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AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION OF BELL PEPPER (*CAPSICUM ANNUUM* L. CV. CALIFORNIA WONDER) WITH *GUS* AND *NPT*-II GENES

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ABSTRACT

Capsicum species show a weak response towards *Agrobacterium*-mediated transformation due to recalcitrancy. Present study was carried out to standardize an efficient system for *Agrobacterium*-mediated genetic transformation of *Capsicum annuum* L. cv. California Wonder. *Agrobacterium tumefaciens* LBA 4404 strain harboring a binary vector pBI121, carrying *npt*-II and *gus* genes was used for co-cultivation with cotyledon and hypocotyl explants. Kanamycin sensitivity test showed that kanamycin concentration as low as 10 mg l^{-1} was inhibitory for the growth of bell pepper cells. Regeneration of putative transgenic shoots was achieved on Murashige and Skoog (MS) basal medium supplemented with 6.0 mg l^{-1} 6-benzylaminopurine (BA), 0.3 mg l^{-1} indole-3-acetic acid (IAA), 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. The elongation of ill-defined rosettes was achieved on MS basal medium containing 2.25 mg l^{-1} BA, 2.0 mg l^{-1} gibberellic acid (GA_3), 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. After fair elongation of the rosettes, root induction was observed on transfer of elongated shoots to MS medium containing 0.25 mg l^{-1} IAA, 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. Polymerase chain reaction (PCR) analysis of the putative transgenic plantlets showed amplification of both neomycin phosphotransferase-II (*npt*-II) and β-glucuronidase (*gus*) genes. Expression of the *gus* gene was studied by GUS assay. Present study could be helpful in facilitating the transfer of desirable genes for biotic and abiotic stress in this recalcitrant crop.
Keywords: *Capsicum, regeneration, Agrobacterium, transformation, gus, npt*-II

[I] INTRODUCTION

Capsicum annuum is the most widely cultivated species in subtropics and temperate regions of the world [1]. It serves as basic ingredient as well as a flavourant and colourant in a variety of food. Due to a rich source of vitamin C, B-complex, minerals like molybdenum, manganese, potassium and other nutritive values it is used as a medicine to treat disorders like dropsy, diarrhea, asthma, arthritis, muscle cramps and toothache [2, 3].

Besides being an important economical crop, biotechnological approaches have not served it much due to its recalcitrant nature and genotypic dependence. Although, significant progress has been made in the field of *Capsicum* improvement using conventional breeding techniques, it still suffers from some limitations for the introduction of new alleles. Genetic transformation holds great potential to overcome constraints like limited gene pool and species barrier posed by conventional

breeding methods. As compared to other solanaceous crops which have been used as model plant systems, *Capsicum* has always been less responsive towards regeneration and transformation [4].

Preliminary reports on transformation in this crop have resulted in few transformed plants [5-12]. Furthur, optimization of transformation parameters such as pre-culture and co-cultivation time using marker and reporter genes could help in increasing the transformation frequency for introduction of biotic and abiotic stress resistant genes. Also, this could amount in an improvement to already published protocols which indicate various inherent problems. So, the present work was undertaken to carryout *Agrobacterium*-mediated genetic transformation of *Capsicum annuum* L. cv. California Wonder with *gus* and *npt*-II genes.

[II] MATERIALS AND METHODS

2.1. Plant material

Certified seeds of bell pepper (*Capsicum annuum* L. cv. California Wonder) were obtained from the Department of Vegetable Science, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan.

2.2. Culture medium

Murashige and Skoog (MS) medium containing 100 mg l^{-1} myoinositol, 3% sucrose and 0.8% agar-agar was used as basal medium [13]. The pH of the medium was adjusted to 5.8.

2.3. *In vitro* germination of seeds

For surface sterilization, the seeds were kept in 3% (v/v) teepol solution for 30 s and then rinsed 4 times with sterilized distilled water. The rinsed seeds were kept in 0.1% (w/v) $HgCl_2$ solution for 1-2 min and subsequently washed 4 times with sterilized distilled water. Finally, the seeds were soaked in sterilized distilled water for 1 h. The soaked seeds were inoculated on half-strength MS basal medium for *in vitro* germination.

