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Optimization of regeneration protocol and *Agrobacterium* mediated transformation in carnation (*Dianthus caryophyllus* L.)

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ABSTRACT

An efficient and reproducible regeneration protocol for carnation genotypes Arka Flame and IIHRS-1 has been developed from leaf and stem explants. Although IIHRS-1 showed slightly higher regeneration (55%) compared to Arka Flame (49.2%), there was no significant difference in their regeneration response. However, significant difference in regeneration potential was observed with leaf explant exhibiting higher regeneration potential (5.5 shoots/explant) as compared to (4.9) stem explant. Among various plant growth regulator combinations tested for regeneration, the best regeneration response and maximum regeneration potential was obtained in MS medium supplemented with NAA (0.1 mg/l) and TDZ (1.0mg /l) for both the explants and genotypes used. The medium also proved suitable for inducing elongation of regenerated shoots. Rooting of *in vitro* formed shootlets could be induced at greater frequency in MS medium supplemented with IAA (1.0 mg/l). Based on this protocol, transformation was carried out in genotype IIHRS-1 using leaf explants with *Agrobacterium tumefaciens* LBA 4404 with binary vector pROK2 containing baculovirus chitinase gene under the control of 35S promoter with *npt* II serving as selectable marker. There was regeneration of putative transformants at a frequency of 28.9%. However, great difficulty was encountered in rooting of shoots. Hence a few shoots regenerated on selection medium at random were tested for transgene integration. Out of the three shoots tested for *npt* II amplification, two shoots tested positive. The presence of transgene was confirmed through PCR amplification of *npt* II gene and dot blot analysis of chitinase gene.

Key words: Carnation, genotype, morphogenesis, *Agrobacterium* mediated transformation

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.) is one of the most important commercial flowers in the world. To date, new carnation varieties have been produced mainly through traditional breeding, and are propagated vegetatively. However, high heterozygosity, a limited gene pool, and almost no knowledge of carnation's genetic makeup, severely restrict breeding programs (Woodson, 1991). Moreover, there are no varieties available in India to match International standards. Carnation breeders at, Indian Institute of Horticultural Research, Bangalore, have recently released a variety Arka Flame, the flower quality of which is on par with International Standards. They have also identified another promising genotype IIHRS-1 that can serve as basic material for future varietal improvement (Fig 1).

Recent developments in plant molecular biology open the way for unprecedented opportunities to use the technique of genetic engineering for improvement and value addition of flower crops. The availability of methods to introduce a useful defined gene(s) would enable the specific alteration of a single trait and broaden the gene pool available for this crop. The most commonly used method for introduction of genes is *Agrobacterium* mediated transformation. An essential step towards development of transgenic plants through *Agrobacterium* mediated transformation is the development of an efficient regeneration protocol. Attempts have been made to regenerate carnation through tissue culture via organogenesis from petals, stem, leaf (Nugent *et al*, 1991;Van Altvorst *et al*, 1994) and somatic embryogenesis (Frey *et al*, 1992; Yantcheva *et al*, 1998). Similarly there are reports on

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Fig.1. Flowers of Arka Flame and genotype IIHRS-1

Agrobacterium mediated transformation in carnation using different explants viz., stem, petals, leaves (Lu *et al*, 1991; Zuker *et al*, 2001a). However, there are differences in the efficiency depending on the explant or genotype used. Keeping this in view, the present investigation was carried out to compare the two genotypes *viz.*, Arka Flame and IIHRS-1 and explants viz., stem and leave for their morphogenetic response and to identify the suitable genotype and explant for *Agrobacterium*-mediated transformation.

MATERIAL AND METHODS

Plant Material

In vitro grown carnation Arka Flame and IIHRS-1 were multiplied through nodal cuttings on MS medium containing BAP 0.25 mg/l, GA_3 0.25 mg/l and NAA 0.1mg/l. These multiplied plants served as source of explants (Fig 2).

