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Genetic Transformation for Pod Borer Resistance in Dolichos Bean [*Lablab purpureus* (L.) Sweet]

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ABSTRACT

Background: *Agrobacterium* mediated genetic transformation experiments were carried out in Dolichos bean Cv. (Konkan Bhushan) showing better regenerability.

Methods: Three *cry* genes *viz*. *cry1Aabc*, *cry1Fa1* and *cry2Aa* were used for the transformation each of which were linked to CaMV35S promoter and *nptII* gene under control of *nos* promoter and terminator. A vector system consisting of the disarmed hyper virulent *Agrobacterium tumefaciens* strain EHA-105 harboring *pBinAR* or *BinBt3* was used. Mature embryo axis with single cotyledon was used as explant. Kanamycin as well as PCR screening was carried out to assess the transformation frequency. Progeny analysis using PCR was also carried out to assess the transgene segregation and stable transformation.

Result: Kanamycin concentration of 500 mg/l was found as optimum for selection of a transgenic turning leaf blades albino. Among five methods of colonization used, the method employing mild injury to explant with dipping in *Agrobacterium* culture for 20 minutes followed by co-cultivation for 48 hours, cefotaxime washing and sowing in soil resulted in maximum survival (74.80%) associated with maximum transformation frequency through PCR analysis (2.13%). Among three *cry* genes, the gene *cry2Aa* was found the most effective in transforming Dolichos bean. The progeny analysis of transformants has shown the 3:1 mendelian segregation ratio confirming stable transformation of transgene.

Key words: *Agrobacterium tumefaciens,* Co-cultivation, Colonization, Survival, Transformation frequency.

INTRODUCTION

Lablab bean [*Lablab purpureus* (L.) Sweet var. typicus], also known as Dolichos bean, popularly known as Val in Maharashtra state, is a member of family fabaceae having chromosome number 2n=22. In India, it is grown mainly in Maharashtra, Uttar Pradesh, Madhya Pradesh, Tamil Nadu andhra Pradesh and Karnataka. In Konkan region, it is grown in Thane, Palghar, Raigad, Ratnagiri and Sindhudurg districts on residual moisture. Lablab bean has the capacity to fix atmospheric nitrogen in the soil and it can do relatively well even under poor soil fertility conditions since it is leguminous crop. Lablab bean yield is limited because of biotic and abiotic factors especially pathogenic, entomological, agronomical, genetic *etc.* The lower productivity of crop is attributed to many factors, among which pest is a major factor. The crop is attacked by as many as 55 species of insects and species of mites. Among them pod borer (*Helicoverpa armigera*) is considered to be the most important group causing loss to the tune of 80 to 100 per cent.

It feeds on buds, flowers and specially pods of plants. *Helicoverpa* have ability to complete a seven generation in a year and is present throughout the year. As there is lot of importance of the dolichos bean in agricultural economy of the Konkan region, it is necessary to apply tissue culture technique for genetic manipulation in plant to receive attention in agricultural research programs. Because of the limited genetic variability in the crop, conventional breeding is not useful. The *Agrobacterium*-mediated genetic transformation is more efficient and results in integration of potentially low copy number, high co-expression of the introduced genes and preferential integration into actively transcribable regions (Gheysen *et al*., 1998). The variety Konkan Bhushan being highly drought tolerant, late maturing and having good yield in Konkan conditions, but susceptible to pod borer, need to be converted as pod borer resistant. Hence the research aiming at standardization of *Agrobacterium* mediated genetic transformation technique in the Dolichos bean Cv. Konkan Bhushan was carried out.

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MATERIALS AND METHODS

The present was carried out at Plant Biotechnology Center, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist- Ratnagiri, during the year 2014-2017. The seeds of popular genotype of Dolichus Bean “Konkan Bhushan” were collected from Vegetable Improvement Scheme, Central

