Australian Journal of Crop Science AJCS

AJCS 5(13):1783-1789 (2011) ISSN:1835-2707

High frequency somatic embryogenesis in mustard crop (*Brassica juncea* L. cv. Pusa Jai kisan): Microscopic and histological analyses

M. Akmal[1], T. Nafis[1], K. J. Mirza[1], P. Alam[1], A. Mohammad[1], A. Mujib[2] and M. Z. Abdin[1]*

[1]Department of Biotechnology, Hamdard University, New Delhi-110062, India
[2]Department of Botany, Hamdard University, New Delhi-110062, India

***Corresponding author: mzabdin@rediffmail.com**

Abstract

A high frequency somatic embryogenesis has been established in mustard crop (*Brassica juncea* L. cv. Pusa Jai kisan), in which embryogenic calli were induced from hypocotyls and cotyledons of *in vitro* germinated seedlings. The hypocotyl derived embryogenic calli (HEC) were transparent and whitish, while cotyledon derived embryogenic calli (CEC) were creamy yellow in colour. Highest embryogenic callusing frequency (98%) was obtained in cotyledons on 2 mg/l 2, 4-D added MS medium. Hypocotyls and cotyledons derived calli were differentiated into somatic embryos at high frequency (90-100%) on 2 mg/l 2ip or 2 mg/l BAP amended medium. Embryo maturation occurred on the same embryo development medium, and germination was best achieved on 2.6 mg/l ABA amended medium. Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and histological studies revealed that the embryos had bipolar structure and developed mainly from the epidermis of explants. Furthermore, the embryonic tissues have stored bodies and numerous cell organelles. Various embryological stages are presented in this short communication. This protocol is much faster and took just six weeks to obtain complete plantlets.

Keywords: Embryogenesis, cotyledon, hypocotyl, callus, cytokinin, MS medium.
Abbreviations: BAP-6-benzylaminopurine, 2,4-D-2,4-dichlorophenoxyacetic acid, TDZ-thidizouron, 2ip-2-isopentenyladenine, SEM-scanning electron microscopy, Kin-Kinetin, TEM-transmission electron microscopy, ABA-abscisic acid, HEC-hypocotyl derived embryogenic calli, CEC-cotyledon derived embryogenic calli, DMRT-Duncan multiple range test, PGR-plant growth regulator, ANOVA-analysis of variance, MS-Murashige and Skoog's (1962) medium, SE-somatic embryogenesis.

Introduction

Brassica juncea L. belongs to the Brassicaceae family. It is a major oil seed crop of the Indian subcontinent and is an alternative source of canola quality oil (Stoutjesdijk et al. 1999). The crop is the best source of edible oils, having essential fatty acids with lowest amount of saturated fat. It also provides oil free meal to animals. *B. juncea* is rich in protein with well balanced aminogram (Agnihotri et al., 2004). It is highly susceptible to a variety of fungal diseases like white and yellow rusts and leaf rot that reduce yield and affect crop quality. Further, the crop is sensitive to various abiotic and biotic stresses. Hence, the primary objectives of research in this crop are to develop *B. juncea* plant tolerant / resistant to stresses and also produce desirable oil quality and yield. A large number of research reports are available on the genetic improvement of oilseed crops (Stoutjesdijk et al., 1999; Potts et al., 1999; Das et al., 2006). Somatic embryogenesis, a useful *in vitro* technique is often used in genetic engineering programme as it offers fast and efficient way of gene transfer. The same embryogenic system also allows recovery of whole plant in one step as opposed to organogenesis, where regenerated shoots need to be subsequently rooted (Thorpe, 1995). Moreover, somatic embryos (SEs) have often been used in developing synthetic seeds that are later used for storage, transport and transplantation (Mujib and Samaj, 2006). Earlier, attempts on induction of SEs in *Brassica juncea* L. were made by several workers (Kirti and Chopra, 1989; Sharma et al., 1991; Kumari et al., 1995, 2000) by using different explants viz. immature zygotic embryos, hypocotyls, young leaves, protoplasts and microspores. However, to the best of our knowledge, there is no report available on high frequency somatic embryogenesis and germination of SE in *B. juncea*. In this investigation, a detailed study of *B. juncea* embryogenesis was conducted in which the role of PGR and carbohydrate was evaluated. TEM, SEM and histological investigations were also conducted at different stages regarding *in vitro* embryogenesis.

