Pak. J. Bot., 36(4): 887-896, 2004.

FACTORS AFFECTING *AGROBACTERIUM TUMEFACIENS* MEDIATED GENETIC TRANSFORMATION OF *VIGNA RADIATA* (L.) WILCZEK

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Abstract

The aim of the present study was to standardize the *Agrobacterium tumefaciens* mediated transformation protocol for *Vigna radiata.* For this purpose some important parameters like sensitivity of explants to kanamycin, pH of co-culture media, age of explants, types of explants, co-cultivation time and optical density of *Agrobacterium* culture medium were studied. *Agrobacterium* strain C58C1 harboring a binary vector p35SGUSINT containing neomycin phosphotransferase (NPTII) gene as selectable marker and β-glucuronidase (GUS) as a reporter gene was used for transformation. Kanamycin at a concentration of 50mg/l was used to select transformed cells. Transient and stable GUS expressions were studied in transformed explants and regenerated calli respectively. Highest transient GUS (70%) expression was observed at pH 5.8 after 3 days of co-culturing in 2-days-old explants. Optical density of 560nm=1 was considered optimal to obtain the highest transformation rate. *Agrobacterium* culture containing both kanamycin and ampicillin had dramatic effect on transformation efficiency. Primary leaves showed higher transformation efficiency (80%) than hypocotyl (60%) or root (40%) explants. Transformed calli were resistant to up to 800mg/l of kanamycin concentration. Transformed shoot were produced on shoot regeneration medium containing 50mg/l kanamycin and 500mg/l cefotaxime.

Introduction

Mung bean (*Vigna radiata* (L.) Wilczek) is an important pulse crop, which is principally grown for its protein-rich edible seeds and sprouts in many tropical and subtropical countries. The seeds are favored due to their easy digestibility and low production of flatulence, rich in phosphorous and pro-vitamin A and are relatively free from anti-nutritional factors. Its high protein levels and high lysine/low methionine amino acid profile complement the high carbohydrate and low lysine/high methionine content of cereals to form a balanced amino acid diet. Moreover, this crop is nitrogen fixing, has a short life cycle therefore, is widely grown as mixed, inter crop or in rotation to improve nitrogen status of soil or to break disease / pest cycle.

Mung bean haploid genome is only 0.48-0.53 pg, one of the smallest plant genome among higher plants. That is why seeds sprouts have been used in various physiological and biotechnological studies (Sharma *et al,* 1998). Despite being an important pulse crop, its production has not been improved significantly during the last three decades in the Indian subcontinent, which accounts for more than two third of total world production. The main reason is its prominent susceptibility to fungal/viral pathogen and insects pest, and salt and drought intolerance (Jaiwal & Gulati, 1995). Consequently, there is a considerable interest in the construction of agronomicaly useful traits into mung bean by breeding and genetic engineering. Classical breeding has met with limited success due to

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lack of desirable genes in other related species leaving genetic transformation as the only way to increase its production to combat shortage in food supplies. *Agrobacterium*-mediated transformation system has been the most efficient and widely employed method for the production of transformed plants with predictable pattern of DNA integration. Though the genetic transformation of grain legume have been difficult and challenging till now (Jaiwal *et al*., 2001), but significant progress has been made in recovery of transformed plants via *Agrobacterium* in soya bean, pea, chickpea, *Vicia narbonensis*, *Phaseolus aconitifolis* (Christou, 1997), *V. unguiculata* (Gracia *et al*., 1986), *Vigna mungo* (Karthikeyan *et al*., 1996) and *Vigna aconitifolia* (Eapen *et al*., 1987). The present report describes the best transformation conditions of *Vigna radiata* by using *Agrobacterium tumefaciens* mediated transformation protocol.

Material and Methods

Seeds of three commercially grown cultivars of mung bean viz., NM92, NCM 209 and NM98 were obtained from National Agriculture Research Center (NARC) Islamabad. Out of these the widely grown NM, 92 showed the best germination and hence was used for detailed studies.

