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In vitro regeneration from callus and cell suspension cultures in Indian mustard [*Brassica juncea* (Linn.) Czern & Coss]

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Abstract *Brassica juncea* is a crucial mustard species cultivated in India for edible oil. An efficient and reproducible plant regeneration protocol is established from callus using mature cotyledons and seeds as an explants and embryogenic cell suspension cultures dervied from embryogenic fraible calli in present investigation. The best callus induction was obtained with MS basal media fortified with 3.0 mgl^{-1} 2, 4-D. Further regeneration *via* embryogenesis/ organogenesis was attained with supplementation of 0.5 mgl^{-1} BAP in combination with 0.5-1.0 mgl^{-1}2,4-D. The superiority of mature seeds as explants was documented clearly points towards that this explant had higher morphogenic potential. Further, effectiveness of regeneration *via* callus was quite significantly correlated with genotype, type (s) and relative concentrations and combinations of plant growth regulators. These protocols may be employed in genetic transformation and *in vitro* selection purposes in future of elite genotype of Indian mustard.

Keywords: Indian mustard, Callus, Cell suspension culture, Somatic embryogenesis, Plantlet regeneration

Introduction

Brassica juncea L., is commonly consumed as spice and oil in India. Besides edible uses, it is also been used in areas of pesticide and biodiesel development. (Kashyap *et al*., 2019). India is an important rape seed mustard producing country in the world, occupying largest area. Traditional breeding advances can be employed to improve the new trait within the species. But conventional breeding programmes alone are not thriving sufficient in *Brassica* attributable to high degree of segregation upon cross-pollination and unavailability of appropriate wild germplasm. Enrichment of genetic variability

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through mutation, somaclonal variation, *in vitro* selection and protoplast fusion can be added in the production of disease resistant/tolerant plants to overcome incompatibility barriers as well as plants with better agronomic characters in *Brassica* spp. (Dubey and Gupta, 2014). Furthermore, *in vitro* regeneration and transformation may have potential to fulfill breeding needs (Khan *et al.,* 2010). In this context an efficient and reproducible plant regeneration protocol for different genotypes of *Brassica* is needed to be established for its exploitation in genetic transformation and *in vitro* selection experiments so as to produce genotypes of necessitated uniqueness.

Optimization of regeneration protocols has been described for most of the *Brassica* species by various researcher throughout world employing an array of explants including embryo (Zisan *et al*., 2015), cotyledons (Narasimhulu and Chopra, 1989; Ratan *et al*., 2001; Bhuiyan *et al*., 2009; Akmal *et al*., 2011; Gerszberg *et al*., 2015; Kumar and Srivastava, 2015; Dhania and Singh, 2016; Biswas *et al*., 2017), hypocotyls (Ratan *et al*., 2001; Bhuiyan *et al*., 2009; Akmal *et al*., 2011; Alam *et al*., 2014; Dubey and Gupta., 2014; Trivedi and Dubey, 2014; Gerszberg *et al*., 2015; Kumar and Srivastava, 2015; Dhania and Singh, 2016; Lone *et al*., 2017; Nasrin *et al*., 2017), leaf segments (Bhuiyan *et al*., 2009; Kumar and Srivastava, 2015), anther (Roy and Saha, 2006), protoplasts (Kirti and Chopra, 1989), cotyledonary petiole (Alam *et al*., 2014; Nasrin *et al*., 2017) and stem (Biswas *et al*., 2017) with diverging extent of triumph. However no intensive work has been done so far with genotypes belonging to this region to establish an efficient and reproducible plant regeneration protocol for further utilization in genetic transformation and *in vitro* selection purposes. An effort has been made to institute protocol for callus and cell suspension cultures pursued by plantlet regeneration, and envisaged the effect of genotype(s), explant(s) and plant growth regulators type (s) and their relative concentration on callus induction and cell suspension cultures leading to an efficient and reproducible plantlet regeneration.

Materials and methods

Experimental materials

Based on performances of diverse morpho-physiological and biochemical parameters (Shyam and Tripathi, 2019) of Indian mustard genotypes, seven promising genotypes *viz.*, CS54, GM2, RB50, NRCDR 2, Rohini, PM30 and LES39 with three explants *viz.,* hypocotyls, mature cotyledon and seeds were chosen for establishment of callus cultures and subsequently raising cell suspension cultures. Experiments were conducted at Plant Tissue Culture Laboratory, Department of Plant Molecular Biology &

Biotechnology, College of Agriculture, Rajmata Vijayraje Scindia Krishi Vishwa Vidalya, Gwalior, Madhya Pradesh, India. Experimental materials were acquired from Zonal Agricultural Research Station, Morena, RVSKVV, Gwalior Madhaya Pradesh., India (All India Coordinated Research Project on Rapeseed and Mustard) and IARI, New Delhi, India.

