Current Synthetic and Systems Biology
ISSN: 2332-0737

Dilshad et al., Curr Synthetic Sys Biol 2016, 4:1
DOI: 10.4172/2332-0737.10001029

Research Article Open Access

Optimization of Conditions for Genetic Transformation and *In Vitro* Propagation of *Artemisia carvifolia* Buch

Erum Dilshad, Hammad Ismail, Waqas Khan Kayani and Bushra Mirza*
Department of Biochemistry, Quaid-i-Azam University, Islamabad, Pakistan

Abstract

The excessive attention in the use of plants as medicine is credited to the occurrence of active principles whose pharmacological activities have been investigated. Due to the limited production and very less quantity of these important metabolites present in the plant cells, their genetic engineering and increased *in vitro* production is the point of focus for many years and can be achieved by *in vitro* transformation and propagation of desired plant. In the current study, we report transformation protocol for *Artemisia carvifolia* Buch with *Agrobacterium tumefaciens* C58C1 harboring β-glucuronidase as reporter and neomycin phosphotransferase as selectable marker gene. We have optimized simple regeneration conditions after transformation involving two different types of explants (leaf and stem) on different media formulations for direct organogenesis and best regeneration response. MS media with 2.5 g/L benzylaminopurine (BAP), 0.25 g/L naphthalene acetic acid (NAA), giving maximum number of shoots was selected. Rooting was obtained on ½ MS medium supplemented with NAA (0.1 mg/L). Transient expression of gus reporter gene was observed in the leaf and stem explants after 2 days of bacterial infection. *Artemisia carvifolia* Buch can be successfully transformed with *Agrobacterium tumefaciens* strain C58C1 by using controlled *in vitro* regeneration conditions. Findings of the current study would be useful for micro propagation and genetic transformation of *Artemisia carvifolia* Buch in future.

Keywords: *Artemisia carvifolia*; Agrobacterium tumefacien; Micro-propagation; Gus expression

Abbreviations: Gus: Glucuronidase; NPTII: Neomycin Phosphotransferase; MS: Murashige and Skoog; PGRs: Plant Growth Regulators; SRM: Shoot Regeneration Medium; BAP: Benzyl Amino Purine; NAA: Naphthalene Acetic Acid

Introduction

Plants *in vitro* cultures are gaining substantial significance owing to their potential applications in the production of useful aromatics with broad range of pharmacological activities [1,2]. They offer a striking substitute to the whole plant for the production of vital plant secondary metabolites [3-5]. Well-built practices are presently accessible to help growers encounter the demand of the pharmaceutical industry. These practices are deliberated to make available prime levels of different nutritional (vitamins, mineral nutrients) and environmental factors (e.g. light, gaseous environment, temperature, and humidity). These techniques also provide the PGRs (Plant growth regulators essential to gain significant regeneration rates of many plant species *in vitro*), to assist commercially worthwhile micro propagation. Distinct tissue and cell culture approaches are also being practiced for the production of numerous significant secondary products [6].

Artemisia genus is an opulent source of terpenoids and other secondary plant products having uses in perfumery and pharmaceuticals [7]. Among the secondary metabolites of genus Artemisia, sesquiterpene lactones and flavonoids are of high therapeutic importance. They show strong anti-inflammatory, antimalarial, antioxidant, antitumor activity, as well as they increase immunity and decrease the risk of atherosclerosis, arthritis and gastrointestinal disorders [8-10]. Due to the limited production and very little quantity of these important metabolites present in the plant cells, their increased *in vitro* production is the point of focus for many years and can be achieved by genetic engineering and *in vitro* propagation of desired plant. There are various reports regarding Agrobacterium mediated transformation of *Artemisia* species, although most of these describe transformation of *A. annua* [11-14]. Some reports are available regarding transformation of other *Artemisia* species such as *Artemisia dubia* [15-18] and *A. absinthium* [19]. Micropropagation and organogenesis of different *Artemisia* species have also been formerly reported such as *A. annua* [20-23], *A. scorpia* [24], *A. vulgaris* L. [25], *A. mutellina vill.* [26]. However, there is no report regarding genetic transformation and tissue culturing of *A. carvifolia* Buch.

In the current study, we have reported simple transformation and regeneration conditions involving two different types of explants for direct organogenesis and best regeneration response. MS media containing different combinations of growth hormones were tested and optimum combination giving maximum number of shoots was selected. *Agrobacterium tumefaciens* strain C58C1 was used for genetic transformation and *gus* reporter gene was used to find the transient β-glucoronidase expression in transformed leaves and stem. Stable integration was confirmed by performing the PCR of kanamycin resistant plants.

