International Scholars Journals

African Journal of Crop Science ISSN: 2375-1231 Vol. 13 (2), pp. 001-005, February, 2025. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Development of a Protocol for Genetic Transformation in Brassica juncea Utilizing Cicer arietinum Lectin

V.V. Singh[1], Vandana Verma[1]*, Aniruddh K. Pareek[1], Monika Mathur[1], Rajbir Yadav[1], Poonam Goyal[1], Ajay Kumar Thakur[1], Y. P. Singh[1], K. R. Koundal[2], K. C. Bansal[2], A. K. Mishra[1], Arvind Kumar[1] and Sandeep Kumar[1]

[1]Directorate of Rapeseed-Mustard Research, Bharatpur, Rajasthan 321 303, India.
[2]NRCPB, IARI, New Delhi, India.

Accepted 15 October, 2024

A simple and easy protocol for regeneration and genetic transformation of *Brassica juncea* (L.) Czern and Coss cv. Pusa bold has been developed. The optimum regeneration was found on combination of BAP (3.0 mg/l) and IAA (0.2 mg/l). The genetic transformation protocol has been standardized using *Agrobacterium tumefaciens* (gv3101). Cotyledonary petioles used as explant were pre-cultured for 48 h and than co-cultivated with Agrobacterium suspension for 2 days. Putative transformants were selected from selection medium containing 20 mg/l Kanamycine and 250 mg/l cefotaxime. Complete plant formed after placing shoots on rooting medium (0.5 mg/l IBA). Confirmation of integration of transgene was done by PCR using *nptII* gene primer.

Key words: Indian mustard, *Agrobacterium tumefaciens*, chickpea lectin gene, cotyledonary petioles, transformation.

INTRODUCTION

Indian mustard [*Brassica juncea* (L)] is a major oilseed crop of India and is grown in around six million hectares, primarily in the north-western part of the country during the winter season. It is also a potential crop in western prairies of Canada, China and Russia. Brassica oilseeds are susceptible to many biotic and abiotic stresses which cause moderate to high level (10 - 90%) losses depending upon infestation and crop growth stage. Although the use of pesticides is quite effective, their excessive and injudicious use leads to imbalances in agro-ecosystem and health hazards (Singh, 2008). Transgenic plants expressing bacterial Bt genes and plant derived insecticidal lectin and protease inhibitor genes provide effective and ecologically sustainable option for insect control (Datta and Koundal, 2003). Plant lectins are a group of carbohydrate binding proteins present in high amount in legume seeds and the toxicity of plant lectins against a wide range of insects is well established (Peumans and Van Damme, 1995). Keeping these facts in view, an *in vitro* regeneration and transformation of *B. juncea var.* Pusa Bold was attempted using chickpea lectin gene construct.

MATERIAL AND METHODS

Plant materials and culture conditions

Seeds of Indian mustard var. Pusa bold were obtained from the Germplasm Unit of DRMR, Bharatpur, where cultivars are being maintained. Approximate one hundred Seeds were surface sterilized with 0.1% $HgCl_2$ for 5 - 6 min and rinsed 2 - 3 times (for 2 - 3 min each time) with distilled water. Sterilized planted seeds were then germinated in culture tubes on half MS medium (Murashige and Skoog, 1962) with 3% sucrose and solidified with 0.8% agar (Himedia). Among aseptically planted seeds, ninety were germinated at 25°C ± 2 in 16 h photoperiod and 1500 lux intensiy. The cotyledonary petioles were excised from 5 day old seedlings and planted on the regeneration medium with their abaxial surface in contact. While plating the explants, it was ensured that the cut end of the petiole remained either in contact or slightly immersed

*Corresponding author. E-mail: bandhanbiotech@gmail.com, vvs_71@yahoo.com

in the growth media.

Bacterial strain and binary plasmid vector

The *Agrobacterium* strain were grown overnight in minimal medium (Chilton et al., 1974) that is liquid YEM (Yeast Extract Manitol) at 28°C with continuous shaking under appropriate antibiotic selection condition (20 mg/l rifampicin and 50 mg/l kanamycin). The disarmed *A. tumifecience* strain gv 3101, carrying the binary plasmid vector pBinAR were used in preliminary transformation experiments. This vector mobilized into *A. tumifecience* strain gv3101.

