durations. While both the explants responded to *in vitro* regeneration, number of shoots regenerated was higher with EA (6.45 ± 0.37) than with DCN (5.24 ± 0.37). Sub-culturing thrice on BAP 1.0 mg L^{-1} 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 soilrite (1:1:1) with 72-80% plantlet survival with successful flowering and viable seed setting on maturity. The study revealed that both explants DCN and EA were equally potential explants for *in vitro* regeneration in Samrat with 1.0 mg L^{-1} BAP for 30 days.**

Key words: Double cotyledonary node, Embryonic axis, *In vitro* regeneration, mungbean

INTRODUCTION

Mungbean *(Vigna radiata* L. R. Wilczek var. *radiata*), is the most widely cultivated species among major crops of *Vigna genus* (Pandiyan *et al.* 2012). India, the largest producer and consumer of mungbean, contributes 60% of the total global production with an area of 3.8 million hectares (Nair and Schreinemachers, 2020). It is known as a farmer's friendly crop due to its low input requirement, wider adaptability towards climate vagaries and adding essential nutrients in vegetarian diet along with income source to farmer families. Seeds of mungbean are particularly rich in (~24 % easily digestible) proteins (Itoh *at al*. 2006; Anwar *et al*. 2007), fibers, antioxidants and exhibit health benefits due to its detoxification bioactivities (Yi Shen *et al.* 2018); thus can be used as a functional food for mitigation of malnutrition and in health programs. The yield potential of mungbean is about 2 tonnes per hectare, while average productivity in most countries is nearly 0.5 tonnes per hectare (Basu *et al*. 2019). This yield gap is primarily due to abiotic and biotic stresses, climate change, non-availability of quality seed and lack of crop management practices (Pratap *et al.* 2021).

Yield loss due to biotic stress (disease and insect infestation) can occur due to infestation at any stage of plant growth and development, and even after harvest (Laosatit *et al.* 2020). Mungbean yellow mosaic disease (MYMD) is a serious disease transmitted by whitefly (*Bemisia tabaci*) (Selvi *et al.* 2006; Mishra *et al.* 2020) that can cause complete yield loss in susceptible cultivars (Marimuthu *et al.* 1981) or even cause mortality of infected plants at early vegetative stages. Abiotic stress such as drought, water logging, heat and salinity are also known to severely affect mungbean productivity (Noble *et al.* 2020).

The mungbean cultivar Samrat (PDM-139) was released in 2001 as a prominent high yielding line tolerant to Yellow mosaic disease, with synchronous maturity, superior vigor quality, halo-tolerant and highly popular among farmers during spring/summer seasons in northern parts of India (Paul *et al.* 2013; Sen *et al.* 2017; Pratap *et al.* 2019). It revolutionized mungbean cultivation amongst farmers, as it yielded ca. 12-14 q/ha within 65 days and earned approximately ₹ 50-60 thousand/ha. (https://icar.org.in/node/250, 2008). Even after two decades, Samrat still finds place amongst the top 10

varieties contributing a major share in mungbean breeder seed indent (Singh *et al.* 2017).

The mungbean hybridization programs face the constraint of narrow genetic base, due to repeated use of few available genetic resources (Kumar 2004, 2011). Therefore, there is dire need to widen the genetic base by incorporating desirable traits using modern biotechnological tools. Distant hybridization aided by tissue culture-based embryo rescue techniques (Palmer *et al.* 2002), *in vitro* mutagenesis (Badere *et al.* 2016), development of trait specific transgenics (Mekala *et al.* 2016; Yadav *et al.* 2012) and genome edited plants (CRISPR/Cas, ZFNs and TALENs) (Bhowmik *et al.* 2021) are a few approaches that have been accepted to aid mungbean crop improvement.

