Pak. J. Bot., 41(6): 3239-3246, 2009.

FACTORS AFFECTING *AGROBACTERIUM TUMEFACIENS* MEDIATED TRANSFORMATION OF *ARTEMISIA ABSINTHIUM* L.

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Abstract

The present work was aimed to study various factors affecting *Agrobacterium tumefaciens* mediated transformation of *Artemisia absinthium* L. *Agrobacterium* strain C58C1 harboring binary vector p35SGUSINT having kanamycin resistance gene (NPTII) as selectable marker and β-glucuronidase (GUS) as a reporter gene was used for transformation. Factors affecting transformation efficiency, such as age and type of explants, presence of ampicillin and kanamycin in *Agrobacterium* inoculum, concentration of *Agrobacterium*, infection and co-cultivation time period of *Agrobacterium*, effects of 2,4-D, pH of co-cultivation medium and effects of cefotaxime in regeneration medium were studied. An amount of 50mg/L each of kanamycin and ampicillin was used to select the pure *Agrobacterium* cultures and 500mg/L cefotaxime along with 20mg/L kanamycin was used to select transformed cells. Results concluded that one week old leaf and root ex-plants of *A. absinthium* inoculated with *A. tumefaciens* for 5 minutes and co-cultivated for 3-4 days in B5 medium with 0.5mg/L of 2, 4-D at media pH 5.8 showed 100% transformation efficiency.

Abbreviations: amp: ampicillin, 2, 4-D: 2,4-dichlorophenoxyacetic acid, GUS: ß-glucuronidase, kan: kanamycin, NPTII: neomycin phosphotransferase,

Introduction

Artemisia, one of the most ancient groups of plants, is a large genus of herbs with about 200 species. It belongs to family Asteraceae (Haq, 1983; Zia *et al*., 2007). In Pakistan, 25-30 species of *Artemisia* have been reported (Abid & Qaiser, 2008; Qureshi *et al*., 2002). *Artemisia absinthium* L., commonly known as 'worm wood' is a perennial herb, growing wildly in northern hilly areas of Pakistan (Ghafoor, 2002). This species has many important medicinal uses, including antibacterial (Kaul *et al*., 1976), antifungal (Maruzzella *et al*., 1960), antimalarial (Hernandez *et al*., 1990) etc. Arial parts of the plant, preferably leaves are used in medicines. The fresh plant is reported to be more effective for treating stomach disorders than dry plant (Dasture, 1952).

Several *Agrobacterium* strains with different binary vectors have been used for the production of transgenic *Artemisia annua* (Vergauwe *et al*., 1996; 1998) and *Artemisia absinthium* (Nin *et al*., 1997). Vergauwe *et al*., (1996) developed a transformation system for *Artemisia annua* by using *Agrobacterium tumefaciens* strain C58C1 Rif^R (pGV2260, pTJK136). In another study, Vergauwe *et al*., (1998) studied various factors affecting *Agrobacterium* mediated transformation of *A. annua*, including explant age and type, different *Agrobacterium* strains and binary vectors.

This study was carried out to optimize various factors affecting transformation conditions of *Artemisia absinthium* by using *Agrobacterium tumefaciens* strain C58C1 with binary vector system, (p35SGUSINT, pGV2260). The conditions optimized in this study can be used later to transform *Artemisia absinthium* in order to enhance the artemisinin content in plant, which is an effective antimalarial drug.

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Materials and Methods

1. Preparation of plant material for transformation: *Artemisia absinthium* seeds collected from Gilgit (Pakistan) were surface sterilized by immersing in 70% (V/V) ethanol for 2 minutes, then in 0.1% (W/V) mercuric chloride ($HgCl_2$) solution for 2-3 minutes and rinsed several times with sterilized distilled water. Seeds were germinated on ½ MS medium containing 3% sucrose for germination in Petri plates. Then seeds were kept at 4ºC in dark conditions for 4 days and then transferred to growth room conditions of 25±1ºC, 16 hours of photoperiod. Plantlets of 1-3 weeks old were used as explants source for genetic transformation.