In vitro regeneration

Leaf and stem explants obtained from 15-20 day old *in vitro* grown cultures were inoculated on MS (Murashige and Skoog,1962) medium containing 3% sucrose with factorial combinations of cytokinins and auxins, viz., benzylaminopurine (BAP) or thidiazuran (TDZ) and naphthalene acetic acid (NAA), respectively. The medium was gelled with phytagel (0.25%) Sigma Chemical Co.(USA) and pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. Cultures were incubated in culture racks provided with cool white fluorescent tubes with a light intensity of 30-40 mmoles $m^{-2}s^{-1}$ under a 16 h photoperiod in a culture room maintained at 25°C ± 2°C. Elongation of shoot buds could be achieved on sub culturing shoot buds to the same regeneration medium. Shootlets obtained were transferred to MS medium (full or half strength) with various auxins or their combinations viz., Indole - 3 - butyric acid (IBA) or Indole-3-acetic acid (IAA) for root induction. Rooted plantlets were transferred to polybags containing autoclaved mixture of sand, soilrite and soil in the ratio of 1:2:1 watered to field capacity and were hardened adopting the closed sachet technique (Ravindra and Thomas, 1995).

Fig 2. Source of explants

Transformation and regeneration

Agrobacterium strain LBA4404 containing pR0K2 vector with baculovirus *chitinase* gene cloned at *Bam*HI site under the control of 35S promoter and selectable marker gene *npt* II under the control of *nos* promoter was used for transformation (Fig 3). Bacterial strain was grown overnight (O/N) in Yeast Extract Mannitol (YEM) medium containing kanamycin at 50 mg/l and collected at log phase, when the absorbance at 600nm was 1.0. The O/N grown culture was centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was discarded and bacterial pellet resuspended in half strength MS medium. For transformation studies, only the leaf explant from genotype IIHRS-1, which showed maximum regeneration potential and the medium in which it was achieved, was used for transformation work.

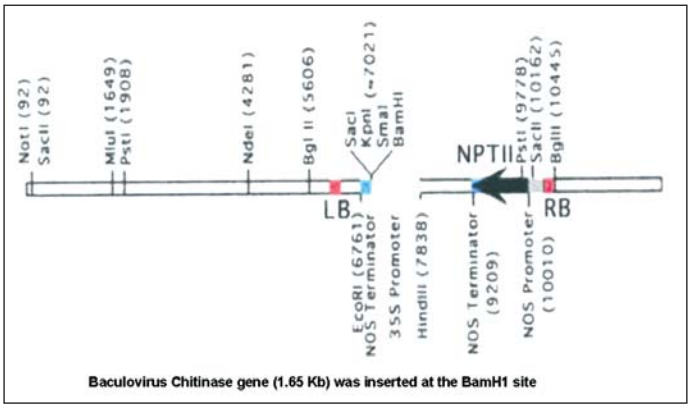

Fig 3. T-DNA of plasmid pROK2 containing 1.65 kb fragment of baculovirus chitinase gene inserted at the multiple cloning site following digestion with *Bam*H1

The leaf explants were infected with *Agrobacterium* culture for 5-30 min. blotted dry with filter paper and placed in the regeneration media for co-cultivation for 1-5 days under 16h photoperiod or under complete darkness for varied periods of time. Thereafter, the explants were transferred to selection medium containing 75mg/l kanamycin and 500 mg/l of cefotaxime. The explants with putative transformed shootlets were transferred to rooting media containing 50mg/l kanamycin and 500 mg/l cefotaxime. The rooted transformed plants were hardened and transferred to pots.