The prerequisite for success of tissue culture-based biotechnological interventions in crop plants is the availability of an efficient *in vitro* regeneration system. The species of *Vigna* genus displays high genotypic specificity and has lesser regeneration potential as compared to other legume crops (Mundhara *et al.* 2006; Atif *et al.* 2013; Pratap *et al*. 2018). Among cytokinins, BAP has been reported to facilitate *in vitro* shoot multiplication in mungbean through shoot bud initiation and continuous adventitious shoots formation (Gulati and Jaiwal, 1992 and 1994; Vijayan *et al.* 2006; Patra *et al.* 2018; Rathore *et al.* 2022). However, the reported systems do not stand amenable to all genotypes. Thus, biotechnological intervention still requires establishment of genotype specific *in vitro* regeneration systems as a prerequisite. Hence, this study was undertaken to develop an *in vitro* regeneration system in mungbean cultivar Samrat, with the objectives to assess (i) *in vitro* regeneration potential of the explants double cotyledonary node (DCN) and embryonic axis (EA), (ii) optimal concentration of BAP for shoot induction and/or regeneration, (iii) optimal duration of BAP treatment, and (iv) initiate rhizogenesis in *in vitro* regenerated shoots.

MATERIALS AND METHODS

Seed sterilization

Breeder's seeds of Yellow mosaic disease tolerant, high yielding mungbean genotype Samrat (Brar *et al.* 2004) was obtained from ICAR-IIPR, Kanpur. The seeds were washed using mild detergent Tween-20 (polyoxyethylene sorbitan monolaurate) for 15 mins and then completely rinsed using tap water. A treatment of 0.2% $HgCl_2$ for 3 mins followed by treatment with 70% ethanol for 1 min was given under laminar air flow. They were then rinsed thrice using autoclaved distilled water to remove any traces of sterilants. Seed were then dried by blotting on sterilized filter paper before being used.

Explant preparation

Surface sterilized seeds were inoculated on MS (Murashige and Skoog, 1962) media with B_5 vitamins (Gamborg *et al*. 1968) (MSB_5) supplemented with 3% (w/v) sucrose and semi-solidified by 0.8% agar, pH adjusted to 5.8. All experiments were done using two types of explants *viz.* EA and DCN. DCN explants were obtained from 4 days old seedling, by excising the epicotyl and hypocotyl (2 mm above and below) at cotyledonary junction. EA was prepared from overnight (~16-18h) soaked seeds in dark condition, by separating one cotyledon resulting in exposed embryo attached to a single cotyledon.

In vitro shoot initiation

Both the explants were inoculated on MSB_5 medium supplemented with three different concentrations of 6-Benzyl amino purine (BAP) viz. 0.5 mg L^{-1}, 1 mg L^{-1} and 2 mg L^{-1} with 0 mg L^{-1} as the control treatment. Explants were kept on shoot initiation media for different durations viz. 20, 30 and 40 days with sub culturing at every 10 days interval. Thereafter, explants were transferred to basal media i.e. MSB_5 without any phytohormone. Sub culturing was done every 7-10 days for removal of dead mass of cells at base of explant and to expose the meristematic tissue for uptake of supplements from media.

Basal media used in experiments was MSB_5 with pH 5.8 ± 0.02 before autoclaving (121 °C and 1.2 kg cm^{-2} for 15 min). Cultures were established at 25 ± 2 °C under a 16:8 h (light, dark) photoperiod with light intensity 25 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by cool white fluorescent tube lights.

In vitro root initiation and acclimatization

In vitro grown shoots attaining a length of 2- 3 cm were transferred to basal media for 1-2 cycles, and then into root initiation media viz. MSB_5 supplemented with 1.0 mg L^{-1} of IAA for both explants. In EA regenerated shoots, rhizogenesis was observed in MSB_5 media alone devoid of any phytohormone.

Regenerated plants with well-developed shoot and root systems were taken out from medium and roots were washed gently under running tap water to remove any traces of agar. The plants were then transferred to mixture of substrates (sterile and moistened sand + soil + soilrite 1:1:1) in small pots. The pots were covered with a perforated transparent polythene sheet to maintain humidity and plants were maintained at 25 + 2 °C with a 16 / 8 h light / dark photoperiod with an irradiance of 30 mmol $s^{-1}m^{-2}$. Unless the plants sowed signs of wilting, they were exposed every day to low humidity by removing the polythene sheet for a daytime period. After 2 weeks, the polythene sheet was completely removed and acclimatized and hardened plants were maintained under ambient temperature and lighting in net house.