***Agrobacterium* strain and vectors:** *Agrobacterium tumefaciens* strain C58C1 (pGV2260) containing binary vectors p35SGUSINT was used for this study (Deblaere *et al*., 1985). T-DNA Of p35SGUSINT (Vancanacyt *et al*., 1990) contains NPTII gene with NOS promoter and NOS terminator and GUS gene with CaMV35S promoter and NOS terminator. This GUS gene contains a portable intron (PIV2) (Vancanacyt *et al*., 1990) and hence does not express in *Agrobacterium* (Fig. 1). *Agrobacterium* strain was grown over night at 28-30°C in YEB medium containing either 50mg/l Kanamycin or 50mg/l of both kanamycin and ampicillin. For solid medium agar was added at a concentration of 1.5 g/l.

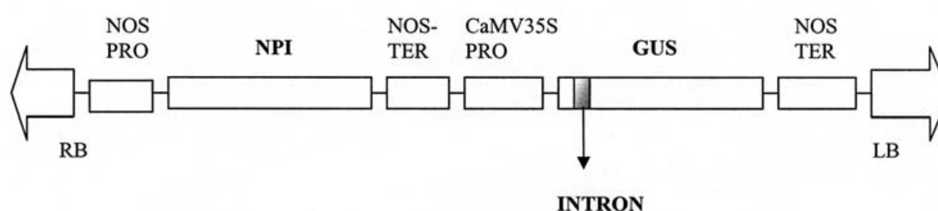

Fig. 1. Schematic diagram of the T-DNA region of transformation vector p35SGUSINT RB, Right border; LB, Left border; NOS-PRO, nopaline synthase promoter; NPT II, neomycin phosphotransferase gene; NOS-TER, nopaline synthase terminator; CaMV 35S-PRO, 35S promoter of cauliflower mosaic virus; GUS, β-glucuronidase gene.

Transformation and regeneration: Explants of hypocotyls, primary leaves with petioles, roots and cotyledonary nodes with both proximal halves attached to the embryonic axis were excised from 2-6 day old *in vitro* dark grown seedlings and were either submerged in bacterial suspension with gentle shaking directly or before infection these explants were cultured on B5 with (0.5mg/l) 2,4-D for three days to allow callus

induction to start (Valvekens *et al*., 1988). Infected explants (1-1.5cm long) were blotted on sterile filter paper and co-cultivated either on B5 or B5 with 2,4-D for three days under dark or light condition at 25°C. After co-cultivation explants were washed 3-4 times with sterile distilled water by vigorous stirring with sterilized forceps and blot dried on sterile filter paper. The hypocotyl, root and primary leaves explants were cultured on callus inducing medium (CIM) containing 2, 4-D (0.5mg/l), BAP (0.1mg/l), kin (0.5mg/l), with kanamycin (50mg/l) and cefotaxime (500mg/l). The explants along with newly initiated calli were transferred to fresh medium after every two weeks for 8-10 weeks. The putative transformed calli were cultured on shoot regeneration medium (SRM) for shoot regeneration (Table 1). Various explants after co-cultivation with *Agrobacterium* were also cultured directly on regeneration medium (SRM) containing 50mg/l kanamycin and 500 mg/l cefotaxime for shoot regeneration.

Table 1. List and composition of shoot regeneration media.

Medium*	Composition
SRM1	2, 4-D (0.5), Kin (0.5)
SRM2	BAP (0.1), Kin (0.5)
SRM3	BAP (0.1), IAA (0.3)
SRM4	BAP (0.1)

*All media contain B5 salt and vitamins. Concentration of each hormone is given in mg/l.

Abbreviation: BAP, 6-Benzylaminopurine; **2,4-D**, 2,4 dichlorophenoxyacetic acid; **NAA,** 1-napthalene acetic acid; **MS,** Murashige and Skoog medium; **B5**, Gamborg's medium; **GUS**, Beta glucuronidase; **nptII**, neomycin phosphotransferase II; **IAA**, indole-3- acetic acid.

GUS assay: Histochemical GUS assay were carried out essentially as described by Jefferson (1987). Transient GUS expression was observed in root, leaf and hypocotyl explants. Stable GUS expression was observed in callus by dipping the small portion of transformed calli into GUS staining solution. The reaction mixture was incubated for overnight at 37°C.