Materials and Methods

Tissue culture media and plant material

Murashige and Skoog (MS) medium [27] was used for micro propagation, organogenesis and transformation of *A. carvifolia*. MS media supplementation with different hormonal combination is given in Table 1. Seeds of *Artemisia carvifolia* were collected from Astore, in the Northern regions of Pakistan (35.3667°N, 74.8500°E; 8,500 ft elevation). No specific permissions were required for the mentioned

***Corresponding author:** Bushra Mirza, Department of Biochemistry, Quaid-i-Azam University, Islamabad, Pakistan, Tel: 051-90643007; 051-9219809; E-mail: bushramirza@qau.edu.pk

Received November 23, 2015; **Accepted** January 30, 2016; **Published** February 02, 2016

Citation: Dilshad E, Ismail H, Kayani WK, Mirza B (2016) Optimization of Conditions for Genetic Transformation and I*n Vitro* Propagation of *Artemisia carvifolia* Buch. Curr Synthetic Sys Biol 4: 129. doi:10.4172/2332-0737.1000129

location for collection of seeds. However, the project was approved by the institutional biosafety committee (IBC) Quaid-i-Azam University Islamabad, Pakistan. Surface sterilization of seeds was carried out with 70% ethanol for 30 sec followed by 0.1% (w/v) mercuric chloride ($HgCl_2$) for different time durations including 20 sec, 40 sec, 60 sec and 2 min. After washing with sterile distilled water, seeds were sown on two types of media i.e. half strength MS medium and plane agar in petri dishes. They were given chill treatment at 4°C for 2 days in dark. Then petri dishes containing seeds were incubated in growth chamber at 25°C 16 h of photoperiod, illumination of 45 µE m-2 s-1 and 60% relative humidity under aseptic conditions.

Bacterial strain and construct used for transformation

Agrobacterium tumefaciens strain C58C1 harboring binary vector p35SGUSINT was used for transformation [28]. T-DNA of p35SGUSINT contained *NPTII* gene with NOS promoter and NOS terminator and β-glucuronidase (*GUS*) gene with CaMV35S promoter and NOS terminator (Figure 1). Prior to infection, bacterial strain was grown overnight in an incubator at 28°C with constant shaking at 120 rpm in Luria broth supplemented with 50 mg/L each of selective antibiotics ampicillin and kanamycin.

Transformation protocol

Transformation of *A. carvifolia* was carried out following the reported protocol [18], with some modifications. Leaf and stem with nodal segments, excised from two month old seedlings of *A. carvifolia* were cut into 0.5-1 cm pieces under laminar flow hood and precultured for 4-5 days on pre-culturing media, after that they were dipped in 50 ml of the overnight grown bacterial culture with optical density 1.0 for varying time durations (5,10,15,20 and 25 minutes). The excess bacterial culture was removed by blotting the explants on autoclaved filter paper, after that they were shifted to co-cultivation medium (Table 1) for 24, 48 and 72 hours to find the best co-cultivation time period. After co-cultivation and washing the explants with diluted antibiotic solution and sterile distilled water, they were cultured on selection media with different hormonal combinations for shooting (Table 1) supplemented with kanamycin 50 mg/l and cefotaxime 300 mg/l. Around 5-6 explants per petri plate were cultured and total 20 petri plates were used for each media formulation in completely randomized block design, the whole experiment was repeated at different time intervals.

Medium	Composition
Pre culturing/Co-cultivation medium	MS+BAP (2.5 mg/l)+ NAA (0.25 mg/l) +200 µM acetosyringone
Shoot regeneration medium (SRM) 1	MS+BAP (0.1 mg/l)+NAA (0.1 mg/l)
Shoot regeneration medium (SRM) 2	MS+BAP (0.5 mg/l)+ NAA (0.1 mg/l)
Shoot regeneration medium (SRM) 3	MS+BAP (1 mg/l)+ NAA (0.1 mg/l)
Shoot regeneration medium (SRM) 4	MS+BAP (2.5 mg/l)+ NAA (0.25 mg/l)
Rooting medium (RM)	MS+NAA (0.1 mg/l)
Antibiotics used for selection	Kanamycin 50 mg/l, Cefotaxime (300 mg/l)

Table 1: Media formulations used for organogenesis of *A. carvifolia*.