Confirmation of the presence of transgene

PCR analysis: DNA was isolated from leaves of control, transformed plants and *Agrobacterium* plasmid following CTAB method (Sambrook *et al*, 1989). The primers of the *npt* II gene used were as follows:

Forward primer (5'GATGGATTGCACGCAGG3')

Reverse Primer (3'GAAGGCGATAGAAGGCG5')

PCR reaction was carried out in 25 ml containing 2.5ml of 100ng of sample DNA, 0.2ml of 10 mM dNTPs mix, 2.5 ml of 10x assay buffer for *Taq* polymerase containing 15mM $MgCl_2$, 0.5 units of *Taq* DNA polymerase, 1 ml of 10mM each of forward and reverse primers. DNA was subjected to initial denaturation of 94°C for 2 min and 35 cycles of 94°C for 1 min, 60°C for 45sec and 72°C for 1.5min with a final extension of 72°C for 10min. Amplified DNA fragments were electrophoresed on 1.5% agarose gel and observed under UV light.

Dot blot assay: Genomic DNA (5 µg) from PCR positive transformed plants and DNA of plasmid pROK2 were blotted on to nylon membrane (Hybond N^+ Amersham pharmacia) and hybridized with a labeled baculovirus chitinase probe, washed and detected as per the manufacturer's instructions of AlkPhos direct labeling and detection kit (Amersham Pharmacia Biotech UK Ltd). The baculovirus chitinase probe was prepared by amplifying it from the plasmid using the gene specific primers as given in Shi *et al* (2000). The PCR product was then labelled and used as a probe.

Statistical analysis

The experiment on *in vitro* regeneration (Tables1 & 2) was carried out in two genotypes using two explants viz., leaf and stem with 5 treatments. For each treatment, 5 tubes were used for each explant and the experiment repeated 6 times in a completely randomized design. The response from the 30 tubes was recorded with 10 tubes representing each replicate. The data indicated in the tables are means of replicated values. The data in Table 1 and 2 were transformed using Arc sine and square root transformation, respectively. The data were analyzed for three way interaction and subjected to analysis of variance (ANOVA). Comparison among treatment means were carried out using LSD values and are reported under "CD" at the end of each table

RESULTS AND DISCUSSION

In vitro *regeneration*

Several factors are known to influence *in vitro* regeneration from cultured plant tissue. Genotypic differences in shoot regeneration ability among cultivated

Table 1. Effect of different plant growth regulator (PGR) combinations on per cent regeneration of shoots from leaf and stem explants of carnation genotypes

Genotype (A)	Plant growth regulators (mg/l) (C)				Explant (B)		Mean
	BAP	NAA	TDZ		Leaf	Stem	
IIHRS-155.0(48.7)	1.0	0.1	-	23.32(28.65)	26.66(30.98)	23.33(28.77)	24.99(29.88)
	1.0	0.3	-	49.12(44.55)	56.60(49.20)	50.00(44.98)	53.33(47.09)
	-	0.1	1.0	75.78(62.20)	86.60(72.76)	73.33(59.19)	79.96(65.97)
	1.0	0.1	0.3	59.97(50.98)	63.30(53.05)	60.00(50.83)	61.65(51.94)
Arka Flame 49.1 (44.5)	1.0	0.1	-		20.00(26.06)	23.33(28.77)	21.65(27.41)
	1.0	0.3	-		46.66(42.98)	43.33(41.05)	44.95(42.01)
	-	0.1	1.0		76.66(61.90)	66.66(54.97)	71.60(58.43)
	1.0	0.1	0.3		60.00(51.12)	56.66(48.91)	58.30(50.02)
Mean					54.5 (48.5)	49.6 (44.7)	

CD (P ≤0.05); A= n.s ; B = n.s ; C = 12.0, (7.89); AxB = n.s ; AxC = n.s ; BxC = n.s ; AxBxC = n.s

n.s.- Not significant

Values in parentheses indicate arc-sine transformed values

Table 2. Effect of plant growth regulator (PGR) on average number of shootlets per explant of carnation genotype