Result and Discussion

Sensitivity to kanamycin: Prior to transformation, an effective concentration of antibiotic for the selection of transformed cells was determined by culturing hypocotyl, leaves and roots (10 explants each) on CIM and B5 media containing various concentrations of kanamycin (0, 25, 50, 75, 100, 150 mg/l). Explants became dry and yellow (Fig. 2a) on B5 containing 50mg/l of kanamycin. Kanamycin at concentration of 75, 100, 150 mg/l caused complete necrosis of the explants after three weeks (Fig. 2b). On kanamycin free CIM medium, explants produced green friable calli while Kanamycin at 50 mg/l concentration caused total inhibition of callus induction in hypocotyls, roots and leaves. These results showed that kanamycin is an effective selection marker for *Vigna radiata.* Hence this concentration was used for the selection of transformed calli. Similar observation has been reported for V. *mungo* by Karthikeyan (1996) while in another study Phogat *et al*., (1999), selected the transformed calli of *Vigna radiata* on 100 mg/l of kanamycin concentration.

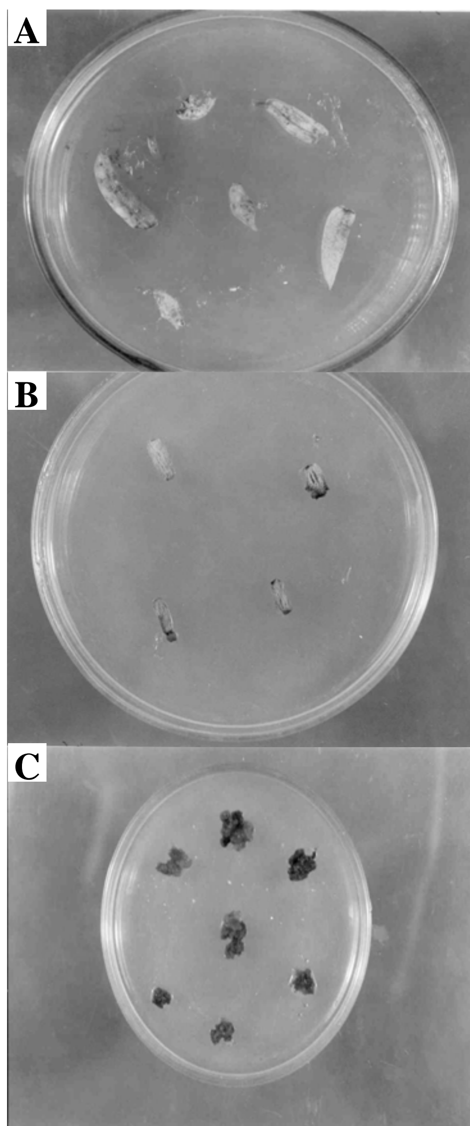

Fig. 2. Kanamycin sensitivity of A. non-transformed leaf explants at 50mg/l concentration. B. non-transformed hypocotyl explant at 75mg/l concentration. C. transformed calli at 1000mg/l concentration.

To evaluate kanamycin resistance of transformed callus, the transformed calli were developed on leaves following inoculation with C58C1 and were checked for their level of kanamycin resistance by sub-culturing transformed calli on CIM medium containing 0, 50, 100, 200, 400, 800, and 1000mg/l kanamycin. It was observed that growth of putative transformed calli appeared normal (green) up to 600mg/l kanamycin, at 800mg/l concentration they appeared pale while 1000mg/l of kanamycin caused blackening of calli within three weeks (Fig. 2c). It would suggest that transformed calli could show

high level of resistance of up to 800mg/l of kanamycin. In a similar study transformed V. *radiata* calli are reported to be resistant to kanamycin up to 800-1000mg/l of kanamycin concentration (Jaiwal *et al*., 2001).

Factors effecting transformation

Effect of optical density (OD) of *Agrobacterium* culture: Explants from 2-day old seedlings were co-cultured with *Agrobacterium* culture of varying optical density (OD_{560}) between 0.8-1.5, keeping pH 5.8 and kanamycin concentration 50 mg/l. Highest %age of GUS expression (36.36%) was observed when OD was 1.0 (Table 2). Transient GUS expression decreased, as optical density was decreased or increased and lowest percentage of GUS expression (5.8%) was observed at the highest OD value recorded i.e. 1.5. This result suggests OD =1.0 is optimal for transformation of *Vigna radiata.* Similar results are also reported by Jaiwal *et al*., (1998, 2001).