2.4. Kanamycin sensitivity test

Prior to transformation experiments, kanamycin sensitivity level of cotyledon explants was established so as to determine the effective concentration for selection. Cotyledon explants were excised from *in vitro* germinated 15 d old seedlings. The explants were cut into small pieces and weighed on a mettler balance inside the laminar air flow cabinet. The initial fresh weight of each explant was recorded. The explants were cultured on MS regeneration medium without and with different concentrations of kanamycin (0, 10, 20, 30, 40, 50, 70 and 100 mg l^{-1}).

2.5. *Agrobacterium* strain

Agrobacterium tumefaciens LBA 4404 strain containing reporter β-glucuronidase gene (*gus*) in binary vector system along with kanamycin resistance gene (*npt*-II) was used for co-cultivation (Fig. 1). Fresh cultures of *Agrobacterium* were prepared by inoculating a colony of bacteria in selective yeast mannitol broth (YMB) medium containing 50 mg l^{-1} kanamycin. The cultures were kept at 28 ºC in orbital incubator shaker for 12 h. A culture with an O.D. of 0.521 at 540 nm (containing 10^8 *Agrobacterium* cells ml^{-1}) was used for co-cultivation.

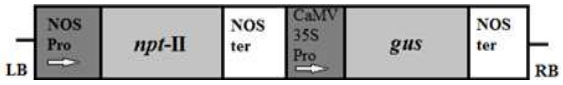

Fig: 1. Structure of expression vector: T-DNA region of pBI 121 containing transcriptional fusion of NOS promoter (NOS Pro) with the coding region of *npt*-II and NOS terminator (Nos ter) and transcriptional fusion of CaMV 35S promoter (CAMV 35S Pro) with the coding region of *gus* and NOS terminator. LB - left border of T-DNA, RB - right border of T-DNA.

2.6. Genetic Transformation

Hypocotyl and cotyledon explants were cut into small pieces and pre-cultured for 24 h, 48 h and 72 h on shoot regeneration medium. Cotyledon explants were pre-cultured on MS medium containing 6.0 mg l^{-1} 6-benzylaminopurine (BA) and 0.3 mg l^{-1} indole-3-acetic acid (IAA). For pre-

culturing of hypocotyl explants, MS medium containing 4.5 mg l^{-1} BA and 0.1 mg l^{-1} IAA was used. After pre-culture for different time intervals, the explants were kept in *Agrobacterium* suspension culture for 20 s and then blotted dry on pre-sterilized filter paper. Subsequently, the infected explants were transferred to shoot regeneration medium for co-cultivation with the *Agrobacterium* strain for 48 h and 72 h. After co-cultivation, the explants were transferred to fresh selective shoot regeneration medium containing cefotaxime (500 mg l^{-1}) and kanamycin (50 mg l^{-1}). All the cultures were kept in culture room at 26±2ºC temperature and 16 h of light period for growth and differentiation. Explants were periodically transferred to fresh selective shoot regeneration medium containing cefotaxime (500 mg l^{-1}) and kanamycin (50 mg l^{-1}) in order to check excessive bacterial growth. The regenerated putative transgenic shoots of bell pepper were transferred to selective shoot elongation MS medium containing 2.25 mg l^{-1} BA, 2.0 mg l^{-1} gibberellic acid (GA_3), 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. After fair elongation of the rosettes, the putative transgenic shoots were transferred to selective root regeneration MS medium containing 0.25 mg l^{-1} IAA, 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin.