Experimental design and statistical analysis

All the treatments had 20 explants and were repeated thrice. Cultures were monitored on daily basis for increase in shoot number, length and callus growth. Regeneration data was recorded as regeneration percentage, numbers of shoots regenerated per explant, shoot length, root length, percentage of rooting and days to rooting at every 10 days interval till hardening. One way analysis of variance (ANOVA) test at significance level of 0.05 was used for data analysis and comparisons between mean values of treatment tests were performed using Tukey's HSD test. The study was conducted in Division of Plant Biotechnology, ICAR-Indian Institute of Pulses Research, Kanpur during 2020-2021.

RESULTS AND DISCUSSION

Shoot regeneration and elongation

Multiple shoot proliferation was directly induced in both explants EA and DCN using BAP. Amongst different BAP concentrations (0.5, 1.0, 2.0 mg L^{-1}) tested, maximum multiple shoot initiation was observed at 1 mg L^{-1} in both the explants DCN (5.24±0.37) and EA (6.45±0.37) (Table 1). Cytokinins regulate cell proliferation and differentiation in plant development by inducing shoot formation, and are hence called shooting hormone (Kieber and Schaller, 2014). In preliminary experiments different shooting hormones viz. BAP, TDZ, BAP/ TDZ in combination with auxins (IAA, NAA) were tested (data not shown); all treatments gave low regeneration frequency and shoot development, except for BAP. BAP has been the phytohormone of choice for shoot regeneration, especially for leguminous crops such as chickpea (Amer *et al.* 2018; Singh *et al.* 2022), urdbean (Singh *et al.* 2020) and soybean (Raza *et al.* 2017) and similar observations were documented in mungbean.

Though at 1 mg L^{-1} BAP shoot regeneration frequency and multiple shoots obtained were higher in both explants, but early initiation of shoot and root with increased shoot length was observed at lower concentration of BAP (0.5 mg L^{-1}). Contrastingly, at 2.0 mg L^{-1} BAP multiple micro shoots formed with large callus at base, but these shoots failed to attain enough length and form complete rooted plantlet, on the other hand, they gradually converted into dead callus. It was thus observed that higher BAP concentrations suppress shoot development affecting further growth and development.

Both the explants responded to BAP fortified medium, however the response varied between the explants, with dose of BAP and also in comparison to control (basal media) within 10- 15 days. Dwarfing of shoots, multiple micro shoots at the surface of callus, callusing (hard and blackening in older cultures), delayed rooting and smaller spindly leaves at higher BAP concentration was recorded. As evident from table 1, percent regeneration was significantly higher in both explants for BAP at 0.5 and 1.0 mg L^{-1}. Also, the number of shoots regenerated per explant using DCN as explant was significantly higher at these doses in comparison to BAP at 2 mg L^{-1}. However, at 1 mg L^{-1} BAP, the response was relatively better. On the other hand, a significantly higher number of shoots regenerated per explant at 1mg L^{-1} BAP using embryonic axis as choice explant. The maximum shoot length attained per explant was, however, higher at 0.5 mg L^{-1} BAP in both explants tested.

BAP stimulates the development of axillary shoots but simultaneously inhibits root formation. At higher doses, BAP can accumulate in plant tissue and in contrast to other cytokinins (kinetin, 2iP or zeatin), BAP conjugates are very stable, and exert a prolonged inhibitory effect on rooting (Podwyszynska, 2003). Complete regeneration response was affected by concentration and duration of BAP exposure, as explants turned into huge brown color calli at high dose for longer period. Hence, optimization of culture duration on medium supplemented with 1 mg L^{-1} BAP was done for both explants by sub culturing them for different days (20, 30, 40) (Fig. 1 and 2). Explants with primary shoots were subsequently transferred to medium