Results

Callus induction

In present study, two different explant types (hypocotyls and cotyledons) were used. Both the cotyledon and hypocotyl explants showed embryogenic calli (98 and 88%) on MS, when fortified with 2,4-D (2 mg/l) and added with 0.5 or 1.5% sucrose (Table 1, Fig. 1a and b). Low frequency calli formation was also induced at higher concentration of 2,4-D (3, 5mg/l).The HEC were compact, transparent and white in colour, while CEC were compact and creamy-yellow in colour. The cut end of both the explants produced more embryogenic calli compared to the other parts of explants. Uncut sides became brownish and failed to produce calli.

Embryo development

The embryos (globular and heart shaped) were induced on both HEC and CEC within ten days of culture on embryo development medium. The frequency of SE development was very high (99.6%) in HEC on 2 mg/l 2ip amended MS medium. High embryo development frequency was also

observed in CEC (i.e.100%), when the embryogenic calli were cultured on MS and supplemented with BAP (2mg/l). The ultrastructural features provided by TEM observations revealed that the embryogenic cells contained smaller vacuoles, large nucleus (Nu) with numerous organelles and stored bodies. These features observed are very common, when a cell starts to convert into an embryo (Fig. 2A). In *B. juncea*, the most distinct feature is the presence of stored lipid bodies. The SEM studies showed the SEs as distinct bipolar structures with radicular and cotyledonary poles and these developed on the entire surface of the embryogenic calli. The globular and heart shaped embryos were developed in higher frequency than the torpedo and cotyledonary embryos on HEC, while the number of heart shaped embryos was more on CEC (Fig. 2B, C). Histological studies revealed that on upper epidermis of hypocotyl, SEs were mostly induced and appeared as bulging structures in which the cells of embryos were smaller and had densely stained cytoplasm. In the developing embryos, inner mass of cells were surrounded by an epidermis. This developing embryo outgrowth was observed on hypocotyl surface from all directions (Fig. 2D, E). The proliferation of SE was continued on these cytokinin containing medium, the globular embryos progressed to heart and then into torpedo. The torpedo embryos subsequently developed into cotyledonary embryos. Cotyledonary, torpedo and advanced heart shaped embryos were more frequently developed on 0.5mg/l TDZ and 2ip containing medium (Table 2 and 3, Fig c, d, e and f).

Embryo maturation and germination

The maturation of SEs occurred on the same embryo induction medium, when induced embryos were regularly sub-cultured after an interval of fifteen days. Maturation percentage of SE developed from HEC was 80% at 0.5mg/l BAP. Maturation percentage was also nearly the same on kinetin and TDZ amended medium. In CEC developed somatic embryos, the maximum maturation percentage was 85% and was noted on medium containing 0.1mg/l BAP or 0.1mg/l kinetin (Table 4, Fig. 1g). On cyokinins omitted medium, loose callusing occurred and induced embryos neither matured nor germinated at all (data not shown). At lower concentration of ABA, the embryo germination frequency was very low, while at higher concentrations embryo desiccation occurred. On 2.6mg/l ABA added medium, the germination frequency was quite high. Hence, it was selected and amended in germination medium. Best embryo germination (58%) was observed on 0.5mg/l TDZ + 2.6mg/l ABA in case of HEC, while the germination percentage of CEC embryos was even higher (60%) in the same medium. Embryos started to germinate within two weeks of culture on embryo germination medium (Table 5, Fig. 1h to m). The radicular ends gave rise to the roots, while shoots were developed from the cotyledonary ends.