Culture media

To instigate with an initial experiment, two different strengthening of basal media *viz*., MS (Murashige and Skoog, 1962) and B_5 (Gamborg *et al.*, 1968) were determined better *in vitro* reaction. All through the itinerary of beginning investigations, MS basal medium was found more amenable than B_5 medium (data not presented). Consequently, MS medium was applied as basal medium for decisive work. Apart from MS basal micro and macro salts and vitamins two different auxins (alone), *namely:* 2, 4- Dichlorophenoxyacetic acid (2,4-D) and Nephtheleneacetic acid (NAA) and a cytokinin (alone) *viz*., 6-Benzyl amino purine (BAP) in varying concentrations as well as 2,4-D and NAA in combination with BAP, 30.0 gl^{-1} sucrose and 7.5 gl^{-1} agar powder was appended to fortify MS media for establishing callus cultures from hypocotyls, mature cotyledons and seeds explants and consequently raising embryogenic cell suspension cultures. The culture media were planned by making final volume to 1000 ml and pH was adjusted to 5.8 ±0.1 with 1N KOH solution. Subsequent to bending the pH, agar powder @ 7.5 gl^{-1} was added to the media as a semi-solidifying agent. Nevertheless, for cell suspension culture, agar powder was not incorporated in culture media. Warm culture media, still in liquid state were poured into baby food bottles (50-60 ml / bottle) and culture tubes (15-20 ml/ tube) pursued by autoclaving at 121^{o}C under 15 psi pressure for 20-25 min. Culture media combinations and other ingredients were short listed on the basis of work conducted by various scientists as well as groundwork experiments of this laboratory. Dehydrated basal medium, plant growth regulators and other ingredients procured from Hi-Media® Laboratories, Mumbai, India were utilized throughout the experimentation.

Establishment of callus cultures

Establishment of contamination free cultures is the foremost footstep in the establishment of an efficient and reproducible regeneration protocol for any plant species. In this stare, healthy and medium sized seeds were taken. The seeds were treated with Tween-20 for 10 min pursued washing with double distilled water. Then treated with 0.25% w/v Bavastin (Carbendazim) for 2 min tracked by a washing with distilled water. The seeds were further treated with70% ethanol for 2 min tracked by washing with double distilled water

pursued by immersing the seed in 0.1% mercuric chloride ($HgCl_2$) for 1-2 min and finishing with sterilized double distilled water. About 7-8 seeds were transferred aseptically with the help of forceps to each culture bottle (7-8 cm diameter) containing MS medium under aseptic condition and incubated at 25 ± 2 ℃ under photoperiod of 16 hours light and 8 hours dark. Three explants *viz*., hypocotyls, mature cotyledons and seeds were utilized for culture establishment. About 0.8-1.0 cm long pieces of hypocotyls obtained from10 to 15 days old seedlings were cut with the aid of sterile blades. Mature cotyledon explants (0.1 cm) from *in vitro* seedling and 1 to 2 mm seeds were also taken. All cultures were incubated in racks inside a culture room with controlled conditions of light, temperature and humidity. The cultures were exposed to photoperiodic cycle of 16 hours cool white fluorescent light and 8 h darkness at 25 ± 2 ℃ and 70% RH.

Establishment of cell suspension culture and induction of somatic embryogenesis

For establishing embryogenic cell suspension cultures, ~2.0 g, six to eight weeks-old morphgenic calli acquired from mature cotyledons and seed cultures were transferred to 250 ml Erlenmeyer flasks containing 50 ml of MS liquid medium. Callus pieces were strained through a stainless-steel mesh (1mm) and were agitated on a horizontal shaker (120 rpm) at 25±2°C under the complete darkness. After 15 days the cultures were sieved aseptically to remove large clumps and 10 ml filtrate was added with 40 ml of fresh medium with replacing the old suspension for subculturing. Relative growth rate was computed on the basis of increment in fresh weight after culturing of morphogenic friable calli on different fortifications of MS liquid medium after 4-5 weeks of primary culture. Cell cultures were examined microscopically within 15 to 35d for somatic embryoiod/ cell clumps induction and determination of developmental pathways.

Plantlet regeneration

Morphogenic calli obtained from callus cultures and cell clumps/embryoids of 2.0 to 6.0 mm Ø gained from 6-8 weeks old liquid suspension cultures were placed onto fortified semi-solid MS regeneration medium amended with BAP alone in addition to combinations with NAA and 2,4-D, 20.0 gl^{-1} sucrose and 7.5 gl^{-1} agar powder. Cultured baby food bottles /culture tubes were subjected to 25±2^{o}C temperature and photoperiod regimes of 60 µ mol m^{-2} s^{-1} luminance provided by cool fluorescent tubes for 16 hr. Per cent of plantlet regeneration was calculated as percentage of calli/cell clumps/embryoids with shootlets from total calli/cell clumps/embryoids plated.

In vitro rooting of regenerants

Regenerants were consequently subjected to MS medium supplemented with different concentrations of IBA (indole-3-butyric acid), 15.0 gl^{-1} sucrose and 7.5 gl^{-1} agar powder for root formation. For rooting, reduced level of sucrose was used on the earlier work conducted by various scientists along with previous experience of this laboratory.

Acclimatization of regenerants

Plantlets with sufficient roots were removed from the culture medium carefully, washed thoroughly till complete agar media was confiscated from the plantlets surface. The rooted plantlets were transferred to the nursery pot containing vermiculate, FYM and sand (1:1:1) for 30 days with maintaining light, humidity and temperature under greenhouse conditions. After 30 days well developed regenerants were transferred in nethouse for 45 days before transfer to field.

Microscopy and photography

Stereo zoom microscope was used for microscopy of callus and somatic embryos of different developmental stages. Photography was taken by Nikon camera of 16 megapixel.

Experimental design and data analysis

Completely Randomized Design (CRD) was used to find out the implication of different genotypes. Each treatment was consisting of two replications. Per replication at least 100-120 explants were cultured on each media of all seven genotypes. The data were analyzed as per method suggested by Snedecor and Cochran (1967).

Results

The analysis of variance (Table 1-5) revealed highly significant ($p<0.01$) differences among the response of different genotypes in terms of different parameters investigated. It indicates the presence of the considerable amount of variability amongst different genotypes along with culture media combinations.