2. Bacterial strains and plasmids: *Agrobacterium tumefaciens* strain C58C1 containing pGV2260 and p35SGUSINT was used for transformation (Jefferson *et al*., 1987). T-DNA of p35SGUSINT contained NPTII gene with NOS promoter and NOS terminator and GUS gene with CaMV35S promoter and NOS terminator (Fig. 1). Prior to infection, *Agrobacterium tumefaciens* strain C58C1 was grown overnight in an incubator at 28ºC with constant shaking at 120 rpm in YEB medium supplemented with 50mg/L each of selective antibiotics ampicillin and kanamycin.

3. Transformation and selection: The leaf, root and hypocotyl explants (0.5-1cm) were cut from one, two and three weeks old seedlings, dipped in 10mL of an overnight grown culture of the *Agrobacterium* and dried on a filter paper to remove excess culture, and were transferred to co-cultivation medium. Co-cultivation medium (Gamborg's B5 medium with 3% sucrose) was supplemented with 0.5mg/L of 2, 4-D. After three days, explants were transferred to selection medium (B5 medium) containing 500mg/L cefotaxime, 50mg/L kanamycin and 0.1mg/L BAP.

4. Histochemical GUS assay: Histochemical GUS assay was carried out essentially as described by Jefferson *et al*., (1987). Hypocotyl, leaf, and root explants were incubated in GUS assay solution at 37°C overnight. The chlorophyll was removed by several washes with 70% ethanol. Transient GUS expression was observed in root, leaf and hypocotyl explants, whereas stable GUS expression was observed in callus by dipping the small portion of transformed calli into GUS staining solution.

Results and Discussions

In the present study, *Agrobacterium tumefaciens* strain C58C1 harboring p35SGUSINT with GUS reporter gene was used to standardized transformation condition for *Artemisia absiunthium*.

1. Age and type of explants: In this study, efficiency of transformation was highest (100%) from both root and leaf explants however hypocotyl explant showed less (80%) GUS expression. Another study also shows that type of explant is very important for getting an efficient transformation (Michalczuk & Wawrzyńczak. 2004). Similarly, one week old explants showed maximum (100%) GUS expression while two and three week old explant showed 90% and 70% GUS expression respectively. These findings are in accordance with earlier findings reported by Vergauwe *et al*., (1996) and Nin *et al*., (1997) showing that transformation efficiency of *Agrobacterium* decreases as age of explants increases.

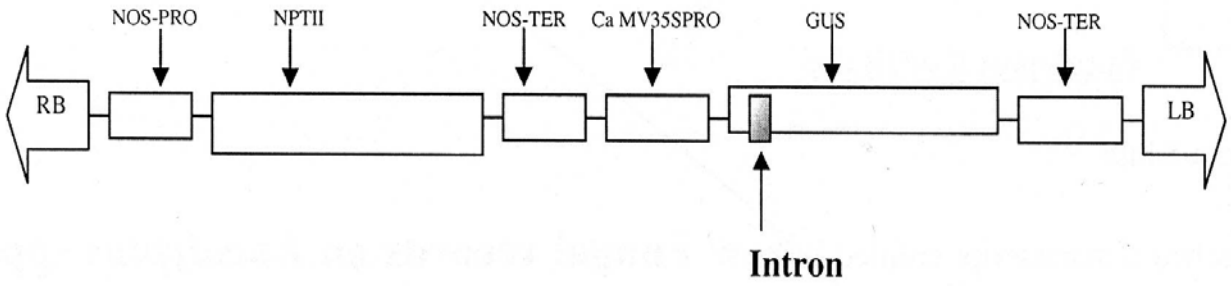

Fig. 1. Schematic diagram of the T-DNA region of transformation vector p35SGUSINT
(RB: Right border, NOS-PRO: nopaline synthase promoter; NPTII: neomycin phosphotransferase, NOS-TER: nopaline synthase terminator, CaMV35S-PRO: 35S promoter of cauliflower mosaic virus, GUS: gene for β-glucuronidase, LB: Left border).