Discussion

In the present study, a high frequency somatic embryo induction was reported in hypocotyl and cotyledon explants from five days old *in vitro* grown seedlings of *B. juncea.* It offers an excellent and fast method of plant regeneration of this valuable oilseed crop. Two different embryogenic calli were induced from two different explants of *B. juncea*, which could be differentiated with non-embryogenic calli in several respects. Embryogenic calli showed organized growth resulting in bipolar structure. Their protein profiles also differed and generally had embryo specific proteins (Sung and Okimoto, 1983). Reprogramming was also noted to be operative during embryogenesis for the development of whole plant from a single cell, thus demonstrating and reconfirming the concept of totipotency in higher plants (Nolan et al., 2003; Imin et al., 2004, 2005). In the present investigation of *B. Juncea,* embryogenic callus was induced on 2,4-D added medium. The use of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) for the induction of SEs on cultured explants was earlier reported and reviewed in angiospermic plants (Yantcheva et al., 1998; Raghavan et al., 2004; Raemakers et al., 2005) including *B. juncea* (Eapen et al., 1989) and other members of Brassicaceae i.e. *B. campestris* (Bhattacharya et al., 1980), *B. nigra* (Narasimhulu et al., 1992), *B. oleracea* (Pareek and Chandra, 1978) and *B. napus* (Turgut et al., 1998). In most cases embryo formation has primarily been observed by culturing explants on high or low concentration of 2, 4-D, either alone or with cytokinins (Gaj, 2004). In *Brassica*, the combined influence of auxins and cytokinins on induction of callus and somatic embryo was earlier described (Kirti and Chopra, 1989, 1990; Sharma et al., 1991; Kumari et al., 1995, 2000). Here, we evaluated the influence of various cytokinins on *in vitro* embryogenesis. We also studied the effect of sucrose on the induction of embryogenic calli in the medium supplemented with 2,4-D. The embryogenic calli obtained from hypocotyls and cotyledons in the above medium produced large numbers of SEs in a short period of time (3 weeks) on cytokinin containing medium. The use of cytokinin alone to obtain SEs from zygotic embryos was also established in a wide range of angiosperms (Maheswaran and Williams, 1984), where exogenous cytokinin was found to enhance the number of embryos in culture (Thorpe, 1995). The polarity once established in the cells, cytokinin present in the medium triggers cell division. Continuous divisions of cells produce different forms of embryos like globular, heart and torpedo. In our experiments, we noted that the presence of different cytokinins is essential for progression of embryo development (from induction to germination phase). In the absence of these cytokinins, a loose organized callus development occurred (data not shown) as the cytokinins are responsible for the establishment and maintenance of apical meristems of embryos (Sugiyama, 1999). We also observed that the embryo proliferation and maturation behaviour of SEs from two different explants, hypocotyls and cotyledons were nearly the same. The germination of cotyledon derived SEs was however, higher as compared to the hypocotyl derived SEs. It was reported that the developed embryos started to reserve metabolic deposition, and these reserve substances are good indicators of maturation (Bewley and Black, 1985). Availability of carbohydrate in medium also appears to be important for both embryo quality and embryo number during embryo development (Thorpe, 1995) and its range usually varies between 3-6%. In the present investigation, 3% sucrose was used in maturation as well as germination medium. In medium, ABA was added for inducing embryo's tolerance to desiccation. In the experiments, ABA promoted germination in embryos derived from both HEC and CEC. Senaratna et al., (1991) used 10-200 μM ABA pretreatment for inducing the tolerance to

Table 1. Callusing frequency of *Brassica juncea* L. cv. Pusa jai kisan from hypocotyls and cotyledons explants.