In this examination, by using hypocotyls as an explant for callus induction from cut edges with diverse genotypes, delayed response was evident. Calli of very small size (Figure 1. A-B) was induced that eventually died when cultured on

MS medium supplemented with 3.0 mgl^{-1} 2,4-D (data not presented). From cultured mature cotyledons, callus induction was started usually from the cut ends (Figure 1. C-E). Higher *in vitro* response in terms of callus induction was evident on MS medium supplemented with 3.0 mgl^{-1} 2, 4-D. Among seven genotypes, genotype NRCDR2 was found to be the most responsive in terms of callus induction (%) resulting with white cream light in colour and compact in texture tracked by genotypes, GM2, RB50, CS54, Rohini, LES39 and PM30.

Mature seeds also were inoculated on MS media fortified with 2, 4-D in range of 1.0- 4.0 mgl^{-1} demonstrated varied response (Table 1). Higher *in vitro* retort in terms of callus induction was very similar to mature cotyledons explants attained on MS medium supplemented with 3.0 mgl^{-1} 2, 4-D (Figure 1. F-H). Genotype GM2 was proved to be the most responsive for callus induction among seven genotypes where white brown in colour and friable in texture callus was induced. Among seven genotypes, callus initiation frequency was evidenced in order of GM2, RB50, CS54, PM-30, Rohini, LES39 and NRCDR2.

To improve callusing and morphogenic potential, mature seed explant subsequently cultured on MS medium amended with 3.0 mgl^{-1} 2,4-D in combinatin with 0.5 mgl^{-1}BA and elevated response documented. It showed differential response in terms of callus induction followed by shootlet development (Table 2). Genotype RB50 was proved to be the most responsive for callus induction among seven genotypes where white and light in colur and compact in texture callus was induced. Among seven genotypes, callus initiation frequency was evidenced in order of RB50, PM30, CS54, Rohini, GM2, LES39 and NRCDR2.The best result for morphogenic calli formation was evident with genotypes PM30 and PM30. The degree of morphogenetic response was tracked by genotypes in order of GM2, PM30, RB50, Rohini, CS54, NRCDR2 and LES39. Consecutively to enhance shoot regeneration efficiency, mature seed explants were subsequently transferred to MS media fortified with BAP in range of 0.5-2.0 mgl^{-1}. The best response was evidenced with application of 0.5mgl^{-1} BAP. Genotypes PM30 chased by RB50 were showed higher degree of shoot proliferating efficiencies (Figure 1. K-N). The shoot differentiation among seven genotypes was in order of PM30 >RB50 > GM2 > Rohini > CS54 >NRCDR2 >LES39 (Table 2). In regard to genotypic response, among seven genotypes, genotype PM30 was proved to be superior for number(s) of shoots/explants intimately pursued by genotypes NRCDR2 and CS54. While genotypes GM2 and LES39 proliferated shootlets in intermediate numbers and genotypes *viz.*, RB50 and Rohini formed shootlets in lesser numbers. Shootlet of higher length was recovered from genotype PM30 intimately trailed by LES39, CS54, RB50, GM2, NRCDR2 and Rohini (Table 2).