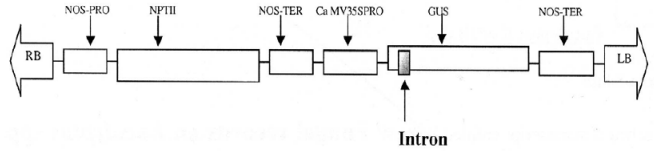

Figure 1: Schematic diagram of the T-DNA region of transformation vector p35SGUSINT: RB: Right border, NOS-PRO: nopaline synthase promoter; NPTII: neomycin phosphotransferase, NOS-TER: nopaline synthase terminator, CaMV35S-PRO: 35S promoter of cauliflower mosaic virus, GUS: gene for β-glucuronidase,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

The explants were transferred to fresh medium after every two weeks for the first month, after which subcultures were made every 3rd week. After eight weeks, the concentration of cefotaxime was reduced to 150 mg/l. The cefotaxime was totally omitted after 12 weeks. Transgenic shoots of 2-3 cm in length were excised and placed on MS rooting medium (Table 1) containing 50 mg/l kanamycin for further selection. Two independent transformation experiments were conducted and 200 explants per transformation event were used. In each transformation experiment, 20-40 control explants were used without co-cultivation with Agrobacterium.

Histochemical GUS assay

Histochemical GUS assay was carried out essentially as reported [28]. The leaf and stem explants after co-cultivation with bacteria were subjected to washing with sterile distilled water and incubated with GUS substrate solution overnight at 37°C. Untransformed explants were also incubated with the GUS substrate solution as control. The chlorophyll was removed by several washes with 70% ethanol after that transient GUS expression was observed.

Molecular analysis

In order to find out the stable integration of *gus* and neomycin phosphotransferase gene, kanamycin resistant plantlets were subjected to molecular analysis, which was performed after extraction of genomic DNA from aerial parts of 2 month old transformed and wild type plants by the CTAB method [29]. The plasmid from C58C1 was isolated by the alkaline lysis method. PCR analysis was performed using a programmed DNA thermal cycler (Biometra, USA). The *nptII* gene forward 5'-AAGATGGATTGCACGCAGGTC-3' and reverse primer 5´GAAGAACTCGTCAAGAAGGCG-3´, GUS gene forward 5´-AACGGCAAGAAAAAGCAGTC-3' and reverse primer 5´-GAGCGTCGCAGAACATTACA-3 were used for PCR analysis. Conditions applied for PCR were as described previously [18]. The annealing temperature for *NPTII* gene was 54°C and for that of *GUS* gene was 56°C.

Results and Discussion

The following research work was performed to optimize regeneration and transformation conditions for *A. carvifolia* Buch by using different explants, various media compositions and *Agrobacterium tumefaciens* strain C58C1 harboring p35SGUSINT with GUS reporter gene.

Effect of sterilization conditions and media on seed germination

Seeds were treated with mercuric chloride ($HgCl_2$) 0.1% (w/v) for different durations of time. No contamination was observed in seeds of all treatments but germination efficiency was found to be different for each treatment (Figure 2a). Germination efficiency was found to be more on half MS medium which was 95% than plane agar on which 75% seeds germinated (Figure 2b). Seeds given chill treatment for 2-3 days were found to be more prone to germination than those without it. Seed germination took not more than one week without requiring any growth hormone for germination. These results are confirmed by the findings of another group who also found seeds germination within 6-7 days and observed that addition of growth hormones to the germination medium does not influence the percentage of seed germination [21]. Later the germinated seedlings were maintained on MS medium with PGRs in order to obtain large no of plantlets for transformation experiment.

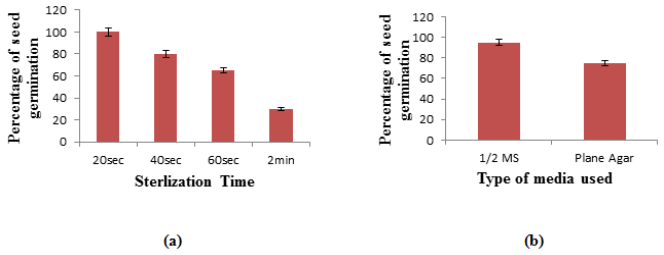

Figure 2: Percentage seed germination: seed germination efficiency with different duration of exposure to 0.1% (w/v) Mercuric Chloride (a) on two different types of media (b).

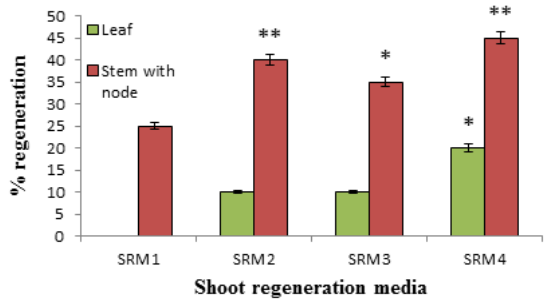

Figure 3: Effect of different media compositions and explants on transformation efficiency: Values are means of two experiments performed independently ± S.E. Asterisk represents the significant difference (*=P<0.05, **=P<0.01) in data compared with the low regeneration efficiencies of explants on different shoot regeneration media.