Experimental Station, Wakawali, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. Fresh and healthy seeds were washed with double distilled water and then dipped in 70% ethanol for 5 min followed by dipping in 0.1% mercuric chloride ($HgCl_2$) solution for 10 min and then washed with double distilled water for several times. These seeds were soaked in distilled water for 15-16 hours and then removed seed coat and isolated the mature embryo axes with single cotyledon (MEASC) as an explant for transformation. Three different strains of *Agrobacterium tumefaciens* having different genes *viz. cry1Aabc*, *cry1Fa1*and *cry2Aa* collected from NRCPB, New Delhi were used for genetic transformation experiments (Fig 1, 2 and 3). The disarmed hyper virulent *Agrobacterium tumefaciens* strain EHA-105 harboring pBinAR or BinBt3 was used as vector system. It contains the respective gene linked to cauliflower mosaic virus (CaMV) 35S promoter and neomycin phosphotransferase (*nptII*) gene under control of nopaline synthase (*nos*) promoter and terminator. The *Agrobacterium* strains were maintained in solid YEMA medium. Sub culturing was done every fortnight in fresh YEMA medium.

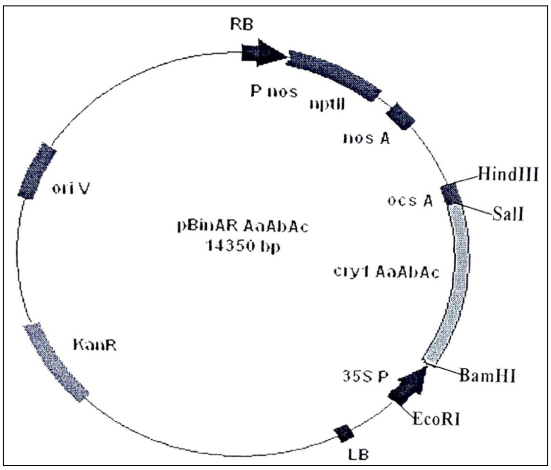


Fig 1: Map of the *Cry1Aabc* gene construct in Ti plasmid.

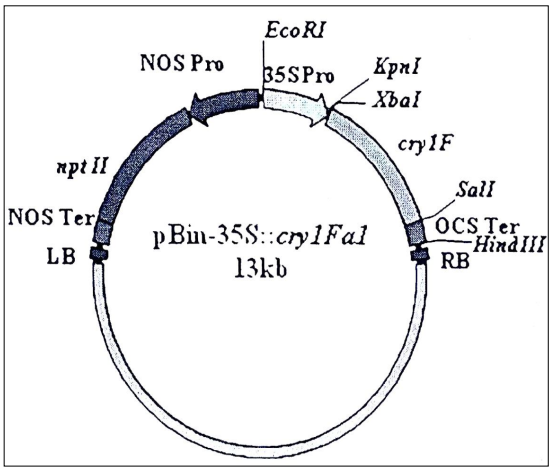


Fig 2: Map of the *cry1Fa1* gene construct in Ti plasmid.

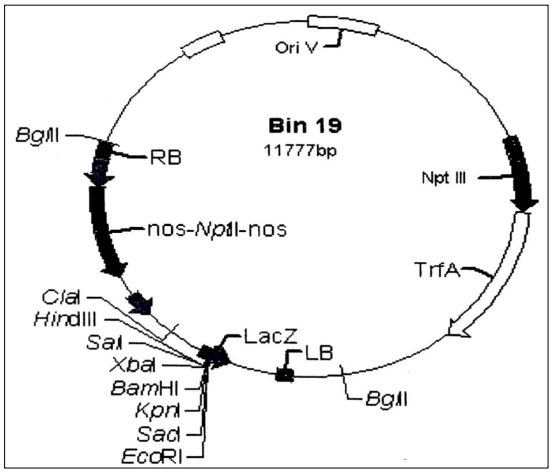


Fig 3: Map of the *cry2Aa* gene construct in Ti plasmid.