Genotype (A)	Plant growth regulators (mg/l) (C)						
	BAP	NAA	TDZ		Explant (B)		Mean
					Leaf	Stem	
IIHRS-15.23 (2.25)	1.0	0.1	-	2.89(1.70)	3.13(1.77)	2.70(1.64)	2.91(1.70)
	1.0	0.3	-	3.67(1.91)	3.97(1.99)	3.67(1.91)	3.82(1.95)
	-	0.1	1.0	7.39(2.71)	7.76(2.78)	6.80(2.62)	7.28(2.70)
	1.0	0.1	0.3	6.80(2.61)	7.57(2.75)	6.23(2.54)	6.90(2.64)
Arka Flame5.14 (2.22)	1.0	0.1	-		3.10(1.75)	2.63(1.62)	2.87(1.69)
	1.0	0.3	-		3.50(1.87)	3.53(1.88)	3.51(1.87)
	-	0.1	1.0		7.60(2.76)	7.40(2.72)	7.50(2.74)
	1.0	0.1	0.3		7.20(2.68)	6.20(2.49)	6.70(2.59)
Mean					5.48(2.29)	4.89 (2.18)	

CD (P ≤0.05); A= n.s; B = n.s ; C = 12.0, (7.89); AxB = n.s ; AxC = n.s ; BxC = n.s; AxBxC = n.s
n.s.- Not significant

Values in parentheses indicate square root transformed values

carnation are known to exist (Firoozabady *et al*, 1995). In the present study, however, no significant difference among the two genotypes was observed although slight differences in the response of genotypes to shoot regeneration was recorded with IIHRS-1 recording higher regeneration (55.0%) followed by Arka Flame with 49.2%. Differences therefore, in regeneration response have been linked to the explant used.

Carnation regeneration has been reported through the use of various explants viz., petal, stem (Nugent *et al*, 1991) and leaf (Van Altvorst *et al*, 1994). Other explants such as anthers, ovule and axillary bud have been occasionally used for regeneration with differences in the regeneration ability. However, leaf and stem are the preferred explants and superior to other explants due to their high regeneration potential as well as better quality of plants regenerated.

Currently most of the work on regeneration of carnation is restricted to these two explants, although there are specific reports on differences in terms of regeneration ability between these two explants. In the present study there was no significant difference in the regeneration response of the explants used, with both leaf (54.6 %) and stem (49.6%) explants giving almost similar regeneration response in both the genotypes tested. However, there was a significant difference between the two explants in their regeneration potential with leaves regenerating more shootlets per explant (5.5) as compared to stem explant (4.9) (Tables 1 and 2, Fig 4 and 5).

Among the growth regulators used, it was found that both regeneration percentage and regeneration potential of the explant was expressed at its maximum level with the use of TDZ and NAA. Incorporation of two cytokinin TDZ and BAP along with NAA proved to be superior (59.9%) over BAP and NAA (23.3%). However, the best regeneration response (75.8%) was obtained in a medium supplemented with NAA (0.1 mg/l) and TDZ (1.0 mg/l) irrespective of the explant and genotype used (Table 1). There was significant reduction in the regeneration response with the use of BAP and NAA. Superiority of TDZ over BAP in regeneration of shoots from explants has been reported by Nugent *et al* (1991). Most of the reports on carnation regeneration have utilized combination of BAP and NAA (Nugent *et al*, 1991; Firoozabady *et al*, 1995; Van Altvorst *et al*, 1996). There are few reports on the use of TDZ along with NAA (Nugent *et al*, 1991 and Sankhla *et al*, 1995). The concentration of BAP and NAA has been found to influence only the regeneration potential of the explant (number of shoots/regenerating explant) and not the regeneration percentage. Highest numbers of shoots were obtained on medium containing 0.9 mg/l BAP and 0.3 mg/l IAA (Van Altvorst *et al*, 1994). However, Sankhla *et al* (1995) reported that prolonged growth in TDZ resulted in hyperhydricity. Hyperhydricity was encountered in the present study as well in both the cultivars of carnation, irrespective of the type of cytokinin used. The problem of hyperhydricity could be checked by incorporation of agar and phytagel in equal proportion in the medium for gelling and supplementing with mannitol (500 mg/l) in addition to plant growth regulators. Complete replacement of phytagel with agar resulted in cracking of the medium and poor response of explants.The regeneration media proved suitable for inducing elongation of the regenerated shoots (Fig 6).