Plant regeneration

To optimize various regeneration parameters for different explants (stems, hypocotyls, petioles, cotyledons and cotyledonary petioles), optimum concentration of hormones for shoot and root regeneration was used. BAP (0.0 - 5.0 mg/l) and IAA (0.0 - 0.5 mg/l) was used for shoot regeneration and IBA (0.0 - 1.0 mg/l) was used for root regeneration. All parameters were tested one after the other, in sequential order. The optimized conditions determined in earlier experiments were used in subsequent experiments. The above parameters (or treatments) were tested in the order as stated below.

For testing of first parameters different explants such as stems from regenerated shoots and hypocotyls, petioles, cotyledonary petioles, cotyledons were used from 5 day old germinated seedlings and transferred on MS media containing different combinations of shoot regenerating hormones. After 2 - 3 weeks, regenerated healthy, individual shoots from explants were excised and transferred on rooting media supplemented with different concentration of IBA (0.0 - 1.0 mg/l). Well developed rooted plants were kept for hardening and acclimatization. Nine plants were kept directly in soil rite: field soil mixture (1:0/1:1/3:1) and three plants were given pretreatment of liquid culture. Initially all plants were hardened in culture room, where they were covered with perforated poly bags. After 20 - 25 days when they had sufficient growth to sustain environmental conditions, they were transferred to the glass house for complete acclimatization.

Plant transformation

Agrobacterium strain gv3101 carrying the binary plasmid vector pBinAR was used for transformation. The vector contains *nptII* (neomycin phosphotransferase) for the selection of transformants. To carry out genetic transformation, cotyledonary petioles (with 2 mm petiole) were precultured on MS medium supplemented with 2, 4-D, BAP and IAA for a period of 48 hrs (Figure 1) (Chakrabarty et al., 2002). After 48 hrs, these petioles were co-cultivated with *Agrobacterium* culture, which was grown overnight in 50 ml liquid YEM containing antibiotics (rifampicin 20 mg/l and kanamycin 50 mg/l) at 28°C (Figure 2). The O.D of culture was maintained at 0.5 with an optimized incubation period (1 - 2 s to 30 min) of explant and *Agrobacterium* suspension. The cut ends of cotyledonary petioles were dipped in the *Agrobacterium* suspension from 1 - 2 s to 30 min and then transferred on co-cultivation medium (for 48 h) containing BAP and IAA. After co-cultivation explants were washed with double distilled water (2 - 3 times for 2 - 3 min) and treated with cefotaxime antibiotic solution (250 mg/l) for 5 - 6 min then blotted on sterile filter paper to remove any traces of bacterial contamination and then placed on selection medium with BAP (3.0 mg/l), IAA (0.2 mg/l) containing cefotaxime (250 mg/l) and kanamycin (20 mg/l). Shoot induction started after 15 - 20 days of co-cultivation. Regenerated shoots were excised and transferred to shoot elongation medium. Healthy shoots with an average height of

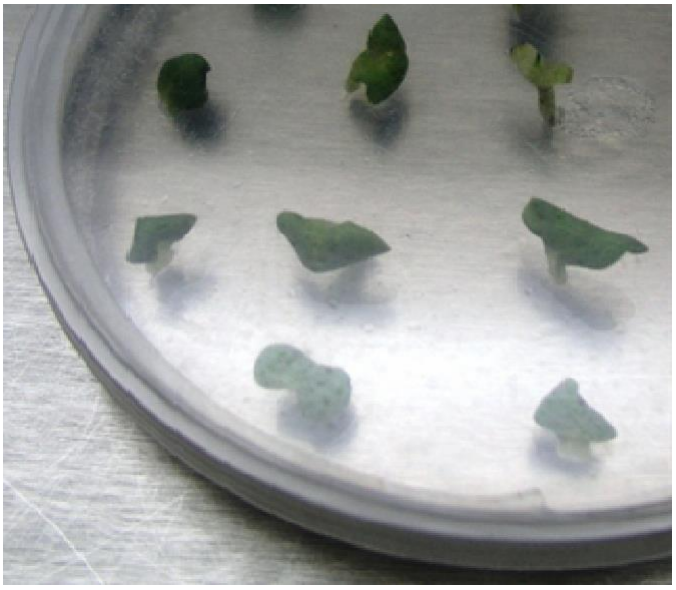

Figure 1. Pre-culturing of cotyledonary petioles of var. Pusa Bold.