Prior to transformation events, kanamycin sensitivity test using leaf blades of normal plants was carried out with kanamycin concentration of 0, 100, 200, 300, 400, 500, 600, 700 and 800 mg/L in MS medium. Observations were recorded on seedlings died, stunted in growth or completely became albino. In Agrobacterium transformation event, initially single *Agrobacterium* colony was taken from the plate with the help of sterilized loop and inoculated into 25 ml YEM liquid medium containing 250 µl kanamycin (10 mg/ml) and was incubated on shaker for 48 hrs at 26°C temperature and fresh culture was used for transformation. The different five methods of colonization and co-cultivation were tried (Table 1). *Agrobacterium* infected explants were co-cultivated on multiple shoot induction medium MS (Murashige and Skoog, 1962) supplemented with 2 mg/l BAP in dark for 2 days. After co-cultivation, explants were washed with 800 mg/L cefotaxime for 20 min to eliminate *Agrobacterium* overgrowth completely and again put on medium MS + 2 mg/l BAP. Observation on plant survival rate was recorded. The leaf discs of regenerated shoots were transferred to selection medium containing kanamycin concentration found effective in kanamycin sensitivity test. Recovery of kanamycin resistant putative transgenic plants was recorded. Recovered plants were subjected to PCR analysis using gene specific primers to confirm the transformation frequency. Survival of plants and transformation frequencies for all the methods using three *cry* genes were compared. T_0 generation plants were grown to maturity to produce seeds from which T_1 generation was raised. Progeny analysis for gene integration was also carried out through PCR in T_1 generation. Chi-square test for Mendelian segregation ratio of 3:1 is used for finding out the goodness of fit with the formula:

$$X^2 = \sum \frac{(O-E)^2}{E} \sum \frac{di^2}{E}$$

Where,
O = Observed value.
E = Expected value.
X^2 = Chi-square value.
d = Deviation.

RESULTS AND DISCUSSION

Optimization of colonization and co-cultivation period

For production of transgenics, the first step is successful infection by bacterium and also successful incorporation in host genome. The site of incorporation of gene is also important for its expression. Bacterial colonization, induction of bacterial virulence system, generation of T-DNA complex, T-DNA transfer and integration of T-DNA into the plant genome are essential steps for transgenic production (Gustavo *et al*., 1998). Difference in susceptibility of the crop to *Agrobacterium* strain was also recorded in legumes such as chickpea (Islam *et al*., 1999), pea (Hobbes *et al*., 1989) and groundnut (Lacorte *et al*., 1991).

In the present transformation study, three stains of *cry* genes were used with five different methods as given in Table 1. Among these, *cry1Fa1* showed maximum (62.50%) survival percentage when MT_1 method was followed (Table 2). However, the frequency of putative transgenics was more in MT_5 (2.13%) when *cry2Aa* strain was used. This could be due to *vir* gene factors which influence the infection by different *cry* genes (Surekha *et al*., 2007).

It could be seen from Table 2 that the infection method employing mild injury to explant and colonization in *Agrobacterium* culture having *cry2Aa* gene for 20 minutes under aseptic conditions followed by co-cultivation for 48 hrs and after cefotaxime washing put for germination in soil (MT_5) showed highest survivability of explants (74.80%) while longer period of colonization (30 min) and 3 days co-cultivation (MT_3) reduced regeneration and survivability (17.69%). However, the lowest survival (6.00%) was observed in method MT_4 with *cry1Aabc* and *cry2Aa* gene in which bacterial suspension culture was injected into explants pre cultured in the medium supplemented with MS + 2 BAP for 2 days and co-cultivated for 48 hrs on same media. These reports are in agreement with Kumar *et al*. (2004); Surekha *et al*. (2007); Sharma *et al*. (2006) and Rao *et al*. (2005). Similar successful transformation in pigeonpea with *cry1Ac* gene using pricked embryo axes as explant was also reported by Kaur *et al*. (2016). It clearly indicates that if axillary buds properly colonized with *Agrobacterium* without disturbing meristematic zone with mild injury, then there will be least harmful effect of co-cultivation on regeneration. Therefore survival percentage was found more in MT_5 method.

Selection and identification of transformed shoots

In kanamycin sensitivity test, the kanamycin concentration of 500 mg/L added to MS medium turned leaf blades of normal plants completely albino (Table 3). Therefore, this concentration of kanamycin was further used for screening of putative transgenic plants. Leaf blade explants excised from well-established putative transgenic plants were subjected to kanamycin screening. Effective selection was observed at 500 mg/l kanamycin turning non transformed plants completely albino. Result of this experiment indicate that stringent selection pressure is required to identify lethal dose of kanamycin for selection of putative transgenic, in case of explants derived from well-established plants. In earlier reports *viz.* Lawrence and Koundal (2001); Kumar *et al*. (2004); Basavanna *et al*. (2008) and Surekha *et al*. (2007) the lower concentration of kanamycin for selection of putative transgenic was mentioned. However, in the present study, at lower concentration only yellowing was observed and complete albino plants recorded at highest concentration. This indicates that dose of kanamycin for effective selection depends on age and type of tissues used for screening. At lower concentrations, tissues show only yellowing at the side of leaves and sometimes it will lead to false selection of transgenic plants. However, at the higher

Table 1: Different methods of colonization.