Fig 4. Regeneration from leaf explant of IIHRS-1

Fig 5. Regeneration from stem explant of IIHRS-1

Rooting of *in vitro* formed shoots was achieved in MS medium supplemented with auxins viz., IAA and IBA. Among these two, IAA (1.0 mg/l) proved to be superior in inducing rooting (81.2%) as compared to IBA (47.9%) at the same concentration. Among the genotypes, highest induction of rooting (70.8%) was noticed in IIHRS-1 compared to Arka Flame (58.3%). Similarly shoots regenerated from stem explants recorded highest rooting (72.9%) as compared to shoots regenerated from leaf (56.2%) (Table 3, Fig 7). There was no difference among genotypes in *ex vitro* establishment of rooted shoots. They established with 75-80% success in *ex vitro* poly bags containing sand, soilrite and soil in the ratio of 1:2:1 and subsequently transferred to earthen pots (Fig 8).

Fig 6. *In vitro* shoot elongation

Fig 7. Rooted shoots

Transformation

The genotype IIHRS-1 was used for transformation work due to its higher regeneration response. Although leaf and stem explants gave almost similar regeneration response, leaf explants were used due to their significantly higher regeneration potential. Further it was found that stem explants produced only callus on selection media and the callus turned brown subsequently without any shoot regeneration. *Agrobacterium* mediated transformation of carnation has been successful with the use of various explants viz., stem (Lu *et al*, 1991; Zuker *et al*, 2001a), leaf (Firoozabady *et al*, 1995) and petals (Van Altvorst *et al*, 1996; Miroshnichenko and Doglov, 2000). Among these, both stem and leaf explants have been widely used. The use of petals for transformation has been less successful, despite the extremely high regeneration potential of these

Table 3. Effect of plant growth regulator (PGR) combinations on percent induction of rooted shoots in carnation genotypes

Genotype (A)	Plant growth regulators (mg/l) (C)		Explant (B)		Mean
			Leaf	Stem	
IIHRS-170.83 (58.7)	IAA (1.0)	81.24 (65.8)	83.33 (66.7)	91.66 (73.4)	87.49 (70.0)
	IBA (1.0)	47.91 (43.8)	50.00 (45.0)	58.33 (49.9)	54.16 (47.4)
Arka Flame 58.33 (50.8)	IAA (1.0)		58.33 (49.8)	91.66 (73.4)	74.99 (61.6)
	IBA (1.0)		33.33 (35.2)	50.00 (45.0)	41.66 (40.1)
Mean			56.24 (49.2)	72.91 (60.4)	

CD (P ≤0.05); A = 9.16, (5.77); B = 9.16, (5.77); C = 9.16, (5.77); AxB = n.s ; AxC = n.s; BxC = n.s ; AxBxC = n.s

n.s.- Not significant

Values in parentheses indicate angular transformed values

explants because of their ability to induce premature flowering *in vitro* and difficulty to transfer *ex vitro.*

There was an increase in the percent regeneration response with increase in inoculation time up to 20 min. There after, there was a decline in percent explant regeneration due to *Agrobacterium* overgrowth and death of explants (Table 4). Hence an inoculation time of 20 minutes with an undiluted *Agrobacterium* culture grown overnight with an O.D of (0.9-1.0) at 600nm followed by 5 days of co-cultivation was required for sufficient infection to take place. It was observed that carnation leaf explants were resistant to infection by *Agrobacterium* as evidenced by the lack of *Agrobacterium* overgrowth even after 3-4 days of co-cultivation. The waxy nature of carnation leaves may be the reason for its resistance to *Agrobacterium* infection. Such a long co-cultivation time has been recommended in carnation (Ahroni, 1996).