2,4-D (mg/l)	Callusing frequency (%)		
	Sucrose (%)	Hypocotyl	Cotyledon
1	0.5	80.8a	90.4a
	1.0	77.2b	80.3d
	1.5	76.2c	85.6c
	2.0	70.5d	87.4b
	2.5	70.7d	80.3d
	3.0	65.4e	80.2d
2	0.5	88.3c	90.0c
	1.0	85.9f	95.0b
	1.5	86.7e	98.2a
	2.0	75.4b	85.5d
	2.5	77.1a	80.2e
	3.0	75.4d	80.2e
3	0.5	75.2a	80.3a
	1.0	67.4d	80.4a
	1.5	66.6e	75.3b
	2.0	62.7c	75.2b
	2.5	70.8b	60.1d
	3.0	75.2a	63.5c
4	0.5	60.5c	80.0a
	1.0	55.2e	75.5d
	1.5	55.5e	77.5b
	2.0	56.3d	76.1c
	2.5	62.5b	75.5d
	3.0	66.2a	70.4e
5	0.5	50.2e	80.2b
	1.0	57.4d	85.2a
	1.5	60.2c	75.3e
	2.0	63.5b	77.5c
	2.5	63.5b	76.6d
	3.0	65.4a	66.3f

Means with common letters within each column are not significantly different at $p \leq 0.05$ according to DMRT

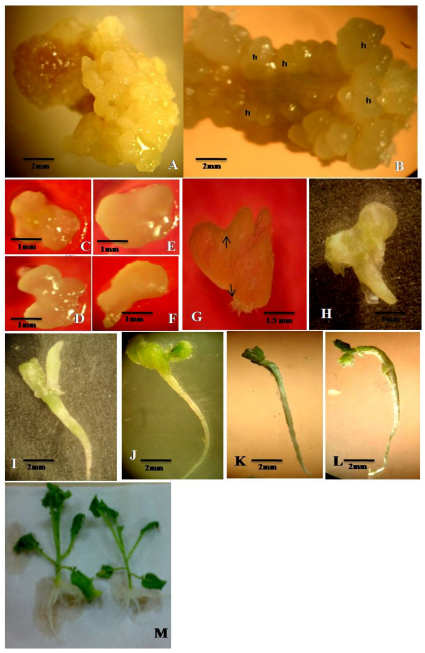

Fig 1. Cotyledon derived embryogenic callus (A), Hypocotyl derived embryogenic callus (B), Globular embryo (C), Torpedo shaped embryo (D), Heart shaped embryo (E), Globular stalked embryo (F), Heart shaped mature embryo (G)(arrow indicating distinct shoot and root pole), Heart shaped embryo with cotyledons and long radical (H), Cotyledonary embryo (I), Germinated seedling like cotyledonary embryo (J), development of emblings (K and L), plantlets developed from the emblings (M).

Table 2. Effect of various concentrations of four different cytokinins on development of somatic embryos from HEC and CEC.

PGR (mg/l)	Somatic Embryogenesis (%)		Number of Embryos /callus mass (100mg)							
	Hypocotyl	Cotyledon	HEC				CEC			
			H	G	T	C	H	G	T	C
BAP										
0.5	80.2c	95.7c	55.2a	20.2c	12.5b	0.0c	67.2a	17.2c	0.0c	6.6c
1.0	82.0b	99.8b	45.3b	33.4b	6.2c	2.6b	58.3b	22.2b	3.4b	8.2b
2.0	98.6a	100.0a	38.5c	37.5a	14.4a	7.2a	55.2c	26.5a	4.4a	12.2a
Kinetin										
0.5	81.5c	97.8b	50.5a	18.5c	0.0c	1.0c	58.8a	20.3b	0.0b	0.0c
1.0	85.2b	96.7c	44.3b	26.6b	12.2b	2.2b	54.5c	24.5a	0.0b	9.5b
2.0	96.6a	98.9a	35.2c	28.9a	18.4a	5.9a	55.5b	24.2a	5.5a	11.5a
TDZ										
0.5	90.7b	85.2a	22.5b	54.3b	26.9a	2.5a	35.2a	54.2c	0.0b	0.0c
1.0	95.5a	77.2c	28.4a	49.5c	16.5b	0.0b	12.5c	56.6b	0.0b	0.0b
2.0	88.2c	78.9b	13.2c	59.2a	2.2c	0.0b	14.1b	58.4a	2.5a	1.5a
2ip										
0.5	95.6b	92.9c	57.3a	34.4c	19.3a	7.6a	66.2a	16.5c	0.0c	0.0c
1.0	95.4b	98.0b	55.5b	40.4a	12.5b	5.2c	57.4b	26.5b	5.5b	5.3b
2.0	99.6a	98.9a	55.2b	38.2b	10.5b	6.5b	45.5c	33.5a	8.2a	10.5a