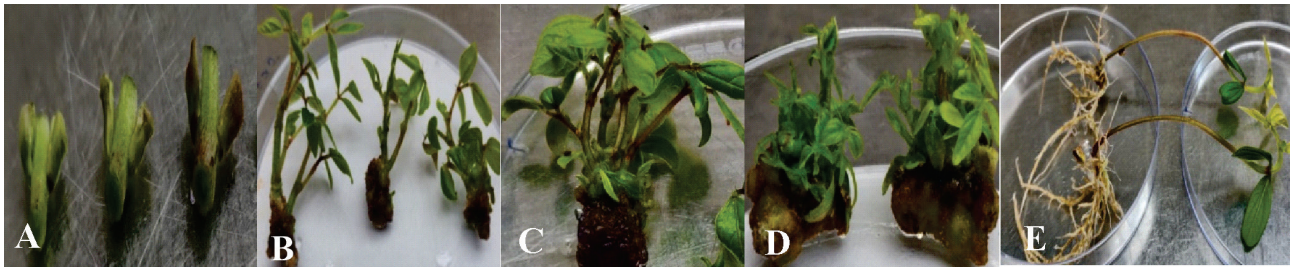

Fig. 1. Effect of BAP 1.0 mg L^{-1} upon shoot differentiation for different time periods from DCN explant: A-0 days, B-20 days, C-30 days, D-40 days, E-Control after 10 days.

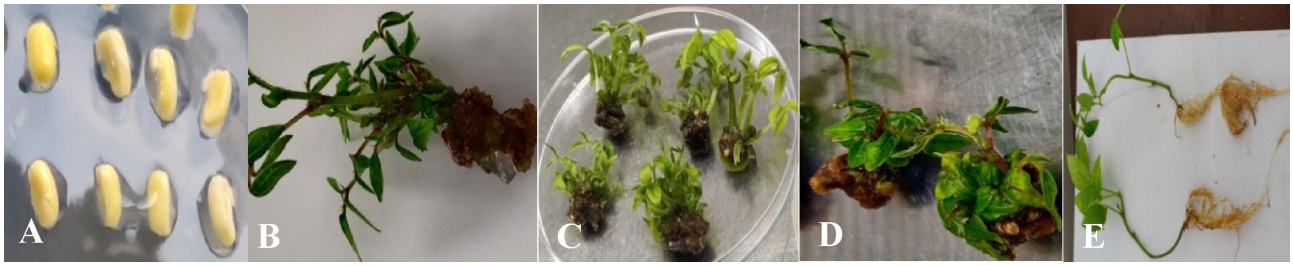

Fig. 2. Effect of BAP 1.0 mg L^{-1} upon shoot differentiation for different time periods from EA explant A- 0 days, B- 20 days, C- 30 days, D- 40 days, E- Control after 10 days.

devoid of any phytohormone for proliferation and elongation of the shoots.

Significant differences were observed in response of explants for multiple shoot regeneration varying with the duration for which they were kept on MS supplemented with BAP at 1 mg L^{-1} (Table 2). Shoot differentiation from both explants was obtained post 20 days of culture on BAP, but maximum number of shoots per explant with DCN (5.52±0.30) and EA (6.97±0.32) was recorded at 30 days. However, when kept for 40 days, stunting of the proliferating shoots was prominent in both explants as evident from shorter shoot lengths in DCN (1.92±0.10) and EA (2.09±0.34). The callus growth from epicotyl region eventually seized growth of the meristematic zones of explant and ultimately turned into a dead mass of cells. The average shoot length significantly decreased upon increasing BAP treatment and the maximum shoot length was recorded in both explants at 20 days post culture. These results are in accordance with previous studies; optimum duration of 30 days in BAP was reported in mungbean cultivar ML267 (Yadav *et al.* 2010).

Rhizogenesis was also affected with increasing durations of explant in 1 mg L^{-1} BAP. It was observed that while cultures kept for 30 days of BAP treatment showed root initiation within 15 days, explants exposed to BAP for 40 days showed first signs of rooting after ca. 30 days.

Earlier reports of *in vitro* studies in mungbean (Hoque *et al.* 2009; Himabindu *et al.* 2014; Sakthi *et al.* 2022) revealed that DCN was choice of explant for establishing regeneration system. This preference is because mature cotyledons, regenerate shoots from the proximal side i.e., from the preexisting meristem or through the proliferation of shoots around pre-existing buds whereas, with immature cotyledons, regeneration of shoot buds obtained from all over the surface of cotyledons (Yadav *et al.* 2010). Shoot differentiation from DCN occurred directly from epidermal and subepidermal cells without callus formation at the nodal region. Fig. 3 depicts the complete regeneration system in *cv.* Samrat from DCN at BAP 1 mg L^{-1} for 30 days as it gave higher no. of shoots with proper shoot elongation and root induction among all 3 concentrations and durations of BAP tested in the cultivars experimented with.