2.7. Characterization of putative transgenic shoots of bell pepper

Transfer and integration of *gus* and *npt*-II genes was analysed by polymerase chain reaction (PCR) using pairs of designed primers for both the genes. Genomic DNA was isolated by modified CTAB method (Doyle and Doyle, 1990) from fresh, young and tender leaves of putative transgenic and non-transgenic (control) bell pepper plantlets [14]. The sequences of forward and reverse primers used for *npt*-II gene are 5' TTGAACAAGATGGATTGCAC 3' and 5' GATGACAGGAGATCCTGCCC 3' respectively and for *gus* gene are 5' TTCGCCTCGGCATCCGCTCAGTGGCA 3'and 5'GCGGACGGGGTATCCGGTTCCTTGGCA 3' respectively. The amplified DNA products separated by agarose gel electrophoresis were documented using Alpha Imager ® EC gel documentation system (U.S.). The transgenic bell pepper shoots characterized by PCR were analysed for expression of *gus* gene using GUS assay [15].

[III] RESULTS AND DISCUSSION

Genetic engineering for obtaining useful traits is dependent on an efficient and reliable regeneration protocol. Bell pepper is considered an extremely recalcitrant species with respect to *in vitro* regeneration [16]. So prior to transformation, regeneration studies were carried out (data not presented). In these studies, high frequency shoot regeneration was obtained from cotyledon explants on MS medium containing 6 mg l^{-1} BA and 0.3 mg l^{-1} IAA. A similar combination of hormones has been used with great efficiency in some earlier reports [17-19].

Kanamycin sensitivity test was shown to affect the growth of cotyledon explants on kanamycin concentration as low as 10 mg l^{-1}. A gradual decline in the fresh weight of explants was recorded every week on medium containing 20 mg l^{-1} and above concentration of kanamycin. The maximum effect was observed in the presence of 100 mg l^{-1} kanamycin. The explants turned brown after 7 days on this concentration. In the presence of 30, 40 and 50 mg l^{-1} of kanamycin, this effect was a little later i.e after 15-20 days. From the experimental data a negative correlation was obtained indicating the inhibition of growth (cell division) with an increase in concentration of kanamycin (Table 1). Cotyledon explants cultured on control medium (i.e shoot regeneration medium without kanamycin) showed normal growth.

During pre-culture, cotyledon explants showed active growth in MS medium containing 6 mg l^{-1} BA and 0.3 mg l^{-1} IAA. The cotyledon explants pre-cultured for 72 h showed better rates of survival on transfer to selective medium (after co-

cultivation), as compared to those pre-cultured for 24 h and 48 h (Table 2).

Callus initiation was found to be delayed after co-cultivation. Callusing was observed in those explants only which were pre-cultured for 48 h and 72 h. No shoot regeneration was observed from the cotyledon explants which were pre-cultured for 24 h, while in case of those pre-cultured for 48 h (co-cultivated for 72 h) only one putative transgenic shoot was obtained (Table 2).

Table: 1. Effect of different concentrations of kanamycin on the relative growth (with respect to the fresh weight) of cotyledon explants of bell pepper at different interval of time.

MS basal medium containing 6mg l^{-1} BA, 0.3mg l^{-1}IAA and different concentrations of kanamycin (mg l^{-1}) ← different concentrations of kanamycin (mg l^{-1}) →								
	0	**10**	**20**	**30**	**40**	**50**	**70**	**100**
No. of days	**Average fresh weight of explants (mg)**							
0	3.390	3.710	3.800	3.440	2.960	2.753	3.127	2.907
7	32.54	27.40	21.76	20.58	20.16	19.34	18.17	16.72
14	78.08	70.72	61.31	55.61	49.26	38.12	32.00	21.24
21	185.5	113.4	99.46	87.37	67.34	49.06	38.62	32.58
28	241.2	192.4	126.9	113.4	89.73	63.42	49.26	39.36
35	526.3	222.4	156.1	136.9	114.4	82.14	62.36	44.91
Factors:			$CD_{0.05}$			SE±		
Effect of Kanamycin concentration (I)			0.10			0.05		
Effect of no. of days (T)			0.12			0.06		
Effect of (T) and (I)			0.11			0.30		

Table: 2. Effect of pre-culturing and co-cultivation time on transformation frequency of cotyledon explants cultured on MS basal medium containing 6.0 mg l^{-1} BA, 0.3 mg l^{-1} IAA, 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin.