Table 2. Effect of optical density of *A. tumefaciens* culture on transient GUS expression after three days of co-cultivation.

OD	Number of explants co-cultured	Number of explants used for GUS staining	Number of explants showing GUS expression	% of explants showing GUS expression
0.8	15	15	1	6.6
1.0	30	11	4	36.36
1.2	30	13	3	23.0
1.3	15	6	1	16.6
1.5	30	17	1	5.8

Effect of pH of co-cultivation media: Explants were co-cultured with *Agrobacterium* for three days using B5 at pH varying between 5.0 - 6.0 keeping optical density OD560 =1.0 and kanamycin concentration 50 mg/l. Table 3 shows the effect of pH on transient GUS expression in explants, 3 days after co-culturing with *Agrobacterium*. Highest %age of GUS expression (23.80%) was observed at pH 5.8, while no GUS expression was found at pH 5.0. This suggests that lower pH value i.e. 5.0 and 5.2 decreased transformation efficiency of *Agrobacterium* and as we go towards higher pH values, transient GUS expression increased. This indicates that pH is an important factor controlling the transformation efficiency and 5.7-5.8 seems to be optimum for getting high transient GUS expression in *Vigna radiata.* In a previous study Hiei *et al*., (1994) reported that high efficiency of gene transfer was achieved at pH 5.2 in rice. Our results are different may be due to difference in crop and /or *Agrobacterium* strain used.

Table 3. Effect of pH on the transient GUS expression after three days of cocultivation with A. *tumefaciens* strain C58C1.

pH	Number of explants co-cultured	Number of explants used for GUS staining	Number of explants showing GUS expression	% of explants showing GUS expression
5.0	30	10	0	0.0
5.2	30	20	1	5.0
5.4	25	20	1	5.0
5.6	30	15	2	13.3
5.8	25	21	5	23.80
6.0	30	20	3	15.0

Co-cultivation time: Co-cultivation duration also affected the transformation efficiency. Extending the co-cultivation time up to three days increased the transient transformation frequency and, subsequently, further increase in co-cultivation time decreased the transformation frequency resulting in bacterial over growth (Fig. 3a).

Age of explant: Age of the explant is a critical aspect in transformation experiment. Explants excised from 2- day -old seedling showed higher transient transformation rate (70%) than those excised from four day (50%) or six-day-old (30%) seedlings. This substantiates the earlier finding that younger explants are more susceptible to *Agrobacterium* than older explants (Fig. 3b).

Explant type: Explant type is also a very important factor affecting plant transformation. We have used three types of explants (leaf, root, and hypocotyl). Transformation efficiency was assessed as the proportion of the blue inclusion in the putative transformed explants. In leaf the transformation efficiency was higher (80%) than that of root (40%) and hypocotyl (60%) (Fig. 3c). The GUS expression was observed near the cut end of the explants however blue spots were also seen near the midrib.

Effect of antibiotics: The pGV2260 of C58C1 strain used in this study contains *vir* genes and an ampicillin resistance gene, while p35SGUSINT contains kanamycin resistant gene. We used two types of *Agrobacterium* culture for infection of explants. In one *Agrobacterium* culture medium we added kanamycin and ampicillin both at concentration of 50mg/l and in second *Agrobacterium* culture medium only kanamycin at concentration of 50mg/l was used. About 20 explants were co-cultured using each of these two culture media. We observed dramatic increase in transformation efficiency (Fig. 3d) by using the culturing media containing both kanamycin and ampicillin. Addition of both antibiotics is recommended as it helps in positive selection of *Agrobacterium* containing both (binary and helper) plasmids.

Gus analysis: To compare transient and stable T-DNA transformation, GUS analysis was done in explants immediately after co-cultivation as well as in calli 4-week after transformation. Transient GUS expression was observed in leaves, hypocotyls and roots explants (Fig. 4a, b and c). For stable GUS expression, four weeks old transformed and untransformed calli were assayed. Calli that developed from untransformed explants showed no detectable GUS activity, while calli of transformed explants stained blue (Fig. 4d). To verify the absence of *Agrobacterium*, transformed calli were transferred to cefotaxime free medium before testing for GUS activity. Compared to the 80% of the explants that showed GUS activity after three days of co-cultivation with C58C1, only 40% explants developed kanamycin resistant calli and showed GUS^+ assay after four weeks. Stable GUS expression was also observed in transformed shoots (Fig. 4e).