Method no.	Treatment
MT_1	Explants were injected by *Agrobacterium tumefaciens* under aseptic condition and co-cultivated for 48 hrs.
MT_2	Explants injured mildly and dipped in *Agrobacterium* for 20 minutes under aseptic conditions and co-cultivated for 48 hrs.
MT_3	Explants dipped for 30 minutes and co-cultivated for 3 days.
MT_4	Bacterial suspension culture was injected into explants pre cultured in the medium supplemented with MS + 2 BAP for 2 days and co-cultivated for 48 hrs on same media.
MT_5	Explants injured mildly and dipped in *Agrobacterium* for 20 minutes under aseptic conditions and after co-cultivation of 48 hrs explants were washed with varied concentration of cefotaxime with several times and blotted it with sterilized blotting paper and inoculated in regeneration media. Some of the explants were directly sown in soil.

Table 2: Effect of co-cultivation of different *cry* genes on survivability and plantlet establishment.

Method	Number of explants co-cultivation			Number of plants established			Survival per cent		
	cry1Aabc	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*
MT_1	250	320	850	150	200	335	60.00	62.50	39.41
MT_2	280	340	1034	170	150	500	60.71	53.57	48.35
MT_3	230	360	650	90	105	115	39.13	29.16	17.69
MT_4	200	210	250	12	18	15	06.00	08.57	06.00
MT_5	290	390	1250	200	270	935	68.96	69.23	**74.80**

concentration non transgenic tissues completely became albino and confirm the integration of transgenic in green and healthy tissues only. The highest number (12) of kanamycin resistant plants was obtained in MT_2 method of transformation followed by MT_1 (Table 4). The benefit of this type of selection was that the original plants were grown without selection pressure so healthy, rooting was observed in rooting medium. It is in agreement with report of S.V. Sawardekar (2007) which emphasizes the importance of relating or even increasing concentration of selection chemical to minimize the number of putative transformed plants.

Recovery of transformed plants

In all circumstances the desired product of transformation must be regenerated to express the introduced gene. It is, therefore important that the cells, tissues and explants selected for transformation exhibit high regeneration. Frequency of the transformed cells is influenced by the method of transformation and the presence of selecting agent (*e.g.* an antibiotic) and other antibiotic used for elimination of *Agrobacterium* in the culture medium (Mhatre, 2011). Sensitivity to the transformation system of different bacterial strains may also affect the transformation frequency. The type of regeneration is also critical in the recovery of transformed plants. Transformed plants are obtained only if regeneration occurs within the region of the explants where the cells can come in contact with *Agrobacterium*.

In the present investigation, among different methods followed, MT_5 method showed highest recovery of putatively transformed plants (2.13%). This indicate that for efficient transformation and regeneration of transgenic plant, one should follow 5 important points: i) Selection of regenerable explants having meristematic tissues ii) pre-culturing of explants and use of growth regulators during co-cultivation iii) Avoidance of longer time co-cultivation and colonization iv) Avoidance of selection pressure immediately after co-cultivation and v) Elimination of *Agrobacterium* from explants.

Table 3: Studies on sensitivity to Kanamycin.

Concentration of Kanamycin (mg/l)	Leaf blade
MS + 0	+++
MS + 100	+++
MS +200	+
MS + 300	+
MS + 400	+
MS + 500	0
MS + 600	0
MS + 700	0
MS + 800	0