G-Globular, H-Heart, T-Torpedo, C-Cotyledon, Data represent s mean with common letters within each column are not significantly different at $p \leq 0.05$ according to DMRT.

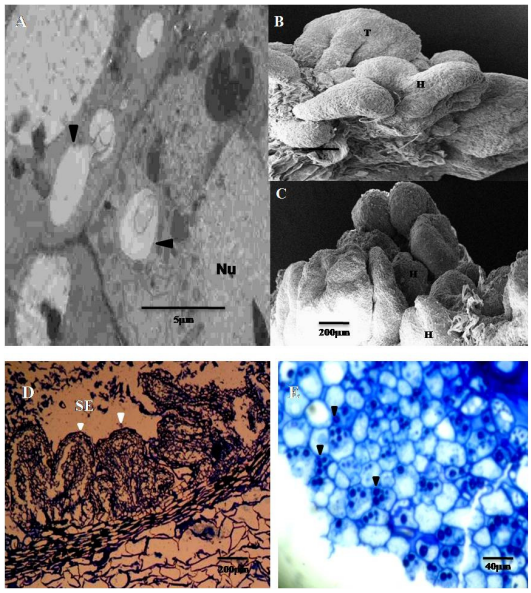

Fig 2. TEM image of embryo cell having stored bodies (A), Cell contain smaller vacuole, large nucleus (Nu) with numerous organelles (B), SEM image of a hypocotyl showing different shapes of embryos (C). SEM image of cotyledon embryos longitudinal section of somatic embryo under Olympus system microscope the embryos formed from upper meristematic cells (D), T.S. of developing embryos clear the presence of stored bodies (arrow) as observed under Olympus system microscope (E).

desiccation in embryos of *B. napus.* Recently, Angoshtari et al., (2009) reported ABA induced somatic embryogenesis and used the same for induction of desiccation tolerance in SEs from *Brassica napus*. In our experiments, 2.6 mg/l of ABA was observed to be very efficient for embryo germination. At lower ABA levels, there was no germination, while at higher concentrations embryos germinated but the germinated emblings dried due to excessive desiccation. To the best of our knowledge, this is the first ever report describing high frequency plant regeneration in *Brassica juncea* L. cv. Pusa Jai Kisan through somatic embryogenesis. This protocol is more rapid than earlier reported methods. It took about six weeks to get complete plantlets and approximatly 2000 plantlets were obtained by employing this method. This protocol can be used to raise transgenic *B. juncea en masse* as it takes less time compared to other methods available.

Materials and methods

Plant material and culture conditions

Seeds of mustard (*Brassica juncea* L. cv. Pusa Jai Kisan) were procured from NRCPB, IARI, New Delhi-110012, India. Mature seeds were thoroughly washed with tap water for 20 minutes and surface sterilized for 8 minutes in 3%

Table 3. Maturation percentage of somatic embryo on various cytokinins containing MS medium.

	Somatic embryo maturation (%)	
PGR (mg/l)	Hypocotyl	Cotyledon
BAP		
0.5	80.2a	75.5d
1.0	72.5d	85.6a
2.0	70.6e	70.8f
Kinetin		
0.5	80.2a	78.2c
1.0	78.5b	85.5a
2.0	75.5c	80.5b
TDZ		
0.5	80.5a	78.6c
1.0	75.7c	76.3e
2.0	70.5e	76.2e
2ip		
0.5	78.5b	70.3f
1.0	70.2e	78.5c
2.0	65.5f	78.5c

Data represent s mean with common letters within each column are not significantly different at $p \leq 0.05$ according to DMRT.