Similar reports have been made earlier about high level of early detection of GUS activity within three days of agrobacterial infection due to transcription and translation of non-integrated T-DNA. However, 28 days after infection the number of explants showing GUS expression decreased, indicating that many of the GUS expressing cells were not stably transformed due to inefficient T-DNA integration into the genome or if integrated gene silencing occurred at later stages (Liu *et al*., 1992; Narasimhulu *et al*., 1996).

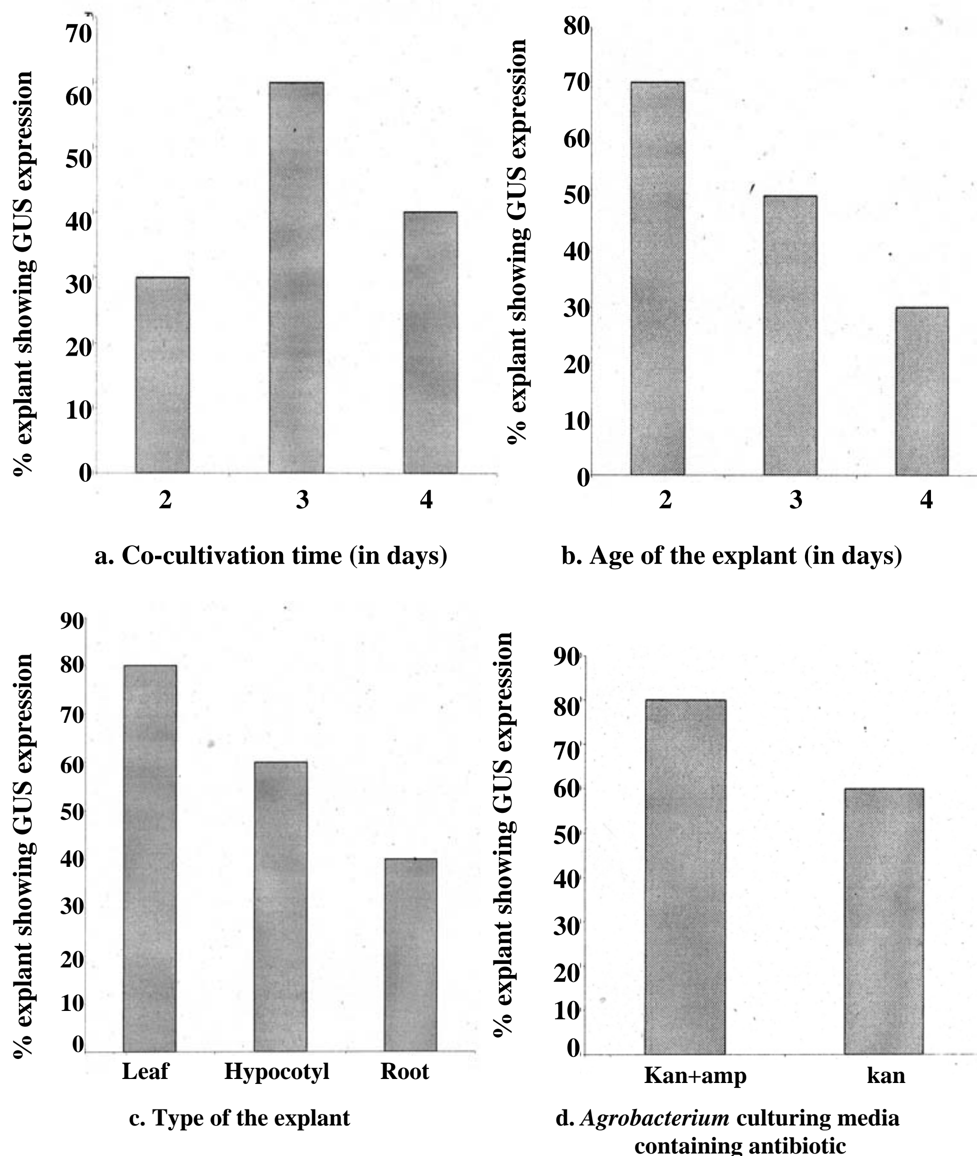

Fig. 3. Influence of a. Cocultivation days b. age of the explant c. type of the explant and d. culturing media on transient GUS expression.