Light was another important factor, which was found to influence the growth of *Agrobacterium* and retention of healthy explants and their regeneration following inoculation with *Agrobacterium.* Incubating the explants after inoculation for 3 days under complete darkness followed by incubation under 16h photoperiod for 2 days resulted in greater number of explant regeneration (51.6%) as compared to incubation of explants under 16h photoperiod (28.3%) or total darkness (33.3%) on all 5 days for co-cultivation. (Table 4) Co-cultivation under 16h photoperiod and total darkness for all 5 days resulted in *Agrobacterium* overgrowth by 3rd day itself as compared to overgrowth appearance on 4th day when co-cultivation was carried out 3 days under complete dark followed by 2 days in 16hour photoperiod condition (Data not shown).

Five days co-cultivated explants were transferred to selection medium containing cefotaxime (500 mg/l) and kanamycin (75 mg/l). There was no need to transfer the co-cultivated explants to cefotaxime medium prior to transfer to selection medium, as there was no *Agrobacterium* overgrowth. In the present study 75mg/l was the concentration at which there was no regeneration from the leaf explant without *Agrobacterium* co-cultivation and hence

Table 4. Effect of inoculation time (min.) and photoperiod (light) during co-cultivation with *Agrobacterium* on percent healthy explants

Time (min)(A)	Photoperiod (B)			Mean (A)
	3 days dark and 16h photoperiod (2days)	Complete dark (5days)	16 h photoperiod (5days)	
		% healthy explants		
5	59.0 (50.1)	55.8 (48.8)	55.8 (48.5)	56.9 (49.2)
10	57.4 (49.4)	55.2 (48.1)	51.2 (46.1)	54.6 (47.9)
15	57.2 (49.2)	54.6 (47.9)	51.2 (45.7)	54.3 (47.6)
20	57.4 (49.6)	55.0 (48.0)	51.8 (46.1)	54.7 (47.9)
30	49.0 (44.4)	46.2 (42.8)	40.6 (39.3)	45.3 (42.1)
Mean (B)	56.0 (48.6)	53.4 (47.1)	50.1 (45.1)	
CD ($P \leq 0.05$); A = n.s ; B = n.s ; AxB = n.s				
n.s.- Not significant				

Values in parentheses indicate angular transformed values

Table 5. Effect of inoculation time (min) and photoperiod (light) during co- cultivation with *Agrobacterium* on percent explant regeneration

Time (min.) (A)	Photoperiod (B)			Mean (A)
	3 days dark and 16 h photoperiod (2 days)	Complete dark (5 days)	16 h photoperiod (5 days)	
		% explant regeneration		
5	15.1 (22.6)	10.0 (18.3)	3.3 (8.6)	12.7 (16.3)
10	25.0 (29.7)	13.3 (21.2)	11.7 (19.9)	15.8 (23.6)
15	38.3 (38.2)	21.6 (27.5)	18.3 (25.1)	26.4 (30.3)
20	51.6 (45.9)	33.3 (35.2)	28.3 (32.0)	35.5 (37.7)
30	33.3 (35.2)	20.0 (26.0)	16.7 (23.3)	23.7 (28.2)
Mean (B)	30.0 (34.3)	21.6 (25.6)	16.9 (21.7)	
CD ($P \leq 0.05$); A = 7.22, (5.12); B = 5.56, (3.97); AxB =12.5, (8.87).				

Values in parentheses indicate angular transformed values

the same concentration of kanamycin was used for selecting transformants.