Table 4. Germination percentage of somatic embryo on MS medium supplemented with various concentrations of cytokinines with 2.6 mg/l ABA.

	Somatic embryo germination (%)	
PGR (mg/l)	Hypocotyl	Cotyledon
BAP		
0.5	50.2d	64.5a
1.0	50.5d	54.6e
2.0	50.3d	45.3g
Kinetin		
0.5	50.2d	50.8f
1.0	45.5e	50.3f
2.0	40.6f	40.7h
TDZ		
0.5	58.2a	60.4b
1.0	55.6b	58.5c
2.0	55.7b	55.2d
2ip		
0.5	52.3c	50.2f
1.0	40.3f	50.3f
2.0	35.2g	45.1g

Data represent s mean with common letters within each column are not significantly different at $p \leq 0.05$ according to DMRT.

(v/v) solution of sodium hypochlorite (Himedia lab, India). Thereafter, these seeds were rinsed three times for 10 minutes with sterilized distilled water, immersed in 0.1% mercuric chloride (Himedia lab, India) for 1 minutes followed by 3–4 washings with sterilized distilled water in the laminar flow cabinet. The seeds were inoculated in 25 ml culture tubes (Borosil, India) containing solidified half-strength MS (Murashige and Skoog, 1962) medium with 1.5% sucrose, pH 5.8. Culture tubes were kept in the dark for 2 days at 25 ± 2˚C , and later kept in illuminated room with white fluorescent tube lights ((100 l mol m^{-2} s^{-1} PFD) from cool-white fluorescent lamps (F40 T12/CW/EG, Phillips, New Delhi, India) in a 16 h photoperiod. All the chemicals used in this study were of analytical grade and of pure quality.

Medium composition

The MS medium contained macro and micro salts, vitamins, 3% sucrose and 0.8% agar-agar. The growth regulators (BAP, 2, 4-D, Kin, TDZ, 2ip and ABA) were filter-sterilized and added to the sterilized culture medium.

Callus induction

Hypocotyl and cotyledon explants were taken from five days old *in vitro* raised seedlings, and cultured on basal MS medium supplemented with various concentrations of 2,4-D (1-5mg/l); and sucrose (0.5-3%). The embryogenic calli, obtained from hypocotyl and cotyledon, were referred as HEC (hypocotyl-derived embryogenic calli) and CEC (cotyledon-derived embryogenic calli), respectively. Ten explants each, in triplicate were cultured on callus induction medium.

Embryo development

After twenty days of inoculation of explants on callus induction medium, the embryogenic calli were cultured on

MS, amended individually with various concentrations of BAP, Kin, TDZ and 2ip (0.5, 1.0 and 2.0 mg/l). In induction medium, the embryogenic calli differentiated into somatic embryos (SEs).

Embryo maturation and germination

After two weeks of culture, these SEs developed further and showed maturation when early staged embryos were sub-cultured on embryo development medium. The composition of germination medium was the same as maturation medium but it was additionally supplied with various concentrations of ABA (0, 1.3, 2.6, 3.9, 5.2 mg/l). The pH of both media was adjusted to pH 5.8. All cultures were incubated in an automated culture room at 25 ± 2˚C and illuminated with white fluorescent tube lights (100 l mol m^{-2} s^{-1} PFD) from cool-white fluorescent lamps (F40 T12/CW/EG, Phillips, New Delhi, India) in a 16 h photoperiod.

Transmission Electron microscopy (TEM)

The study of ultra structure of embryogenic calli was done using TEM. Embryo regenerating tissue samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. They were postfixed in 1% OsO_4 in the same buffer solution. Embedding was made in Epon–Araldite resin in flat molds with proper orientation to obtain cross sections of embryos. Resin blocks were cut on an UC6 Leica ultramicrotome with a diamond knife and 60-nm sections were stained with uranyl acetate followed by lead citrate. Stained sections were examined with a Philips MORGAGNI 268 transmission electron microscope. The microscope was operated with an electron beam at 70 kV.