Regeneration of transgenic plants: The hypocotyl, primary leaves and roots that were co-cultivated with *Agrobacterium* for three days under dark condition were grown on CIM containing 50mg/l kanamycin and 500mg/l cefotaxime. Callus initiated at the base of petioles, wounded edges of leaves, at both cut ends of hypocotyl and roots after 15-20 days of culture (Fig. 5a, b, c). Calli were separated from explants and were sub-cultured after every 2 weeks on selection media. The growth of kanamycin resistant calli was doubled during one week.

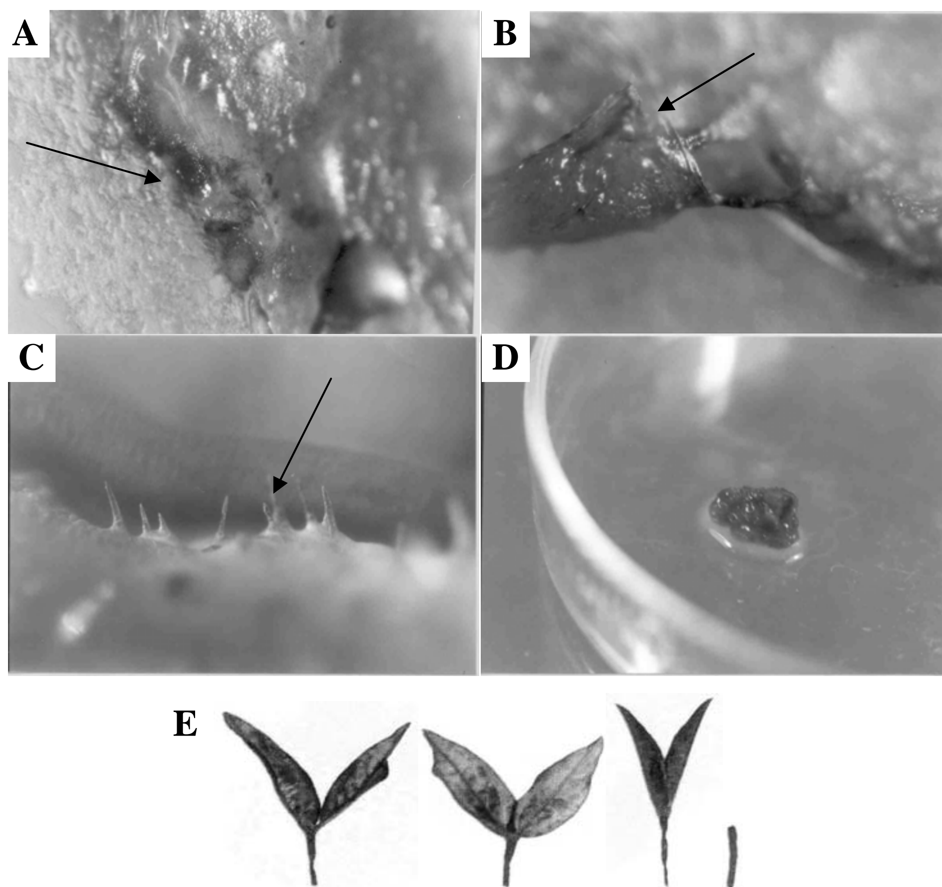

Fig. 4. Transient GUS expression A. in two-day-old leaf explant. B. in two day old hypocotyl explant. C. in four-day-old root explant. Stable GUS expression D. in transformed callus E. in transformed shoot and hypocotyl of *Vigna radiata*.

Regeneration of transgenic GUS^+ calli of NM92 was tried by sub-culturing on various shoot regeneration media. The calli proliferated and regenerated shoots after long incubation of four weeks. The shoot regeneration frequency from transformed calli was very low. Only one shoot regenerated out of 25 calli on SRM2 medium (Fig. 5d). Calli cultured on other SRM did not produce any shoots.