Higher rate of shoot regeneration was obtained from embryonal axis attached to the adjacent part of cotyledon than with DCN at all treatments, this might be due to the exposed and highly proliferating preexisting and predetermined meristem, as soon as it gets slight induction of BAP, cell division boosts at tip and creates a zone of micro shoots (Fig. 4 d). The presence of several shoot primordia are likely to be originated from the meristematic structures which commenced by cell division and helps in formation of direct shoot from explants tissue from one or few cells that actually originated from single cell (Broertjes and Keen, 1980; Hansen and wright,

Fig. 3. Stages of regeneration from double cotyledonary node (DCN) on MSB_5 with 1.0 mg L^{-1} BAP. a. Aseptically germinated seedlings in MSB_5 media. b- DCN explant from 4 day old seedlings, c- After 10 days of inoculation MSB_5+BAP (1.0mg L^{-1}), d- After 20 days of inoculation MSB_5+BAP (1.0 mg L^{-1}), e- Shoot proliferation after 30 days in BAP and 10 days in MSA and segregation of shoots from single explant, f- Root initiation in MSB_5+ IAA 1.0 mgl^{-}, g- Establishment of plantlets in soil after primary hardening.

Fig. 4. Stages of regeneration from embryonic axis (EA) explant on MS with 1.0 mg L^{-1} BAP. **a.** Embryonic axis attached with cotyledon. **b-** After 10 days of inoculation in MSB_5 + BAP (1.0 mg L^{-1}) **c-** After 20 days in same media. **d-** Shoot proliferation after 30 days in BAP and 10 days in MSA. **e-** Shoot elongation and root initiation in MSB_5. f- Rooted shoots from single explant. **g-** Establishment of plantlets in soil after primary hardening.

Table 1. Effect of different concentrations of BAP on multiple shoot regeneration from DCN and EA explants

MSB_5 + BAP (mg L^{-1})	% regeneration* (Mean ± SE)		No. of Shoots / Explant (Mean ± SE)		Shoots length (cm) (Mean ± SE)	
	DCN	EA	DCN	EA	DCN	EA
0.5	95.8±1.16 [a]	97.4±0.89 [a]	3.73±0.29 [ab]	4.12±0.23 [b]	4.62±0.16 [a]	5.25±0.18 [a]
1	97.8±0.32 [a]	98.8±0.18 [a]	5.24±0.37 [a]	6.45±0.37 [a]	3.73±0.14 [b]	4.47±0.13 [b]
2	89.3±0.66 [b]	90.0±0.20 [b]	3.33±0.17 [b]	4.43±0.27 [b]	2.10±0.10 [c]	2.51±0.20 [c]

Data represent mean ± SE of three independent experiments
Means followed by the same letter under different treatments within a column are not significantly different (P<0.05) from each other

Table 2. Effect of duration of (BAP) 1 mgl $^{-1}$on shoot regeneration from DCN and EA

Culture duration in days	DCN			EA		
	Shoot number/Explant (Mean ± SE)	Shoots length (cm) (Mean ± SE)	Days to rooting (days)	Shoot number/Explant (Mean ± SE)	Shoots length (cm) (Mean ± SE)	Days to rooting (days)
20	4.07±0.20 [b]	4.26±0.11 [a]	10-12	5.02±0.34[b]	5.03±0.31 [a]	≥10
30	**5.52±0.30** [a]	**3.77±0.11** [b]	**15-20**	**6.97±0.32** [a]	**4.00±0.32** [b]	**10-15**
40	3.07±0.15 [c]	1.92±0.10 [c]	25-30	3.49±0.18 [c]	2.09±0.34 [c]	20-25

Data represent mean ± SE of three independent experiments
Means followed by the same letter under different treatments within a column are not significantly different (P < 0.05) from each other

Table 3. Effect of BAP on self -induction of roots from DCN and EA (on basal media)