Scanning electron microscopy (SEM)

SEs on embryogenic calli were cleaned with 0.1 M phosphate buffer (pH 7.4) and fixed for 18 hour at 4ºC in modified Karnovsky's fluid made in 0.1 M Phosphate buffer (pH 7.4). The specimens were dehydrated in a graded acetone solution. Critical Point Drying was done with liquid CO_2 using Polaron Jumbo Critical Point Dryer, and Gold Sputter Coating was carried out under reduced pressure in an inert argon gas atmosphere (Agar Sputer Coater P 7340). After sputter coating, the tissues were examined under Scanning Electron Microscope (Leo 435VP) operated at 15 kV (David et al., 1973).

Histological studies

Embryogenic calli with developing embryos were fixed in FAA (v/v, formaldehyde/100% ethanol/acetic acid, 95:5:5), dehydrated in an alcohol series (30-100% ethanol), and then embedded in pure paraffin wax. Paraffin blocks containing the embedded samples were sectioned to 10 µm thickness with a microtome. The sections were deparaffinized in xylol, stained with 1% (w/v) toludine blue for 2 min, viewed under a compound microscope (Olympus CX41RF) and photographed.

Statistical analysis

The data on the effects of growth regulators on different stages of embryogenesis were analyzed by one-way analysis of variance (ANOVA). Values are means of five replicates, and the presented mean values were separated using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

Acknowledgements

The first author is thankful to Council of Scientific and Industrial Research (CSIR) for providing the research fellowship for carrying out this research. We are thankful to Dr. R.K Katiyar, IARI, New Delhi for providing seeds of *Brassica juncea* cv. Pusa Jai kisan, used in this study.

References

Agnihotri A, Prem D, Gupta K (2004) Biotechnology in quality improvement of oilseed *Brassicas* In: Srivastava PS, Narula A, Srivastava S (eds) Plant Biotechnology and Molecular Marker Anamaya Publishers New Delhi, pp, 10.

Angoshtari R Afshari RT, Kalantari S, Omidi M (2009) Effects of abscisic acid on somatic embryogenesis and induction of desiccation tolerance in *Brassica napus*. Asian J Plant Sci 8:276-284.

Bewley JD, Black M (1985) Seeds: germination, structure and composition, in physiology of developmental and germination, Bewley JD and Black M, Eds. Plenum Press, New York, 1.

Bhattacharya NM, Sen SK (1980) Production of plantlet through somatic embryogenesis in *Brassica campestris*, *Z. pflanzenphysiol* 99:357-365.

Das B, Goswami L, Ray S, Ghosh S, Bhattacharyya S, Das S Majumder AL (2006) *Agrobacterium*-mediated transformation of *Brassica juncea* with a cyanobacterial (*Synechocystis* PCC6803) delta-6 desaturase gene leads to production of gamma linolenic acid. Plant Cell Tiss Org Cult 86:219–231.

David GFX, Herbert J, Wright CDS (1973) The ultra structure of the pineal ganglion in the ferret. J Anat 115:79–97.

Eapen S Abraham V Gerdemann M Schieder O (1989) Direct somatic embryogenesis, plant regeneration and evaluation of plants obtained from mesophyll protoplasts of *Brassica juncea*. Ann Bot 63:369-372.

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Reg 43:27-47.

Imin N, De Jong F, Mathesius U, Noorden G, Saeed NA, Wang XD, Rose RJ, Rolfe BG (2004) Proteome reference maps of *Medicago truncatula* embryogenic cell cultures generated from single protoplasts. Proteomics 4:1883–1896.

Imin N, Nizamidin M, Daniher D, Nolan KE, Rose RJ, Rolfe BG (2005) Proteomic analysis of somatic embryogenesis in *Medicago truncatula* explant cultures grown under 6-benzylaminopurine and 1-naphthaleneacetic acid treatments. Plant Physiol 137:1250-1260.