Sr. No.	**Pre-culture time (in h)**	**Time of co-cultivation (in h)**	**Explants cultured**	**Callus formation**	**Explants formed shoots**	**Per cent shoot regeneration**
1.	24	48	150	-	0.00	0.00(0.00)*
2.		72	150	-	0.00	0.00(0.00)
3.	48	48	150	+	0.00	0.00(0.00)
4.		72	150	+	1.00	0.66(1.08)
5.	72	48	350	+	3.00	0.85(1.16)
6.		72	350	+	8.00	2.28(0.88)
Factors:			CD0.05		SE±	
Effect of pre-culturing (T)			0.0039		0.0017	
Effect of co-cultivation (I)			0.0031		0.0014	

The values in the parenthesis are square root transformed values.

\+ Callus formation

\- No callus formation

Shoot regeneration from the cotyledon explants (pre-cultured as well as co-cultivated for 72 h) was observed after 40-45 days of transfer of explants to selective medium (Fig. 2A). The shoots obtained from cotyledon explants pre-cultured for 72 h and co-cultivated for 48 h were found to be chlorophyll deficient.

Though the duration of survival of the hypocotyl explants on the selective medium was greater as compared to the cotyledon explants, as some even survived for 2 months but per cent shoot regeneration was less (1.32%) as compared to cotyledon explants (2.28%). Many of the hypocotyl explants died after few days of transfer to selective medium. Initiation of callus from hypocotyl explants was observed after 20-22 days and shoot regeneration after 40 to 45 days on the

treatment following 72 h of pre-culture and 72 h of co-cultivation. It was interesting to note that some of the explants died after callusing on the same medium while other underwent proper callusing and adventitious shoot regeneration as well. In many of the earlier reports, the use of hypocotyl explants did not give significant results (as in the present studies) except that of Ashrafuzzaman *et al*. (2009) who have reported a high frequency of shoot regeneration from hypocotyl explants as compared to cotyledon explants [20].

For both the explants, the best results regarding transformation frequency were obtained on the treatment following 72 h of pre-culture and 72 h of co-cultivation (Table 2).

It has already been reported that bell pepper is recalcitrant with respect to regeneration especially at shoot elongation stage [21, 22]. Hyde and Phillips (1996) have used GA_3 and BAP in combination with $AgNO_3$ for shoot elongation [23]. Whereas, in some other reports only GA_3 have been used [24, 25]. In the present study, the shoots elongated about 1-1.5 cm in length after 30-35 d of culture on transfer to selective shoot elongation medium containing 2.25 mg l^{-1} BA, 2 mg l^{-1} GA_3, 500 mg l^{-1} cefotaxime and 50mg l^{-1} kanamycin (Fig. 2B).

Initiation of roots from the elongated shoots was observed after 20-23 d on transfer to selective root regeneration MS medium containing 0.25 mg l^{-1} IAA, 500mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin (Fig. 2C). It took 45-50 days in total for and the formation of complete putative transgenic plantlet with well-developed shoot and root system (Fig. 2D).

Genotypic characterization of transgenic shoots was done using PCR and phenotypic characterization was done by studying the expression of *gus* gene using GUS assay. Out of the 5 randomly selected putative transgenic plantlets, only 2 plantlets showed an amplified band of 750 bp and 800 bp, indicating the presence and integration of *npt*-II and *gus* genes respectively (Fig. 3A, B).

The β-glucuronidase activity measured in terms of nmole of p-nitrophenol liberated/h/g fresh weight of the leaf explants (taken from the two transgenic plantlets which showed amplification of the *npt*-II and *gus* gene) was found to be 2828.57 ± 0.04 and 2114.28 ± 1.15. Whereas, no β-glucuronidase activity was found in control (non-transformed) plantlets.