Days of BAP exposure	% of Rooting (Mean ± SE)	
	DCN	EA
20	81.0±0.80 [a]	88.3±0.46 [a]
30	64.7±0.77 [b]	76.0±1.14 [b]
40	29.2±0.49 [c]	50.0±1.06 [c]

Data represent mean ± SE of three independent experiments
Means followed by the same letter under different treatments within a column are not significantly different (P < 0.05) from each other

Table 4. Effect of auxin on root length from DCN and EA derived shoots after 50-55 days of culture initiation

Media	Root Length (cm) after durations of BAP treatment (Mean ± SE)					
	20 days		30 days		40 days	
	DCN	EA	DCN	EA	DCN	EA
MSB5	5.87±0.18 [b]	6.55±0.20 [b]	4.07±0.13 [b]	5.78±0.20 [b]	3.38±0.14 [b]	4.07±0.17[b]
IAA 1mg L^{-1}	7.14±0.36 [a]	8.48±0.51 [a]	6.64±0.41[a]	7.09±0.64 [a]	5.04±0.24 [a]	6.34±0.50 [a]

Data represent mean ± SE of three independent experiments
Means followed by the same letter under different treatments within a column are not significantly different (P < 0.05) from each other

1999). Present experiment reveals that embryonic axis (EA) produced a significantly higher number of shoots per explant, hence, it is the most amiable explant for regeneration (~98%) in *cv.* Samrat. Also, in vitro regenerated shoots from EA explant took relatively lesser days to rhizogenesis in comparison to those regenerated from the DCN explant (Fig. 4).

Rhizogenesis and acclimatization

In vitro root initiation is highly dependent on type of explant, media supplement and the duration after which explants are exposed to rooting media. A significantly higher root induction percentage (88.3%) was recorded in embryonic axis derived shoots from 20 days of BAP treatment without any auxin supplement followed by 64.77 % from 30 days culture and 29.20 % from 40 days culture (Table 3). A similar trend was observed in rooting from shoots regenerated using DCN explants this decrease in rooting percentage with increasing culture duration indicates that presence of a cytokinin for longer duration creates hindrance in root induction. Also, *de novo* rhizogenesis was observed in explants placed on basal media alone without any phytohormone supplement. Here, rooting initiated within 10 days on culture, the roots formed were relatively robust and revealed formation of lateral roots. These results validated the detrimental effect on rooting of explant on long exposures to BAP. Hence optimization of dose of shoot multiplication hormone and duration of its exposure was essential.

A similar observation was recorded for root length of regenerated shoots from both EA and DCN; there was a decrease in average root length with an increase in duration of exposure of explants to BAP in shoot regeneration media. The trend was similar in both explants under both rooting conditions viz. in absence of rooting hormone and presence of IAA at 1 mg L^{-1} (Table 4). The highest average root length was recorded in explants exposed to BAP for 20 days and with IAA as rooting hormone in both explants DCN (7.14 ± 0.36) and EA (8.48 ± 0.51). Overall, the maximum root length was obtained in EA explants exposed to 20 days of BAP and to IAA in rooting media.

The rooted plantlets were gently taken out from the culture vessels, washed and transferred to plastic cups containing sterile soilrite and covered with polythene bags to ensure high humidity and

placed in controlled environment. After 2 weeks, the polybags were gradually removed and plantlets were transferred to the greenhouse. Subsequently, the plantlets were established in larger pots. The percentage of successful hardening recorded was 72-80%.

CONCLUSION

The present study demonstrates a reproducible protocol for *in vitro* plantlet regeneration in mungbean cultivar Samrat using dicotyledonary node and embryonic axis as explants. An increased regenerative capacity of EA explants by adventitious shoot formation in 1.0 mg L^{-1} BAP, its subsequent elongation and rhizogenesis was demonstrated.

It is also proposed that *in vitro* multiple shoot regeneration using EA as explants can pave way for a genotype independent regeneration system as EA is largely a meristematic tissue amenable for both *in vitro* regeneration and possibly genetic transformation studies using contemporary techniques like genome editing.

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CONFLICT OF INTEREST

The authors declare no competing financial/personal interest related to this study.

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