There was regeneration of 28.9 % of putative transformants on selection medium (Fig 9) and 50% of the regenerated transformants showed elongation in the same medium (Fig 10). However, great difficulty was encountered in rooting of these shoots. Incorporation of antibiotics in selection media has been reported to reduce multiplication and rooting rates (Cassels, 1991). The rooting ability of transgenic carnation plants was enhanced dramatically with rolC gene from *A. rhizogenes* (Zuker *et al*, 2001c). It was also observed that induction of rooting was considerably reduced in shoots regenerated from leaf explants (56.2%) as compared to shoots regenerated from stem explants (72.9%) in control plants. This may be another reason for encountering difficulty in rooting of shoots as leaf explants were used for transformation. Zuker *et al* (1999) on the other hand have developed a highly efficient procedure for *Agrobacterium* mediated transformation following wounding of stem explants through particle bombardment. This procedure gave rise to 20% transformation efficiency.

Hence, few shoots regenerated in selection medium at random were tested for transgene integration through PCR analysis of the *npt* II gene using the specific primer sequence. Out of the 3 shoots tested for *npt* II amplification 2 shoots tested positive with a single 750bp band amplifying in the both plasmid and transformed plants but absent in control plant (Fig 11) and these transformed shoots tested positive in dot blot assay as well (Fig 12).

Hitherto, the focus of carnation breeding has been on the development of novel floral traits, although the grower would desire carnation plants with improved agronomic performance especially for resistance to diseases. Among the various diseases afflicting carnation, wilt disease caused by *Fusarium oxysporum* f.sp. *dainthi* is a major one. The methods currently used to control this soil borne fungus are very hazardous as well as ineffective and costly. Classical breeding efforts to identify and select for resistant phenotype based on extensive and costly screening in infected soil is proving difficult. Under these circumstances, development of transgenic lines by incorporating specific genes that could

Fig 8. Hardened plants

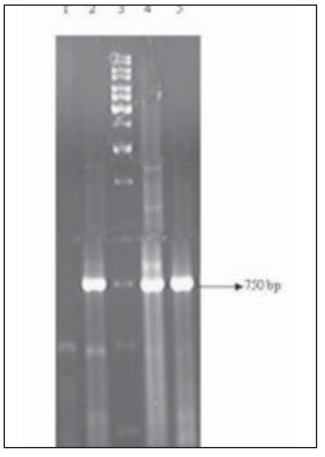

Fig 11. Amplification of *npt*II gene in putatively transformed plants (IIHRS-1)

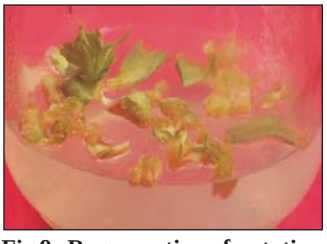

Fig 9. Regeneration of putative transformants

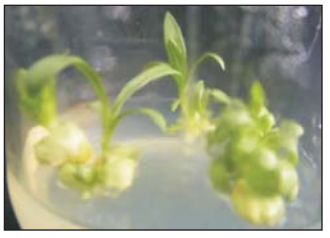

Fig 10. Elongation of putative transformants

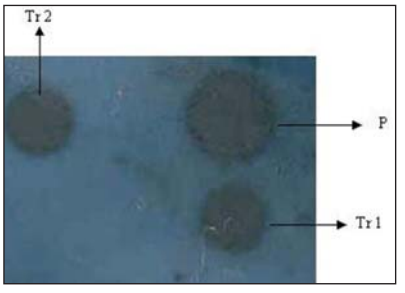

Fig 12. Dot blot assay of transgenic (Tr1,Tr2)plants and plasmid pROK2 (P). Membrane probed with Alk Phos labelled baculovirus chitinase gene (1.65 Kb) PCR fragment

impart resistance to the target organism would be a more effective approach. One of the candidate genes for this purpose is that of the defence protein viz., chitinase. Using this approach, Zuker *et al* (2001b) have produced transgenic lines of carnation cv White Sim showing high levels of resistance to *Fusarium oxysporum* f.sp. *dianthi* under glasshouse testing. The present report on the transformation of carnation with a chitinase gene can thus be utilized for the development of fungal resistant carnations.

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