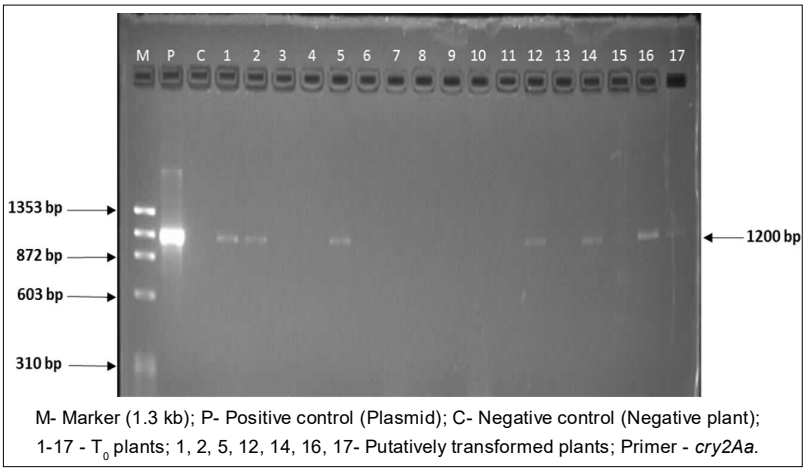
+++: Fresh green; +: Pale green; 0: Albino.

Molecular characterization: Integration and expression

One of the easiest ways to detect the integration of transgenic is through polymerase chain reaction (PCR). Among the different methods followed MT_5 method of transformation showed highest transformation frequency of 2.13 per cent (Table 4). Results obtained from PCR assay

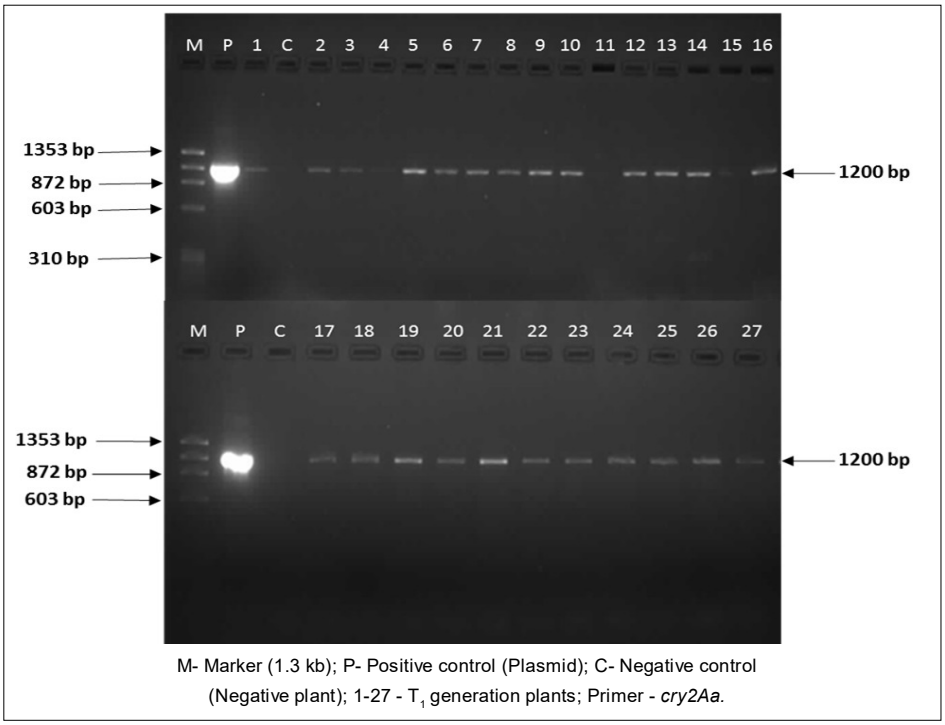
Table 4: Frequency of putative transgenics.

Method	Number of plants established			No. of Kanamycin resistance plants			PCR positive plants			Transformation frequency (%)		
	cry1Aabc	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*
MT_1	150	200	335	6 (4.00%)	4 (2.00%)	7 (2.08%)	2	1	3	1.33	0.05	0.89
MT_2	170	150	500	8 (4.70%)	7 (4.66%)	12 (2.40 %)	1	2	5	0.58	1.33	1.00
MT_3	90	105	115	4 (4.44%)	3 (2.84%)	3 (2.60%)	1	0	2	1.11	0.00	1.73
MT_4	12	18	15	0	0	0	0	0	0	0.00	00.00	0.00
MT_5	200	270	935	-	-	-	2	4	20	1.00	1.48	**2.13**



M- Marker (1.3 kb); P- Positive control (Plasmid); C- Negative control (Negative plant); 1-17 - T_0 plants; 1, 2, 5, 12, 14, 16, 17- Putatively transformed plants; Primer - *cry2Aa.*

Fig 4: PCR assay of T_0 generation plants.