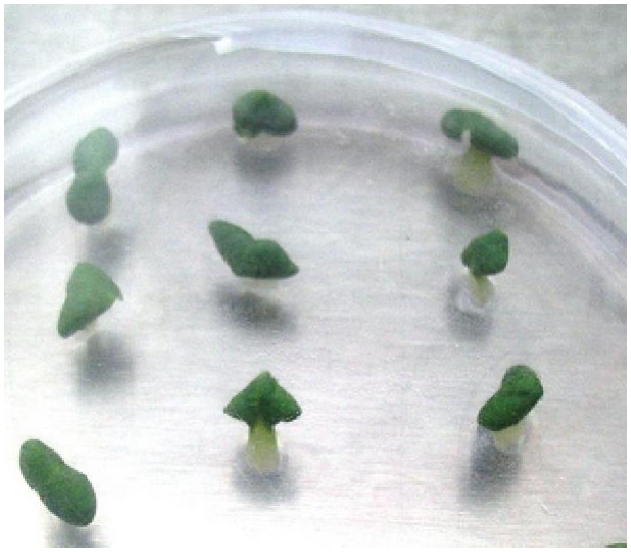

Figure 2. Co-cultivation of pre cultured Cotyledonary petioles

1.5 cm were transferred to the rooting medium, after 3 - 4 weeks when roots grown sufficiently they were transferred to hardening medium containing soil rite. With this method primary transgenic were hardened to get T1 seeds.

Molecular analysis of putative transformants total genomic DNA was isolated from leaves of *in vitro* growing putative transformants and control plants using modified SDS DNA extraction method (Dellaporta et al., 1983). Gene specific primers *npt II* were used to amplify integrated transgene of 700bp. Each PCR reaction was carried out in 25 l reaction mixture containing 1.5 mM $MgCl_2$, 200 M dNTPs, (1.25 U) Taq DNA polymerase, 50 ng genomic DNA and oligonucleotide primers. The sequence of these oligonucleotide primers is as follows: Forward primer (F): 5'GAG GCT ATT CGG CTA TGA CTG 3'.Reverse primer (R): 5'ATC GGGAGC GGC GAT ACCGTA3'.

The amplifications were carried out under the following conditions: 94°C for 1 min (one cycle), 94°C for 30 s (denaturation), 56.1°C for 30 s (annealing), 72°C for 2 min (for 35 cycles) and final extension at 72°C for 5 min (one cycle). Analysis of amplified product was carried out on 1.5% agarose gel containing ethidium bromide (0.01%). DNA bands were visualized in UV light.

RESULTS AND DISCUSSION

Regeneration

Present study was conducted on *B. juncea* var. Pusa Bold with a view to optimize regeneration and transfor-mation protocol using cotyledonary petioles. In this study, cotyledonary petioles, hypocotyls, petioles of 5 day old seedlings and stems from back cultured shoots were used and inoculated on MS medium with varying concentrations of BAP (0.0 - 5.0 mg/l) and IAA (0.0 - 0.5 mg/l).The optimum regeneration frequency of 58.65% was obtained on combination of BAP and IAA (Table 1). Although hypocotyls and stems showed 60 - 70% regeneration frequency and comparatively gave good response than cotyledonary petioles, but in further experi-ments of transformation, both stems and hypocotyls gave very poor transformation frequency (3 - 5%). Therefore cotyledonary petioles were used as an explant in transformation experiments. Our results are in conformity with earlier reports of regeneration obtained in *Brassica juncea* var. PJK and Rai-1 using cotyledonary petioles (Sharma et al., 1990; Babu et al., 2003). In contrast, genetic transformation in *B. juncea* was done by using hypocotyl as an explant source and successfully transformed RLM198, Pusa Bold, Skorospieha II, Pusa Jaikisan cultivars of Indian mustard (Babu et al., 2003). Presence of BAP was found to be critical in this study as reported earlier in Indian mustard. In absence of BAP, there was no shoot regeneration. At BAP concentration of 1.0 mg/l and 2.0 mg/l, regeneration frequency of 5.78 and 21.50 was observed. Maximum shoot regeneration was observed at a BAP concentration of 3.0 mg/l when supplemented with 0.2 mg/l IAA (Table 2). Beyond optimum shoot regeneration level there was decline in regeneration frequency. In addition, BAP at concentration of 3.0 mg/l gave maximum number of shoots (6- 7 per explants). In contrast to this, lower range (2 - 4 shoots/explant) was obtained at other concentration. These results are also in accordance with earlier reports of Babu et al. (2003) and Sharma et al. (2004) in *B. juncea*. Since best results were achieved by using cotyledonary petioles as explants on MS medium containing 3.0 mg/l BAP and 0.2 mg/l IAA. This combi-nation was used for transformation study. Regenerated shoots were kept on root inducing medium having diffe-rent concentration of IBA (Table 3).