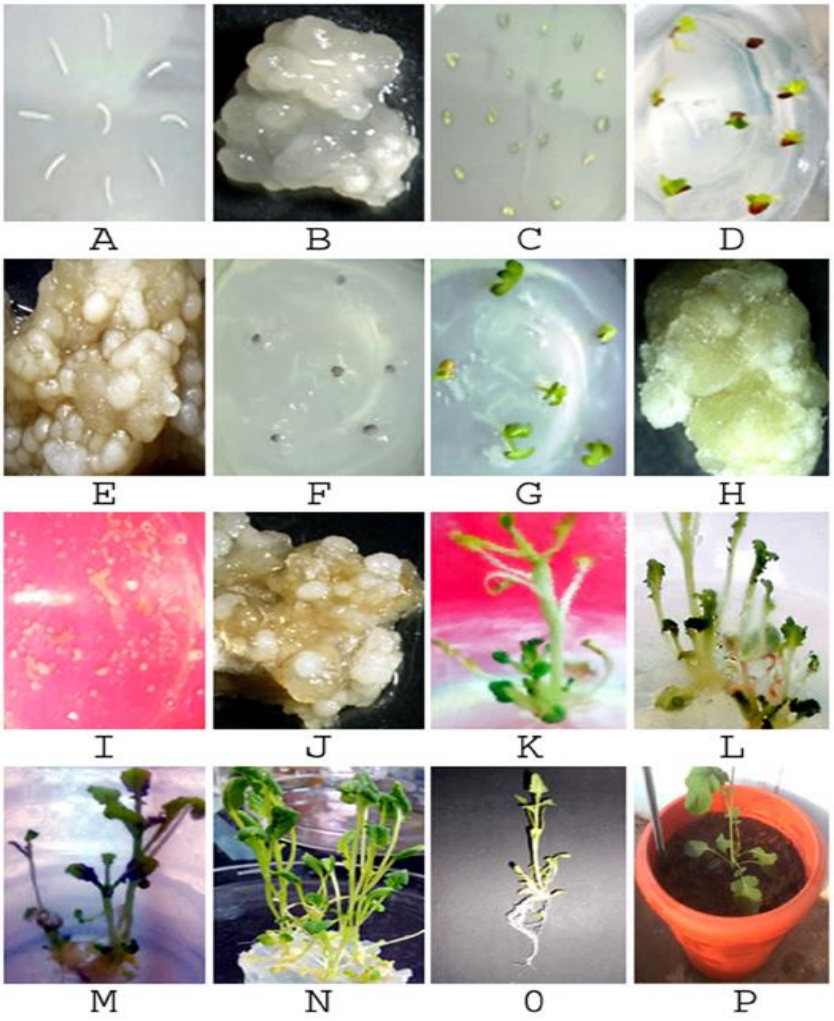

Figure 1. Plant regeneration from callus and cell suspension cultures in Indian mustard: **A.** Cultured hypocotyls after 7-10 days in culture; **B.** Callus induction from cultured hypocotyls; **C.** Cultured mature cotyledons after 7-10 days in culture; **D.** Cultured mature cotyledons after 14-21 days in culture; **E**. Callus induction from cultured mature cotyledons; **F.** Cultured mature seeds after 7 days in culture; **G.** Cultured mature seeds after 14-21 days in culture; **H.** Callus induction from cultured mature seeds; **I.** Initiation of cell clumps and embryoid formation from cell suspension cultures ; **J.** Globular stage somatic embryoid formation in liquid medium; **K.** Single shoot formation *via* direct organogenesis**; L.** Multiple shoot formation *via* direct organogenesis; **M-N.** Multiple shoot formation *via* indirect organogenesis; **O**. Rooting of regenerants and **P.** Hardening of regenerants in greenhouse

Table 1. Effect of genotypes on callus induction from cultured mature cotyledons and seed on MS3D (MS+ 3.0mgl^{-1}2,4-D) medium

S. No.	Genotypes	Mature cotyledons		Mature seed	
		Callus induction (%)	Callus features	Callus induction (%)	Callus features
1.	CS54	81.80	Dark brown and friable	72.72	Cream and light compact
2.	GM2	83.30	Cream and friable	83.33	White brown and friable
3.	RB50	81.30	White cream and friable	77.58	White cream, friable and granular
4.	NRCDR2	84.60	White cream and light compact	51.11	White and friable
5.	Rohini	80.00	Brown, friable and granular	69.23	White cream and friable
6.	PM30	50.00	Cream and light compact	71.11	White and loose
7.	LES39	71.4	Brown and friable	65.78	White brown and loose
C.D. (0.05)		**0.255**		**0.070**	
SE(m)		**0.072**		**0.020**	
SE(d)		**0.102**		**0.028**	
C.V.		**0.134**		**0.040**	

For raising embryogenic cell suspension culture, ~ 2.0 g embryogenic callus having good, friable nature and white brown in colour were subsequently transferred to liquid MS medium amended with 3.0 mgl^{-1} 2,4-D. Friable calli when agitated were easily broken and dispersed into clumps of ~ 2.0-5.0 mm Ø sizes. Further, agitation fragmented these clumps into small cell aggregates (Figure 1. I-J). After 40- 45 days, effect of genotypes on cell growth was recorded. Maximum relative growth rate was attained by genotypes GM2 chased by RB50, Rohini, PM30, NRCDR2, CS54 and LES39 (Table 3). Embryogenic cell suspension cultures showing embryoids of two developmental stages *i.e*., globular and heart shaped (Figure 1. J). After transferring cell clumps/embryoids on different concentrations and combinations of auxins and cytokinins, plantlet regeneration was attained on MS media supplemented with BAP and NAA from genotype PM30 trailed by genotypes Rohini, RB50, CS54, GM2, NRCDR2 and lowest in LES39. However, in combination of BAP with 2, 4-D, maximum plantlet regeneration was recorded form the genotype GM2 tracked by genotypes PM30, CS54, RB50, LES39, NRCDR2 and Rohini (Table 4).

Table 2. Effect of genotypes on callus induction, formation of morphogenic calli and plantlet regeneration efficiency from cultured mature seed on MS.5B (MS+ 0.5 mgl^{-1}BAP) medium

S. No.	Genotype	Callus induction (%)	Callus features	Morphogenic calli (%)	Shoot regeneration (%)	Average Number(s) of shoots/explants	Mean shoot length
1.	CS54	87.40	Cream and compact	76.60	74.00	8	6.5
2.	GM2	83.30	White and compact	85.60	75.50	6	6.0
3.	RB50	88.80	White and light compact	83.20	80.00	5	6.5
4.	NRCDR2	75.00	White and compact	66.50	57.70	8	5.5
5.	Rohini	85.70	Light green and compact	80.20	74.20	4	3.3
6.	PM30	87.50	Cream and light compact	85.00	82.00	9	7.5
7.	LES39	71.40	Cream and light compact	60.00	56.60	6	7.2
C.D. (0.05)		**0.198**		**0.641**	**0.212**	**0.203**	**0.203**
SE(m)		**0.056**		**0.182**	**0.062**	**0.060**	**0.060**
SE(d)		**0.080**		**0.257**	**0.088**	**0.085**	**0.085**
C.V.		**0.100**		**0.334**	**0.115**	**1.262**	**1.409**

Table 3. Effect of genotypes on initiation of embryogenic cell suspension cultures from callus raised from cotyledon explants on MS3D (MS+ 3.0 mgl^{-1}2,4-D) liquid medium

S.No.	Genotypes	Embryogenic cell suspension culture	
		Increment in fresh weight (FW in g) *	**Relative growth rate (RG in%)**
1	CS54	5.90 ± 0.31	295
2	GM2	9.30 ± 0.39	465
3	RB50	9.10 ± 0.36	455
4	NRCDR2	6.50 ± 0.27	325
5	Rohini	8.40 ± 0.34	420
6	PM30	4.70 ± 0.21	235
7	LES39	7.30 ± 0.30	365

- Evaluation was made after 45 days in culture.
- **FW**: Fresh Weight; **RG**: Relative growth.
- *****Mean of five readings ± standard deviation.
- Initial fresh weight was taken 2.0 g friable callus per flask containing 50ml liquid media

Table 4. Effect of genotypes and plant growth regulators on plantlet regeneration efficiency from cultured cell clumps/ embryoids obtained from cell suspension cultures