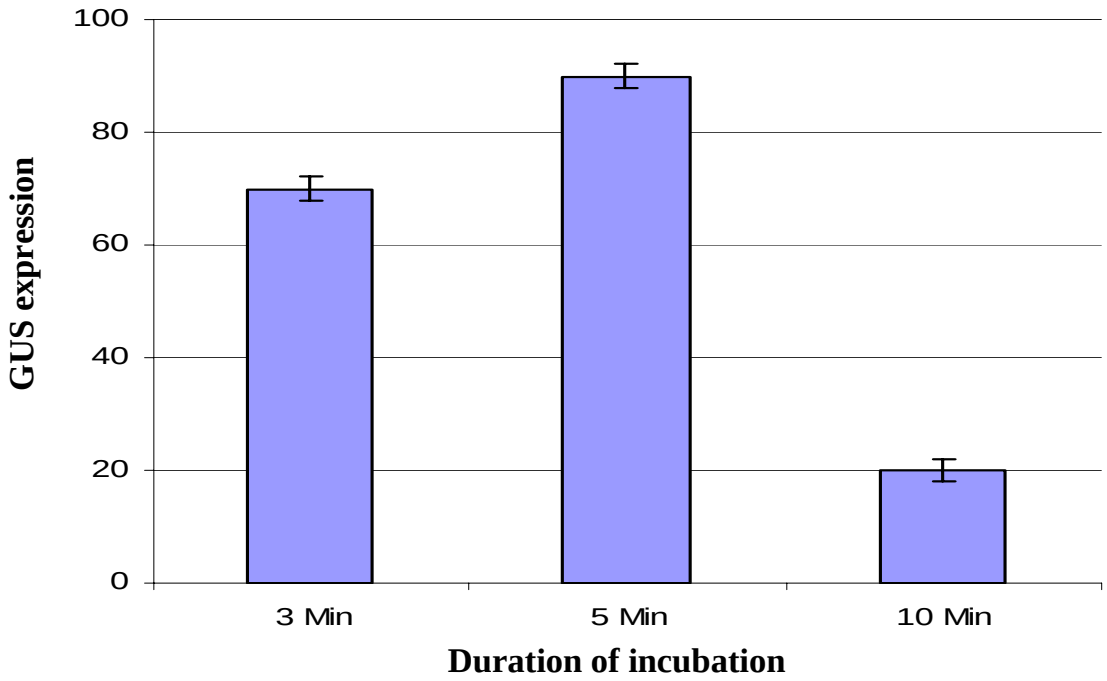

Fig. 2. Effect of incubation period of *Agrobacterium*.

2. Infection and co-cultivation conditions: A specific time period is required by *Agrobacterium* for its attachment and for transfer of its T-DNA to explant. Less infection time period, of course produces low number of transformed explants where as more time period for explants in infection medium may cause hypertonic conditions that bursts the cell or it may be due to hyper activation of defense mechanism that may be lethal to cell and hence results low frequency of transformation. Plant cell requires some time to adopt this foreign DNA making cocultivation time period important. Less time period does not ensure integration and working of transferred DNA while higher time period can also be lethal due to overgrowth of *Agrobacterium* that behaves as parasite with explants and decreases nutrients supply. In this study, *Agrobacterium* infection for five minutes and co-cultivation time period of three to four days gave maximum positive GUS expression (Figs. 2 & 3). These results are in accordance with Vergauwe *et al*., (1998); they co-cultivated *Artemisia annua* for 48 to 60 hours and found maximum transformation efficiency. According to their findings, longer co-cultivation period does not help the transformation efficiency.