For direct organogenesis transformed as well as un-transformed ex-plants were cultured on various SRM in the presence of kanamycin and cefotaxime. After every two weeks explants were transferred to fresh selection media. About 3% of the co-cultivated explants produced transformed shoots on SRM2 after five weeks (5e, f), while the non-transformed explants failed to produce any shoots on selection media. These result are in agreement with the previous reports that regeneration frequence of legumes is very low (Gulati and Jaiwal, 1994; Lakhanpaul *et al*., 2000). Jaiwal *et al*., (2001) recovered only 10 green shoots on selection medium from 1050 co-cultivated explants. A well-defined

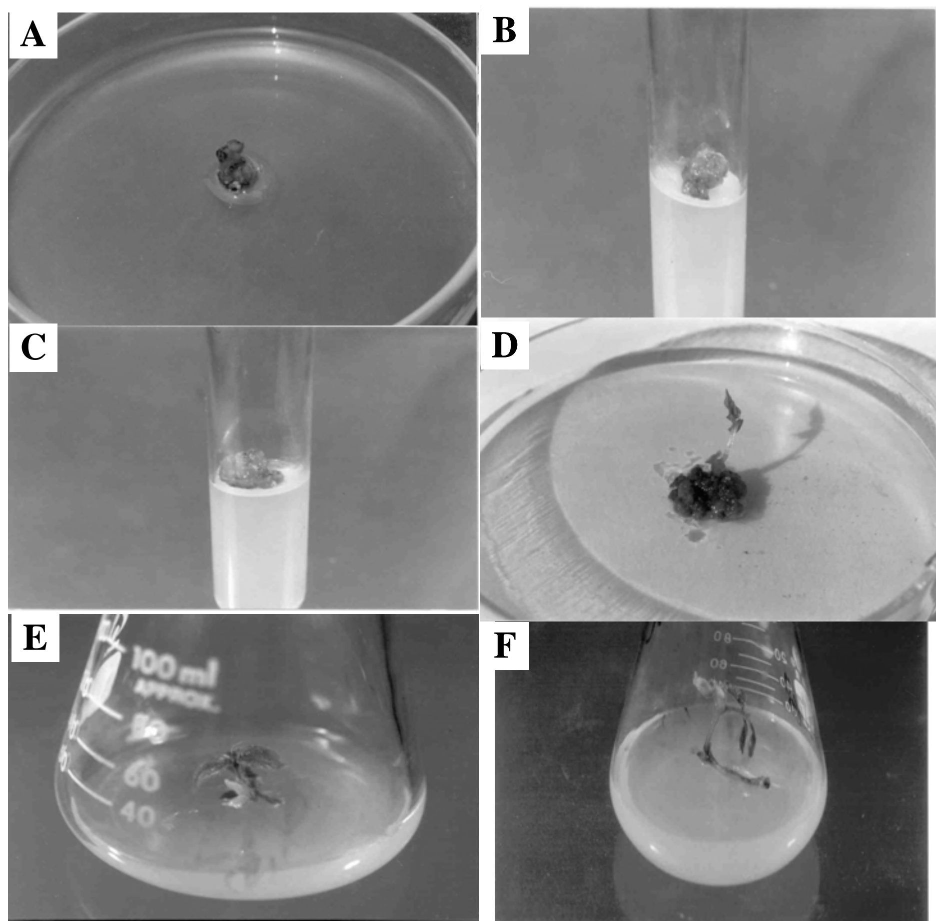

Fig. 5. Transformed callus from a. two day old leaf explant b. four day old hypocotyls explant c. two day old root explant. Regeneration of shoot from d. transformed callus e. two day old transformed leaf explant f. four day old transformed hypocotyls explant.

preferably simple shoot regeneration protocol is a pre-requisite for the production of transgenic plant. Grain legumes are least amenable group to transformation among dicotyledonous crop. As shown by this report they are susceptible to *Agrobacterium* infection and we have identified optimal transformation conditions, however further work is required to improve regeneration protocol for *V. radiata*.

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(Received for publication 25 November 2003)