S.No.	Culture media Genotypes	Shoot regeneration (%)	
		MS.5D.5B (MS+0.5mgl^{-1}2,4-D+0.5mgl^{-1}BAP)	MS.5N.5B (MS+0.5mgl^{-1} NAA+0.5mgl^{-1}BAP)
1.	CS54	30.00	37.50
2.	GM2	50.00	33.30
3.	RB50	28.50	42.80
4.	NRCDR2	20.00	25.00
5.	Rohini	16.60	44.40
6.	PM30	33.30	66.60
7.	LES39	25.00	14.20
C.D. (0.05)		**0.193**	**0.328**
SE(m)		**0.057**	**0.096**
SE(d)		**0.080**	**0.136**
C.V.		**0.276**	**0.362**

Table 5. Effect of genotypes on *in vitro* rooting

S.No.	Genotypes	Root induction (%)	Average number(s) of roots/explant	Mean root length
1.	CS54	75.00	7.00	4.50
2.	GM2	77.34	5.00	5.50
3.	RB50	66.6 0	4.00	6.00
4.	NRCDR2	68.9 0	4.00	4.70
5.	Rohini	70.6 0	3.00	3.80
6.	PM30	65.60	5.00	5.80
7.	LES39	63.84	6.00	5.50
C.D. (0.05)		**0.180**	**0.203**	**0.280**
SE(m)		**0.053**	**0.060**	**0.082**
SE(d)		**0.075**	**0.085**	**0.116**
C.V.		**0.091**	**1.695**	**2.287**

Observations were recorded after 30 days transferring in rooting media

The best result for root formation was recorded on MS medium supplemented with 0.5 $mg1^{-1}$ IBA (Table 5). Among seven genotypes, genotype GM2 and CS54 were formed maximum roots chased by genotypes *viz*., NRCDR2, Rohini, RB50, PM30 and LES39. Average number (s) of roots/explants was attained with genotypes CS54 tracked by LES39, GM2, PM30, RB50, NRCDR2 and Rohini, while root of higher length was noted maximum in genotypes RB50 itimately pursued by PM30, GM2, LES39, NRCDR2, CS54 and Rohini.

Discussion

Underneath suitable and defined circumstances of nutrient media, type (s) and concentration (s) of plant growth regulators, it is promising to induce callus from any explants received from any living part of plants. However, the frequency of callus induction varied with kind (s) and magnitudes (s) of plant growth regulators along with nature of explants. Present investigation was carried out in sequence to select responding explants type (s), genotype (s) and concentration (s) and combination (s) of plant growth regulators for establishment of callus and cell suspension cultures tracked by plantlet regeneration. In our experiment cultured hypocotyls had not been responded well, while, Dubey and Gupta (2014) attained callus induction from hypocotyls explants on medium containing $0.5mgl^{-1}$ 2, 4-D in combination with $0.5mgl^{-1}$ NAA. Subsequently Lone *et al*. (2017) reported formation of callus in higher frequencies from cultured hypocotyls with supplementation of culture medium with 2,4-D in range of 0.5- 2.5 mgl^{-1}.

Higher *in vitro* response, from cultured mature cotyledons for callus induction was evinced on MS medium supplemented with 3.0 mgl^{-1} 2, 4-D. Earlier Akmal *et al*. (2011) and Lone *et al*. (2016) also documented maximum callus induction with application of 2,4-D in range of 2.0-2.5 mgl^{-1}. Subsequently Alam *et al*. (2014) and Nasrin *et al*. (2017) have reported that nature and color of callus was significantly influenced by concentrations of exogenous levels of 2, 4-D could be an accordance of present investigations. However, plantlet regeneration from these calli has not been acquired in optimum frequencies. In contrast to findings of Thakur *et al*. (2013) who have established an efficient plant regeneration protocol in higher frequencies in *Brassica juncea* var. NRCDR2 by culturing cotyledonary petiole explants. To accomplish better *in vitro* response, mature seeds were also considered as an explant.

Higher *in vitro* retort regarding callus induction was evidenced from mature seeds as compared to mature cotyledons explants attained on MS medium fortified with 3.0 mgl^{-1}. Analogous to present inspection, Dubey and Gupta (2014) also documented callus induction pursued by shoot proliferation using seedling explants. Response to callus induction applying 2, 4-D was more pronounced in terms of type, nature and colour of callus proliferated. However, plant regeneration has not been evidenced on medium amended with 2, 4-D as sole from cultured mature seeds in optimum frequencies. Consecutively to augment shoot regeneration efficiency, mature seed explants were subsequently inoculated on MS media fortified with BAP. The best response was confirmed with application of 0.5mgl^{-1}BAP. The present findings are supported by previous verdicts of Thakur *et al*. (2013). These workers also advocated higher numbers of shoots per explant with fortification of regeneration medium with 1.0 mgl^{-1} BAP in combination with 0.1 mgl^{-1} NAA and 10.0 mgl^{-1} $AgNO_3$. Parrell findings also documented by Gerszberg *et al*. (2015) as they attained shoot regeneration in higher frequencies with supplementation of 8.88 μMBAP in combination with 0.53 μM NAA. Similarly, Kumar and Srivastava (2015) recovered shootlets in higher numbers with application of 3.5 mgl^{-1} BAP in combination with 0.019 mgl^{-1} NAA. While Ahmad *et al*. (2016) found maximum shoot induction with 3.0 mgl^{-1} BAP as alone. Although, Lone *et al*. (2016) reported shoot regeneration with 5.0 mgl^{-1} BAP in combination with 0.5 mgl^{-1} 2, 4-D.