Plant transformation

In the present study, cotyledonary petioles were dipped for 1 - 2 s in *Agrobacterium* suspension and co-cultivated for 48 h on co-cultivation media. This treatment which was given for 1 - 2 s showed higher transformation frequency of 15 - 20%. After a period of 12 - 15 days, cut tips of cotyledonary petioles initiated shoot regeneration and started differentiating into green or white color shoots. The transformation frequency was calculated on the basis of the number of green shoots obtained out of the total number of shoots formed on the selection media. Transformation frequency varying between 15 - 20% was obtained from 10 independent batches, each having approximately 95 explants. Green shoots thus obtained were separated and sub cultured on same media.

Kanamycin level (20 mg/l) was maintained in sub cul-turing and rooting to rule out any possibilities of escapes. Multiplied shoots of 3 - 4 cm length were excised and cultured on medium containing IBA (0.5 mg/l) for rooting. Rhizogenesis started after 2 - 3 weeks of inoculation and shoots having well developed roots was obtained in 3 - 4 weeks (Figure 3) . The rooted plant-lets were then transferred to pots filled with hardening media that is soil rite (Figure 4) . However, rooting in putative transformants was found to be difficult. This may be due to insertion of that lectin gene in root forming zone. Insertion resulting into hormonal imbalances might be responsible for preventing root induction. Half MS supplemented with IBA was showing high frequency of root induction than normal MS. While on basal medium it was showing less frequency of root induction and non- fibrous roots. There-fore, in all our experiments half MS medium con-taining IBA was used for root induction from regenerated shoots. During hardening of putative transformants, they were pretreated with liquid culture so that they able to acclimatize rapidly according to environmental conditions. Further during hardening process soil rite: field soil (3:1) ratio found to be most appropriate for plants. Trans-formants were hardened and acclimatized to get T1 seeds. Molecular analysis of putative transformations was performed to confirm the presence of chickpea lectin gene using *nptII* primers. Results were showing that some of transformants succeed (l-4 in Figure 5) to get that gene of 0.7 kb.

Table 1. Shoot regeneration frequency in *Brassica juncea* cv Pusa Bold at various combinations of BAP & IAA.

BAP/IAA	0.0	0.1	0.2	0.3	0.4	0.5
0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.0	10.25	13.24	19.75	13.00	8.95	10.95
2.0	22.63	19.43	26.18	21.73	17.14	22.85
2.5	21.30	24.50	32.25	23.50	20.15	19.52
3.0	47.25	50.10	58.65	52.21	43.50	25.56
3.5	30.19	34.45	32.11	28.50	23.60	21.63
4.0	22.85	20.36	28.45	21.85	24.65	19.25
4.5	13.62	10.95	19.52	16.34	15.64	13.55
5.0	9.06	14.52	10.23	13.26	14.26	8.08

Table 2. Transformation table of *Brassica juncea* cv Pusa Bold.

Cultivars	No. of explants Co-cultivated	Total no of shoots developed on selection media	Total no. of green shoot	No. of explant on rooting	No. of explant for hardening
Pusa Bold	95	65	12	3	2
	80	40	7	2	3
	105	75	15	3	1
	95	75	14	1	1
	110	66	10	1	0
	70	56	11	0	3
	66	43	8	4	2
	101	65	2	2	0

Table 3. Effect of various concentrations of auxins (IBA) on formation of roots.