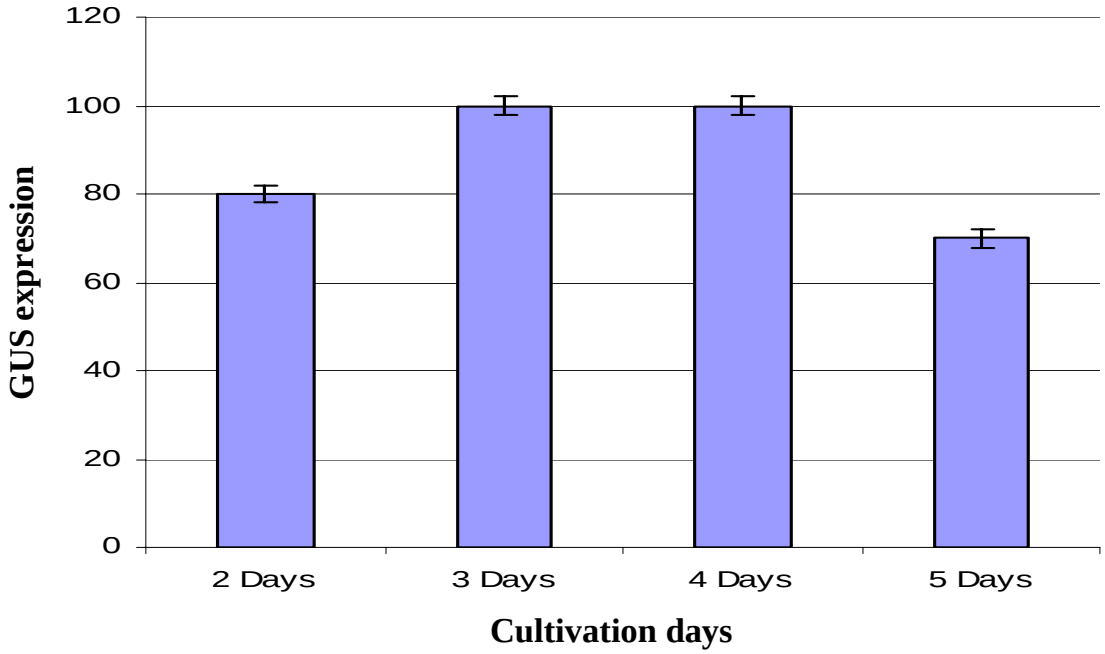

Fig. 3. Effect of cocultivation period on GUS expression.

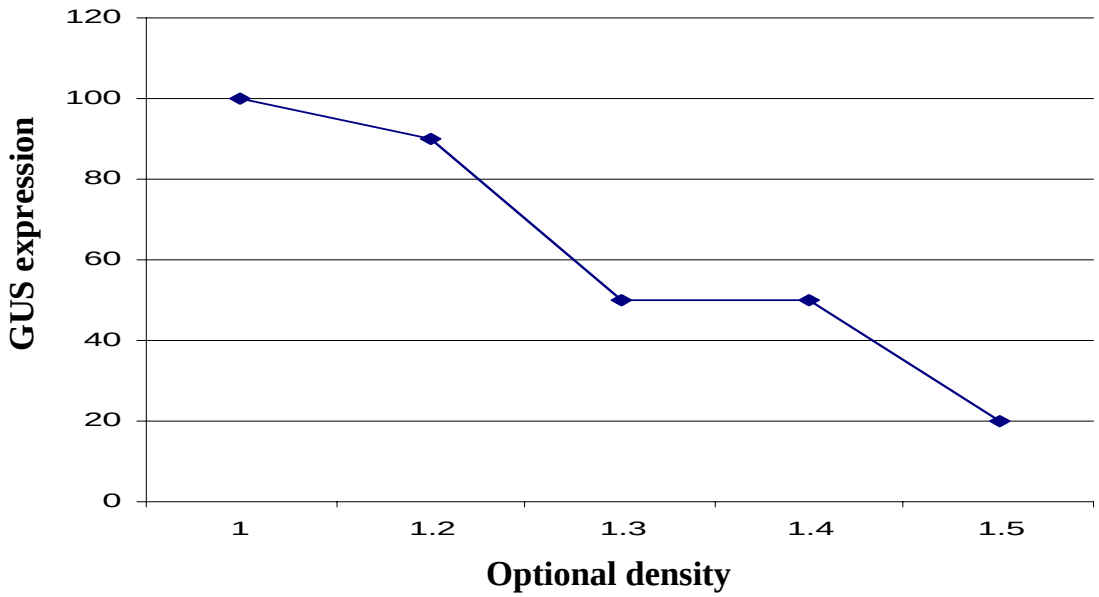

Fig. 4. Effect of optical density of *Agrobacterium tumefaciens* culture on transient GUS expression.