The present results sustained by former statements undoubtedly point towards role of BAP concentration for initiation of callus and morphogentic calli and further shoot differentiation. Failure to induce organogenesis from callus tissue derived from hypocotyls and mature cotyledons explants is quite evident. Shoot organogenesis was observed from callus mass proliferated from mature seed explants. The inclusion of BAP was found the most effective for inducing gamogenesis. This noticeably shows that cytokinin BAP utmost required to induce shoot differentiation from callus cultures. Callus having 1-2 green shoot primordia illustrated an array of morphogenetic changes during subsequent growth and development. The green shoot primordia appeared on surface of callus and transferred to fresh media resulted 1-4 shoots after 4 weeks. The removal of these shoots and subsequent transferring on MS media supplemented with BAP resulted multiple shoot formation, elongation and development. The present observations are supported by earlier observations, where use of BAP evoked shoot differentiation. Munir *et al*. (2008) reported callus induction pursueed by shoot differentiation with application of 1.0 mgl^{-1}

BAP. Trivedi and Dubey (2014) with 0.5, mgl^{-1} BAP, Ahmad *et al*. (2016) with 0.4 mgl^{-1} BAP, Nasrin *et al*. (2017) with 1.0 mgl^{-1} BAP and Khan *et al*. (2010) and Biswas *et al*. (2017) with 2.0 mgl^{-1}BAP. The present results regarding BAP influenced shoot differentiation are supported by earlier reports of Thakur *et al*. (2013) and Kumar and Srivastava (2015) for numbers of shoots proliferating explants, Dubey and Gupta (2014) and Dhania and Singh (2016) for numbers of shoots per explant and shoot length and Abrha *et al*. (2013) for shoot length where BAP has influenced better shoot regeneration.BAP promotes adventitious buds in excised organs and tissues *in vitro* (Bhojwani and Johri, 1971). Present finding also revealed that BAP favored adventitious multiple shoots. The regeneration of multiple adventitious shoot buds in different concentrations of BAP has also been adressed in case of soybean (Tiwari *et al*., 2005; Mishra *et al*., 2021), liquorice (Sharma *et al*., 2010), citrus (Vibhute *et al*., 2012) and *Withania* (Saini and Jaiswal, 2000; Jhankare *et al*., 2011).

Embryogenic callus was transferred to liquid media supplemented with 3.0 mgl^{-1} 2,4-D. During a prelude experimentation, it was examined that in liquid culture, calli could not disintegrate to form suspension of cells or small cell clumps, could probably owing to higher lignifications. Comparable surveillances have been made and addressed by Tiwari *et al*. (2007) for onion, Jhankare *et al*. (2011) for *Withania*, Bairwa *et al*. (2012) for muskmelon and Sharma *et al*. (2018) for grape cell suspension cultures in liquid medium. To obtain small cell clumps, calluses were agitated mechanically. Friable calli when agitated were easily broken and dispersed into clumps of ~ 2.0-6.0 mm Ø. Further, agitation fragmented these clumps into small cell aggregates. Strong genotypic differences were documented regarding relative cell growth during present investigation as earlier were experimented by various scientists including Tiwari *et al*. (2007) for onion, Jhankare *et al*. (2011) for *Withania*, Bairwa *et al*. (2012) for muskmelon, Sharma *et al*. (2018) for grape and Mishra *et al*. (2021) for soybean liquid cultures. Liquid medium fortified with 3.0 mgl^{-1} 2,4-D facilitate higher growth rate is an accordance to verdicts of Patidar *et al*. (2017) as recorded highest growth rate of embryogenic tissues with application of 2.0-3.0 mgl-1 2,4-D in combination with 0.5 mgl-1 BAP. On transferring cell clumps/embryoids embryogenic cell suspension cultures showed embryoids of two developmental stages *i.e*., globular and heart shaped. The present results are in line with earlier observations of Akmal *et al*. (2011) who also documented globular and heart shaped embryoids development after ten days of culture on embryo development medium. Higher plantlet regeneration was achieved on MS media supplemented with BAP and NAA as compared to

BAP in combination with 2, 4-D suggested that NAA is more useful than 2,4-D in terms of plantlet regeneration. This is an accordance of findings of Sharma *et al*. (2018) for grape and Mishra *et al*. (2021) for soybean who also reported efficient plantlet regeneration form cell clumps/embryoid acquired from embryogenic cell suspension culture with addition of each of 0.5 mgl^{-1} of BAP, TDZ[1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron)] and NAA.

In regard to genotypic response strong genotypic responses were evident for regeneration of shootlets. Within each media treatment, the variation observed for the *in vitro* response resulted from genetic and physiological differences between genotypes as the culture condition were standardized. In addition, a difference between the endogenous hormone levels (Norstog, 1970) amongst the genotypes also contributes to variability. Earlier genotypic responses have also been observed in soybean (Tiwari *et al*., 2005), citrus (Vibhute *et al*., 2012) and an array of other crop species (Tripathi *et al*., 2019) for *in vitro* response.

An efficient root formation was observed on MS medium supplemented with 0.5mg1^{-1}IBA. Alam *et al*. (2009; 2014) supported the present observations as they realized higher root induction on medium supplemented with 1.0 mgl^{-1} IBA in combination with 0.5mgl^{-1}. Biswas *et al*. (2017) also attained highest percentage of root induction with supplementation of 2.0 mgl^{-1} IBA in combination with 0.5 mgl^{-1} NAA whereas Nasrin *et al*. (2017) documented an efficient rooting on medium amended with 1.0 mgl^{-1} IBA. Strong genotypic differences were observed for different phases of *in vitro* rooting is an accordance of earlier reports have been advocated by Khan *et al*. (2010) for number (s) of roots per shoot and Abrha *et al*. (2013) and Thakur *et al*. (2013) for number (s) of roots per shoot and root length. Among auxins, IBA was the most effective than any other synthetic auxins in the most of the cases apparently because it is not destroyed by IAA oxidase or other enzymes and therefore persists longer. The above results are in conformity with the earlier findings of Vibhute *et al*. (2012) and for citrus and Tripathi *et al*. (2019) for various *species*.