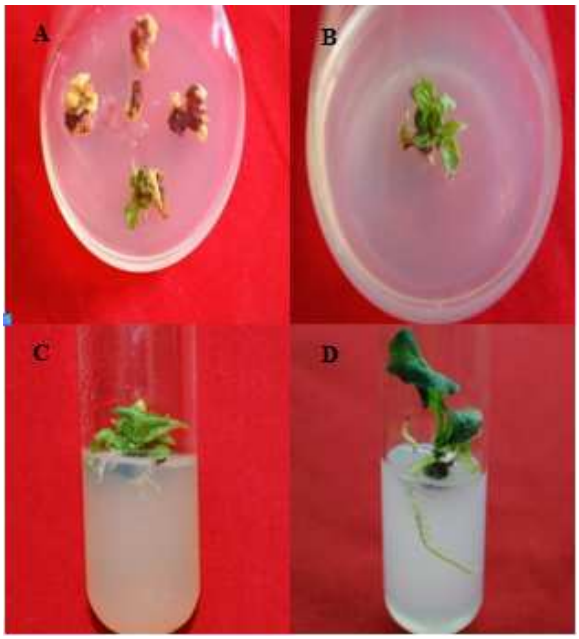

Fig: 2 (A-D). *In vitro* regeneration of putative transgenic plantlets.

(A) Cotyledon explants (pre-cultured and co-cultivated for 72 h) transferred to selective shoot regeneration medium **(**MS full strength basal medium containing 6.0 mg l^{-1} BA, 0.3 mg l^{-1} IAA, 500mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin) showing regeneration of putative transgenic shoots as well as dead non-transformed explants after 40-45 d in culture.

(B) Elongated putative transgenic shoots on selective shoot elongation medium (MS full strength basal medium containing 2.25 mg l^{-1} BA, 2.0 mg l^{-1} GA_3, 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin) after 30-35 d in culture.

(C) Putative transgenic shoot showing initiation of root on root regeneration medium (MS half strength basal medium containing 0.25 mg l^{-1} IAA, 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin) after 20-23 d.

(D) Development of complete putative transgenic plantlet after 40-45 d of transfer to selective root regeneration medium (MS half strength basal medium containing 0.25 mg l^{-1} IAA, 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin).

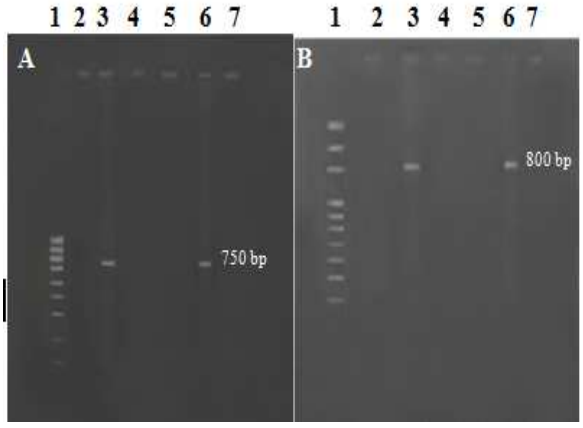

Fig: 3 (A-B). Molecular characterization of putative transgenic plantlets.

(A) PCR analysis showing amplification of (0.75kb) *npt*-II specific fragment in regenerated transgenic plantlets of bell pepper: lane 1 -100 bp DNA ladder; lane 2 - control (non-transformed) sample; lanes 3-7 -independent lineages of putative transgenic plantlets.

(B) PCR analysis showing amplification of (0.8 kb) *gus* specific fragment in regenerated transgenic plantlets of bell pepper: lane 1 -100 bp DNA ladder; lane 2 - control (non-transformed) sample; lanes 3-7 - independent lineages of putative transgenic plantlets.

Present communication reports successful standardization of pre-culture and co-cultivation time for *Agrobacterium*-mediated genetic transformation in *Capsicum annuum* L. cv. California Wonder. Standardization of this system is expected be of great value for the transfer of agronomically important genes. The presence of an easily identifiable marker linked to an agronomically desirable gene would facilitate efficient selection in this recalcitrant crop.

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