3. Concentration of *Agrobacterium* in solution: The number of *Agrobacterium* cells in the inoculum is considered to be a critical factor in the efficiency of transformation. An excessive number of bacteria can stress plant cells and affect their regeneration potential, whereas low concentration can reduce the frequency of T-DNA transfer. Highest percentage of GUS expression was observed when optical density (O.D.) of *Agrobacterium* inoculum was kept 1.0 at 560nm. Further increase in O.D. resulted in a decrease in GUS expression. These results suggest that an O.D. of 1 is optimum for transformation of *Artemisia absinthium* (Fig. 4). Jaiwal *et al*., (1998; 2001) also reported such results; they also got the highest transient transformation efficiency in *Vigna radiata* at O.D. of 1.0.

4. Effect of pH and 2, 4-D in the co-cultivation medium: Presence of growth regulators influence transformation efficiency of *Agrobacterium*. It was found that addition of 0.5mg/L of 2, 4-D to the cocultivation medium yielded higher transformation efficiency of explant. This is in agreement with previous work, where improved transformation frequencies were obtained by using tomato cell feeder layers on a medium high in auxins during the cocultivation period (Pena *et al.,* 1997; Cervera *et al.,* 1998; Dominguez *et al.,* 2000). While pH of co-cultivation medium also effects expression of vir genes and T-DNA transfer. Acidic pH is considered optimum for this purpose depending on type of plant and explant. Hiei *et al*., (1994) obtained high efficiency of gene transfer through *Agrobacterium* strain at pH 5.2 in rice. For *Artemisia absinthium* 5.8 pH proved best as 100% transient GUS expression was noticed whereas decrease in pH showed lower transformation efficiency (Fig. 5).

5. Presence of kanamycin and ampicillin in the inoculum: Selection of *Agrobacterium* prior to transformation is recommended to get rid of contaminants that may interfere T-DNA transfer. *Agrobacterium* grown in the presence of 50mg/L each of kanamycin and ampicillin showed highest results (100%) whereas only presence of kanamycin in the medium decreased the results to 20%. It is important to note that p35SGUSINT has kanamycin resistant gene while *vir* containing plasmid (pGV2260) contains ampicillin resistant gene and addition of both antibiotics helps in positive selection of *Agrobacterium* containing both plasmids.

6. Effect of antibiotics on *Agrobacterium* in regeneration medium: After transformation, two important factors are considered; first elimination of *Agrobacterium* that hinders the growth of explant and secondly regeneration in medium with appropriate growth regulators and selective antibiotic for growth of transformed cell into callus or tissue. Elimination of *Agrobacterium* is achieved by induction of cefotaxime in the medium and selection of transformed cell is achieved by kanamycin to confirm that nptII gene is transformed to the plant. So concentration of both antibiotics is important because higher level may put the explant in stress and so retard the growth (Ling *et al.,* 1998). Different doses of cefotaxime and kanamycin were used to optimize the standard dose which could control the overgrowth of *Agrobacterium* and also did not inhibit the regeneration of transformed explants. Cefotaxime and kanamycin at a concentration of 500mg/L and 20mg/L respectively controlled overgrowth of *Agrobacterium* with effective selection of transformed cell. Even though this concentration of cefotaxime retarded the callus formation, but regenerated explants were able to grow successfully. This is in agreement with Vergauwe *et al*., (1996), who used cefotaxime as decontaminating antibiotic and reported that it results in retardation of callus formation and inhibition of the shoot inducing capacity in *Artemisia annua*.