To Sum up, the results of the present investigation extend that the best callus induction was obtained with MS media supplemented with 2,4-D. Further regeneration *via* organogenesis was accomplished with application of BAP in combination with lower concentration of an auxin. The superiority of mature cotyledons and seeds as explants was observed during present study clearly points towards that these two explants had higher morphogenetic potential. Additionally, effectiveness of regeneration *via* callus was quite

significantly correlated with genotype, explants type and relative concentrations and combinations of plant growth regulators. Furthermore, it was evidenced during present investigations that type and that quantity of callus produced was positively correlated with the subsequent shoot induction. This protocol provides a successful and rapid technique that can be used for mass *in vitro* propagation of elite genotype and could permit genetic transformation studies, *in vitro* selection at cell level for screening useful somaclonal variants which so far have been limited in Indian mustard.

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References

Abrha, G. T., Mekbib, F. and Admassu, B. (2013). *In vitro* plant regeneration from callus of hypocotyls and cotyledonary explants in Ethiopian mustard (*Brassica carinata* A. Braun) yellow dodolla cultivar. Asian Journal of Plant Sciences, 12:262-270.

Ahmad, K., Jalal, A., Rajab, H., Ullah, M. and Khan, M. S. (2016). Screening of promising B*rassica napus* L. genotypes for callus induction and regeneration. International Journal of Biology and Biotechnology, 13:203-215.

Akmal, M., Nafis, T., Mirza, K. J., Alam, P., Mohammad, A., Mujib, A. and Abdin, M. Z. (2011). High frequency somatic embryogenesis in mustard crop (*Brassica juncea*L.) cv. Pusa Jai kisan: Microscopic and histological analyses. Australian Journal of Crop Science, 5:1783-1789.

Alam, M. A., Haque, M. A., Hossain, M. R., Sarker, S. C. and Afroz, R. (2009). Haploid plantlet regeneration through anther culture in oilseed *Brassica species*. Bangladesh Journal of Agricultural Research, 34:693-703.

Alam, S. S., Khaleda, L. and Al-Forkan, M. (2014). *In vitro* plant regeneration from hypocotyl and cotyledon explants of bari sarisha-13 (*Brassica napus* L.). Asian Journal of Agriculture and Food Science, 2:39-45.

Bairwa, S. K., Tripathi, M. K., Kushwah, S. S., Tiwari, S. and Baghel, B. S. (2012). Somatic embryogenesis and plantlet regeneration from embryogenic suspension culture in muskmelon (*Cucumis melo* L). Indian Journal of Horticulture, 69:338-347.

Bhojwani, S. S. and Johri, B. M. (1971). Morphogenetic studies on cultured mature endosperm of *Croton bonplandianum*. New Phytologist, 70:761-766.

Bhuiyan, M. S. U., Min, S. R., Choi, K. S., Lim, Y. P. and Liu, J. R. (2009). Factors for high frequency plant regeneration in tissue cultures of Indian mustard (*Brassica juncea* L.). Journal of Plant Biotechnology, 36:137-143.

Biswas, S. K., Tareq, M. Z., Ahmmed, S., Hoque, A. B. M. Z. and Rahman, M. T. (2017). Callus derived regeneration of some selected *Brassica* genotypes. The Agriculturists, 15:01-10.

Dhania, G. and Singh, R. P. (2016). Standardization of *in vitro* shoot regeneration protocol for Indian mustard, *Brassica juncea (L)* using cytokinin, BAP as inducer. Biological Insights, 1:5-12.

Dubey, S. K. and Gupta, A. K. (2014). Callus induction and shoot proliferation from seedling explants of different mustard genotypes. International Journal of Current Microbiology and Applied Sciences, 3:858-864..

Gamborg, O. L., Miller, R. A. and Ojima, K. (1968). A nutrients requirement of suspension cultures of soybean root cells. Experimental Cell Research, 50:151-158.

Gerszberg, A., Hnatuszko-Konka, K. and Kowalczyk, T. (2015). *In vitro* regeneration of eight cultivars of *Brassica oleracea* var. capitata. *In Vitro* Cellular & Developmental Biology, 51:80-87.

Jhankare, A., Tiwari, G. Tripathi, M. K., Baghel, B. S. and Tiwari, S. (2011). Plant regeneration from mature cotyledon, embryo and hypocotyls explants of *Withania somnifera* (L.) Dunal. Intenational Journal of Agricultural Technology, 7:1023-1035.

Kashyap, S., Tharannumm, S. and Taarinim R. (2019). Influence of formulated organic plant tissue culture medium in the shoot regeneration study of *Brassica juncea* (L.) -Indian mustard. Journal of Plant Biotechnology, 46:114-118.

Khan, M. M. A., Khan, A. H., Robin, A. B. M., Nazim-Ud-Dowla, M. A. N., Talukder, S. K. and Hassan, L. (2010). *In vitro* regeneration potentiality of *Brassica* genotypes in differential growth regulators. *Bangladesh.* Journal of Agricultural Research, 35:189-199.

Kirti, P. B. and Chopra, V. L. (1989). Rapid plant regeneration from organogenesis and embryogenesis from protoplasts of *B. juncea*. Plant Cell, Tissue and Organ Culture, 20:65-67.