M- Marker (1.3 kb); P- Positive control (Plasmid); C- Negative control (Negative plant); 1-27 - T_1 generation plants; Primer - *cry2Aa*.

Fig 5: PCR assay of T_1 generation plants.

Table 5: Chi-square test for Mendelian segregation of *cry2Aa* gene.

T_0 plant	Total plants established in T_1	Observed no. of plants (O)		Expected no. of plants (E)		$(O-E)^2/E$		$X^2 = \sum \frac{(O-E)^2}{E}$
		PCR+ve	PCR-ve	PCR+ve	PCR-ve	PCR+ve	PCR-ve	
1	9	6	3	6.75	2.25	0.08	0.25	0.33
2	7	4	3	5.25	1.75	0.30	0.89	1.19
3	5	3	2	3.75	1.25	0.15	0.45	0.60
4	8	5	3	6.00	2.00	0.17	0.50	0.67
5	7	4	3	5.25	1.75	0.30	0.89	1.19
6	4	3	1	3.00	1.00	0.00	0.00	0.00
7	4	2	2	3.00	1.00	0.33	1.00	1.33
8	6	4	2	4.50	1.50	0.06	0.17	0.22
9	6	4	2	4.50	1.50	0.06	0.17	0.22
10	5	3	2	3.75	1.25	0.15	0.45	0.60
11	4	3	1	3.00	1.00	0.00	0.00	0.00
12	7	5	2	5.25	1.75	0.01	0.04	0.05
13	10	7	3	7.50	2.50	0.03	0.10	0.13
14	9	6	3	6.75	2.25	0.08	0.25	0.33
15	9	7	2	6.75	2.25	0.01	0.03	0.04
16	10	6	4	7.50	2.50	0.30	0.90	1.20
17	6	4	2	4.50	1.50	0.06	0.17	0.22
18	7	4	3	5.25	1.75	0.30	0.89	1.19
19	7	5	2	5.25	1.75	0.01	0.04	0.05
20	4	3	1	3.00	1.00	0.00	0.00	0.00
Total	134	88	46	100.50	33.50	1.55	4.66	6.22

X^2 Table value at 1% level of significance and 1 d.f. = 6.635.

Comparison = The calculated value of X^2 is less than the table value.

Result = The differences are not significant.

Conclusion = The fit is good for 3:1 ratio at 1% hence the assumption that the segregation on the basis of 3:1 ratio is correct.

for detection of *cry2Aa* gene construct (1.2 kb) in T_0 generation transformed plants is presented in Fig 4. Amplification at the range of plasmid of respective gene construct indicates the stable integration of gene under study.

In other pulse crops it ranged from less than 0.1 per cent (Kumar *et al*., 2004; Basavanna *et al*., 2008 and Surekha *et al*., 2007) in *Agrobacterium*-mediated and other methods of transformation.

In this study, transgenic dolichos bean was produced by *Agrobacterium*-mediated transformation using mature embryo axes with single cotyledon (MEASC) as an explant. The ability to regenerate dolichos bean in *in vitro* as well as use of positive selection system may provide a means for recovery of genetic transformation. Till now, limited studies were conducted, but it has been not commercialized. However the present study showed that *Agrobacterium mediated* transformation is a possible approach to develop and commercialize transgenic plants in dolichos bean.

Progeny analysis

In the present investigation, emphasis was given for the development of heritable pod borer transgenic dolichos bean. The results obtained from study conducted on progeny analysis by chi-suare test indicated that most of these plants showed Mendelian segregation pattern (Table 5). Some plants also showed non mendelian segregation pattern. In all the progenies PCR positive plants were identified (Fig 5). Same kind of segregation pattern was reported by many scientists in different crops including pulses as Kaur *et al*. (2016) in pigeonpea being one of the recent ones; Sawardekar *et al*. (2007) in chickpea, Budar *et al.* (1986) in tobacco, Feldman and marks (1987) in *Arabidopsis etc.* It indicates the heritable transformation of *cry2Aa* gene in dolichos bean cv. Konkan Bhushan.

CONCLUSION

The present study showed that dolichos bean can be effectively transformed by *in vitro Agrobacterium*-mediated transformation. In dolichos bean we report 2.13 per cent transformation frequency.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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