Kirti PB, Chopra VL (1989) A simple method of inducing somatic embryogenesis in hypocotyl explant culture of mustard, *Brassica juncea* (L.) Czern & Coss. Plant Breed 102:73-78

Kirti PB, Chopra VL (1990) Rapid plant regeneration through organogenesis and somatic embryogenesis from cultured protoplasts of *Brassica juncea*. Plant Cell Tiss Org Cult 20:65-67.

Kumari A, Cheema GS, Munshi SK (1995) Induction, growth and development of somatic embryos from hypocotyl explants in mustard (*Brasica juncea* L. Czern & Coss). Cruciferae Newslett 17:30–31.

Kumari A, Cheema GS, Munshi SK (2000) A hypocotyl-derived somatic embryogenic system in *Brassica juncea* Czern & Coss and its manipulation for enhanced storage lipid accumulation. Plant Cell Tiss Org Cult 63:109–120.

Maheswaran G, Williams EG (1984) Direct somatic embryoid formation on immature embryos of *Trifolium repens, T. pratense* and *Medicago saliva* and rapid clonal propagation of *T. repens*. Ann Bot 54:201−211.

Mujib A, Samaj J (2006) Somatic embryogenesis. Springer-Verlag, Berlin, Heidelberg, New York.

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

Narasimhulu SB, Kirti PB, Prakash S, Chopra VL (1992) Somatic embryogenesis in *Brassica nigra* (Koch) J Exp Bot 43:1203-1207.

Nolan KE, Irwanto RR, Rose RJ (2003) Auxin up-regulates MtSERK1 expression in both *Medicago truncatula* root-forming and embryogenic cultures. Plant Physiol 133:218–230.

Pareekh LK, Chandra N (1978) Somatic embryogenesis in leaf callus from cauliflower (*Brassica oleracea*). Plant Sci Lett 11:311–316.

Potts DA, Rakow GW, Males DR (1999) Canola-quality *Brassica juncea*, a new oilseed crop for the Canadian prairies. In: Proc. 10th International Rapeseed Congress, Canberra, Australia, September, pp, 23-29.

Raemakers K, Pereira I, Putten HK, Visser R (2005) Indirect somatic embryogenesis in cassava for genetic modification purposes. Methods Mol Biol 318:101-109.

Raghavan V (2004) Role of 2,4-D in somatic embryogenesis on cultured zygotic embryo of *Arabidopsis*: Cell expantion, cell cycling and morphogenesis during continuous exposure of embryo to 2,4-D. Am J Bot 91(11):1743-1756.

Senaratna T, Laima K, Beversdorf WD Mc Kersie BD (1991) Desiccation of microspore derived embryos of oilseed rape *(Brassica napus* L.). Plant Cell Rep 10:342-344.

Sharma KK, Bhojwani SS, Thorpe TA (1991) The role of cotyledonary tissue in the differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. Plant Cell Tiss Org Cult 24:55-59.

Stoutjesdijk PA, Hurlstone C, Singh SP, Green AG (1999) Genetic manipulation for altered oil quality in *Brassicas*. In: Proc. 10th International Rapeseed Congress, Canberra, Australia, September, pp, 23-29.

Sugiyama M (1999) Organogenesis *in vitro*. Curr. Opin. Plant Biol 2:61–64.

Sung ZR, Okimoto R (1993) Coordinate gene expression during somatic embryogenesis in carrots. Proc Natl Acad Sci USA 80:2661-2665.

Thorpe TA (1995) *In Vitro* embryogenesis in plants. Kluwer academic publishers, Netherlands. ISBN 0-7923-3149-4

Turgut K, Barghchi M, Scott R (1998) Efficient shoot regeneration and somatic emhryogenesis from immature cotyledons of *Brassica napus* L. Plant Breed 117:503-504.

Yantcheva A, Vlahova M, Antanassor A (1998) Direct somatic embryogenesis and plant regeneration of carnation (*Dianthus caryophyllus* L.). Plant Cell Rep 18:148-153.