7. Transient GUS expression: The leaves, roots and hypocotyl explants were cocultured with Agrobacterium for three days, washed with sterile distilled water and put into GUS substrate overnight at 37°C in incubator. Un-transformed explants kept as control showed no GUS+ expression whereas transformed explants showed GUS+ results in the form of blue dots scattered in the explants that could be seen with the naked eye (Figs. 6, 7 & 8).

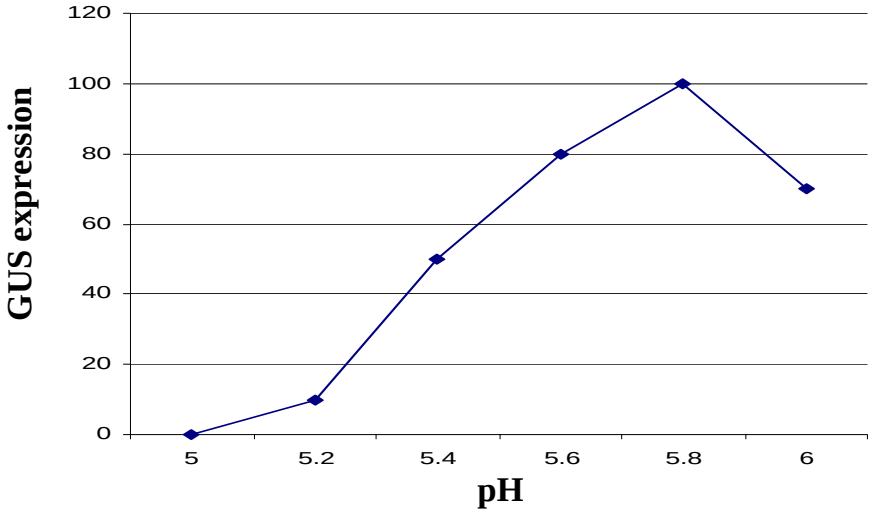

Fig. 5. Effect of pH on the transient GUS expression.

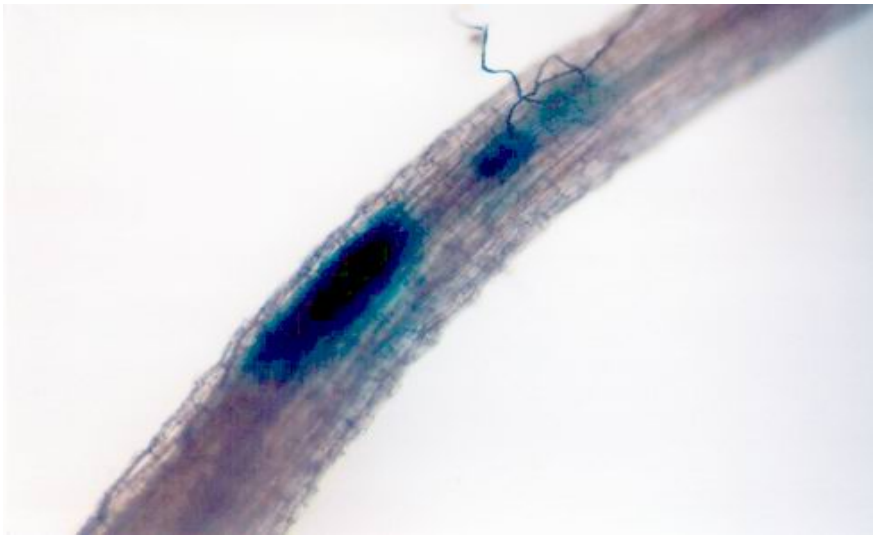

Fig. 6. GUS expression in transformed root explant.