Kumar, P. and Srivastava, D. K. (2015). High frequency organogenesis in hypocotyl, cotyledon, leaf and petiole explants of broccoli (*Brassica oleracea* L. var. italica), an important vegetable crop. Physiology and Molecular Biology of Plants, 21:279-285.

Lone, J. A., Gupta, S. K., Wani, S. H., Bhat, M. A. and Lone, R. A. (2016). *In vitro* regeneration studies in *Brassica napus* with response to callus induction frequency and regeneration frequency. International Journal of Agriculture, Environment and Biotechnology, 9:755-761.

Lone, J. A., Gupta, S. K., Wani, S. H., Sharma, M., Lone, R. A. and Shikari, A. B. (2017). Efficient callus induction and regeneration in *Brassica juncea* for environment friendly agriculture. International Journal Pure and Applied Bioscience, 5:135-141.

Mishra, N., Tripathi, M. K., Tiwari, S., Tripathi, N., Ahuja, A., Sapre, S. and Tiwari' S. (2021). Cell suspension culture and *in vitro* screening for drought tolerance in soybean using poly-ethylene glycol. Plants, 10:517-536.

Munir, M., Rashid, H., Rauf, M., Chaudhry, Z. and Bukhari, M. S. (2008). Callus formation and plantlets regeneration from hypocotyl of *Brassica napus* by using different media combinations. Pakitan Journal of Botany, 40:309-315.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15:473-497.

Narasimhulu, B. S. and Chopra, V. L. (1989). Shoot regeneration from cotyledons of three diploid species of *Brassica*. Plant Breeding, 99:49-55.

Nasrin, F., Khaleda, L. and Forkan, M. A. (2017). *In vitro* plant regeneration from callus of hypocotyls and cotyledonary explants of some indigenous mustard varieties. Indian Journal of Plant Sciences, 6:21-30.

Norstog, K. (1970). Induction of embryo like structures by Kinetin in cultured barley embryos. Developmental Biology, 23:665-670.

Patidar, S. L., Tripathi, M. K., Tiwari, G., Patel, R. P. and Ahuja, A. (2017). Standardization of an efficient and reproducible embryogenic cell suspension culture protocol for production of secondary metabolites in *Plumbago zeylanica* Linn. Ecol. Env. Conserv., 23:373-380.

Ratan, S., Ranwah, B. R. and Singh, R. P. (2001). Tissue culture studies in Indian mustard *(Brassica juncea* L. Czern & Coss). Indian Journal of Agricultural Research, 35:259-262.

Roy, A. and Saha, P. K. (2006). Isolation of low erucic acid-containing genotype of Indian mustard (*Brassica juncea* Czern. and Coss.) through F_1 hybrid anther culture. African Journal of Biotechnology, 5:2092-2096.

Saini, R. and Jaiwal, P. K. (2000). *In vitro* multiplication of *Peganum harmala* –An important medicinal plant. Indian Journal of Experimental Biology, 38:499-503.

Sharma, D. K., Tripathi, M. K., Tiwari, R. Baghel, B. S. and Ahuja, A. (2018). Somatic embryogenesis and plantlet regeneration *via*embryogenic suspensions of grape (*Vitis vinifera* L.). Asian Journal of Microbiology, Biotechnology and Environmental Science, 20: S112-125.

Sharma, P., Tripathi, M. K. Tiwari, G. Tiwari, S. and Baghel, B. S. (2010). Regeneration of liquorice (*Glycyrrhiza glabr*a L.) from cultured nodal segments. Indian Journal of Plant Physiology, 15:1-10.

Shyam, C. and Tripathi, M. K. (2019). Biochemical studies in Indian mustard [*Brassica juncea* (Linn.) Czern & Coss] for fatty acid profiling. International Journal of Chemical Studies, 7:338-343.

Snedecor, G. W. and Cochran, W. G. (1967). Statistical methods. VI Ed. Oxford IBH Pub. Co. Delhi.

Thakur, A. K., Singh, B. K., Verma, V. and Chauhan, J. S. (2013). Direct organogenesis in *Brassica juncea* var. NRCDR-2 and analysis of genetic uniformity using RAPD markers. National Academy Science Letter, 36:403-409.

Tiwari, S, Tripathi, M. K., Khare, U. K. and Rana, R. (2007). Initiation of embryogenic suspension culture and regeneration in onion (*Allium cepa* L.). Indian Journal of Biotechnology, 6:100-106.

Tiwari, S. and Tripathi M. K. (2005). Comparison of morphogenic ability of callus types induced from different explants of soybean *(Glycine max* L. Merrill). Legume Research an Internationl Journal, 28:115-118.

Tripathi, M. K., Mishra, N., Tiwari, S., Singh, S., Shyam, C. and Ahuja, A. (2019). Plant tissue culture technology: sustainable option for mining high value pharmaceutical compounds. International plant Journal of Current Microbiology and Applied Sciences, 8:102-110.

Trivedi, N. and Dubey, A. (2014). Efficient callus regeneration and multiple shoot induction in *Brassica juncea* var. Pusa Jaikisan. Research Journal of Recent Sciences, 3:16-19.

Vibhute, M., Tripathi, M. K., Tiwari, R., Baghel, B. S. and Tiwari, S. (2012). Interspecific morphogenic ability differences in citrus. International Journal of Agricultural Technology, 8:625-638.

Zisan, S., Robin, A. H. K., Hoque, A. and Hossain, M. R. (2015). *In vitro* callus induction and plantlet regeneration is influenced by the maturity status of embryos of *Brassica rapa* varieties. Journal of Bioscience and Agriculture Research, 6:518-529.

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