IBA (mg/l)	% of Root Induction	No. of roots/shoot
0	20	3
0.1	40	2
0.2	30	4
0.3	50	5
0.4	40	4
0.5	80	12
0.6	60	4

Figure 3. Rooting in putative transformants

Figure 4. Hardening of putative transformants.

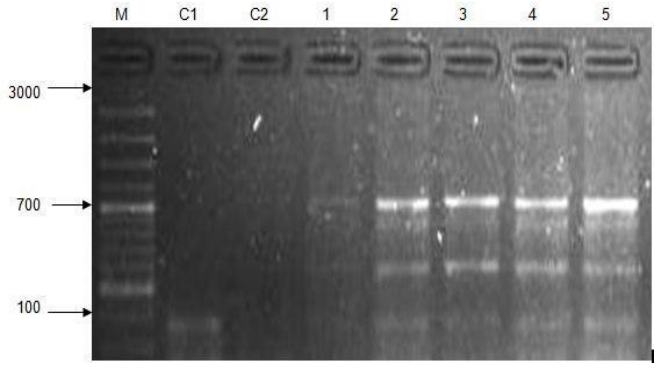

Figure 5. Molecular analysis of putative transformants through specific primers (M = Marker, C = Controls, 1 - 5 Putative Trans-formants).

Present study clearly demonstrates *in vitro* regeneration and *Agrobacterium* mediated transformation of *B. juncea* variety Pusa Bold. The present process of transformation is simple and easy in operation. However, selection of appropriate explant, choice of *Agrobacterium* strain with proper media and hormone combination are the major determinants for the success for any trans-formation and regeneration system. The development of aphid resistant

transgenic *Brassicas* will definitely be a boom to mustard growing farmers of the country.

ACKNOWLEDGEMENT

Authors gratefully acknowledge the financial grant and gene construct received under Network project on Transgenics in Crops, ICAR, New Delhi. We are also thankful to Dr. K.C. Bansal, Coordinator, NPTC and Dr. Rekha Kansal, NRCPB, New Delhi for their all round help and support to carry out this experiment.

REFERENCES

Babu BS, Brijmohan SB, Sabharwal V, Chaudhary A, Lakshmikumaran M (2003). *In vitro* regeneration & genetic transformation of *B. juncea* via Agrobacterium using cotyledonary petiole explants. *Brassica*. 5(1-2): 16-23.

Chakrabarty R, Viswakarma N, Bhat SR, Kirti PB, Singh BD, Chopra VL (2002). Agrobacterium mediated transformation of cauliflower: Optimization of protocol and development of Bt-transgene cauliflower. Indian Acad. Sci. 27: 495-502.

Chilton MD, Currier T, Farrand S, Bendich A, Gordon MP, Nester EW (1974). *Agrobacterium tumifecience* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc .Natl. Acad. Sci. USA. 71: 3636-3672.

Datta S, Koundal KR (2003). Genetic transformation of *B. juncea* (l.) using Insecticidal Lectin gene from cowpea (*Vigna unguiculata* (L. Walp). *Brassica 5* (1-2): 33-40.

Dellaporta SL, Wood J, Hicks JB (1983). Plant DNA minipreparation version II. Plant Mol.Biol.Rep. 1: 19-21.

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

Peumans WI, Van Damme ELM (1995) Lectins as plant defense proteins. Plant Physiol. 109: 347-352.

Sharma KK, Bhojwani SS (1990) Histological aspects of in vitro root and shoot differentiation from cotyledon explants of *Brassica juncea* (L.) Czern. Plant Sci. 69: 107-214.

Sharma M, Sahni R, Kansal R, Koundal KR (2004). Transformation of Oilseed mustard *B. juncea*. var. PJK with Snowdrop lectin gene. Ind .J. Biotech. 3: 97-102.

Singh D (2008). Breeding for Aphid Resistance in Rapeseed–Mustard. In Sustainable production of oil seeds. A.Kumar, J.S.Chauhan and C.Chattopadhyay (eds).Agrotech Academy Publishing Udaipur (India), pp: 142-154.