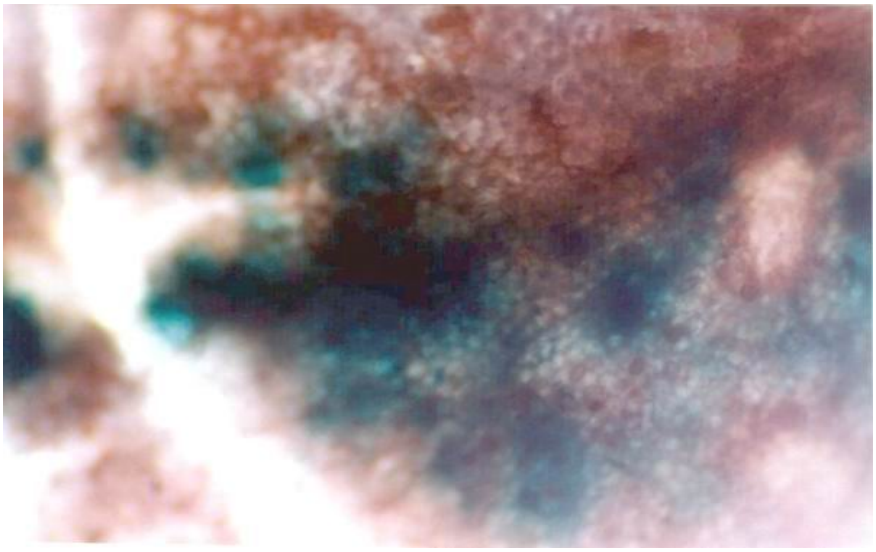

Fig. 7. GUS expression in transformed leaf explant.

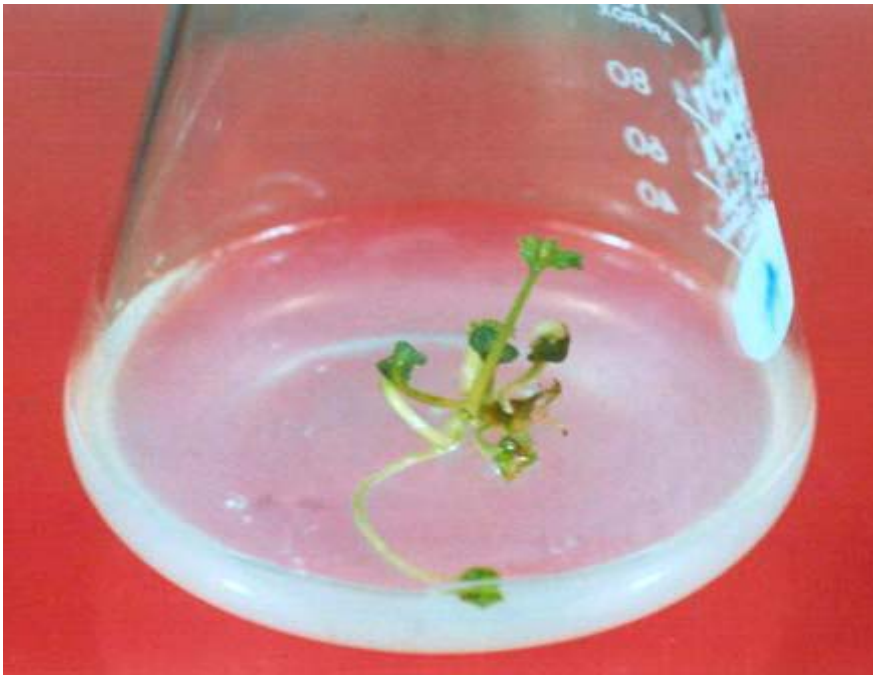

Fig. 8. Regenerated *Artemisia absinthium L.* showing transgenic shoots.

The results from the present studies can be combined with that of Vergauwe *et al*., (1998) to produce an enhanced protocol for the transformation of *Artemisia absinthium* L. The protocol includes incubation of the explants for five minutes in *Agrobacterium* solution, containing 50mg/L each of ampicillin and kanamycin having a pH of 5.8 and an O.D. 1 of *Agrobacterium* at 560 nm, followed by a three-four days co-cultivation period on medium supplied with 2, 4-D, and then transfer of the explants to selective regeneration medium containing 500mg/L cefotaxime and 20mg/L kanamycin with appropriate growth regulators.

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(Received for publication 20 October 2008)