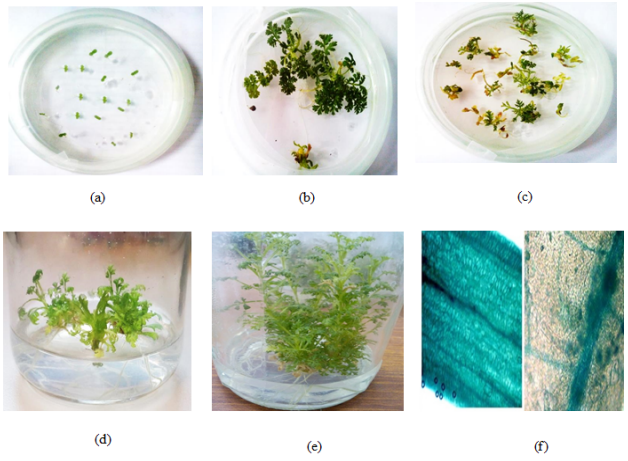

Figure 4: Seeds germination, regeneration of explants and gus expression: seeds germination (a), giving rise to plantlets used for transformation (b). Regeneration of leaf and stem explants on selection media (c), kanamycin resistant plants on shoot regeneration media SRM4 (d) and rooting media (e). Expression of gus gene in stem and leaf explants after bacterial infection.

Effect of media formulation and explants on shoot organogenesis and transformation

In vitro regeneration of *A. carvifolia* after transformation was achieved successfully (Figures 3 and 4). The best shoot regeneration results were obtained on SRM4 (MS+2.5 mg/l BAP+0.25 mg/l NAA) followed by SRM2 (MS+0.5 mg/l BAP+0.1 mg/l NAA) (Figure 3), both were found to be optimal (P<0.05) for regeneration of maximum no. of transgenics. Whereas SRM1 was found to be best for shoot elongation. The regeneration response obtained on shoot induction medium (SRM4) was in accordance with the previous report of [30], who were able to regenerate transgenic plants of *A. annua* under similar growth hormone concentration. There is another report stating that nodal explants of *Artemisia annua* on 2.5 mg/l BAP gives highest regeneration response [20]. Direct organogenesis takes less time for the rapid production of transformed plants [23] in contrast to callus tissue which needs to undergo few months of specialized culture before differentiating into shoot and root primordial [31]. Rooting was obtained on half strength MS medium supplemented with NAA at concentration of 0.1 mg/l which is supported by the results of Nair et al. [32] and Jun-Li et al. [17] who were working with *Agrobacterium tumefaciens* mediated transformation of *Artemisia annua* and found rooting on 0.05-2.0 mg/l NAA and 0.1 mg/L NAA gave the best results.

For the selection of transformed plants 50 mg/l kanamycin and 300 mg/l cefotaxime were used in the selection media. Various reports have shown that 50 mg/l kanamycin is sufficient for the selection of transformed plants [33-35]. The control of Agrobacterium growth at 300 mg/l cefotaxime has been shown by some other groups as well [36-38].

The highest transformation efficiency was shown by stem explants with nodal regions (50%) on all types of media as compared to leaf explants (25%). The type of explant contributes significantly towards efficient transformation system [39]. The increased transformation efficiency of stem explants as compared to leaf explants has also been reported [40-44]. Analysis of variance showed highly significant interaction (P<0.01) between different types of media and explants used for transformation (Table 2).

The age of explant is also considered to be critical factor for transformation. In our report one week old explants were found capable of being transformed sufficiently. These findings are also supported by previous findings [45,46] showing that transformation efficiency of *Agrobacterium* decreases as age of explants increases.

Effect of infection time and co-cultivation period

The bacterial suspension used for transformation at the optical density of 1.0 was found to be optimum for efficient transformation of *A. carvifolia*. Reports are available showing that bacterial solution with 1.0 OD gave maximum number of transformants of *A. absanthium* [19] and *Vigna radiata* [47,48]. Bacterial density higher than this does not increase transformation efficiency but affects regeneration potential of explants due to stress encountered [19].

Infection and co-cultivation time greatly affect transformation efficiency. Infection time of 10-15 minutes was sufficient for getting maximum regeneration response on selection media, longer infection time results in the death of the explants (Figure 5a). The time duration of co-cultivation also positively influences the transformation efficiency, probably it allows the T-DNA transfer, integration, transcription and sufficient enzyme production leading to the expression of kanamycin resistant and GUS phenotypes. It is reported that co-cultivation of

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value	Prob
Factor A (explants)	1	9009.375	9009.375	1221.6102	0.0000
Factor B (media)	3	1741.125	580.375	78.6949	0.0000
AXB	3	829.458	276.486	37.4896	0.0000
Error	16	118.000	7.375		
Total	**23**	**11697.958**			
Coefficient of Variation: 10.77%.					

Table 2: ANOVA for the effect of shoot regeneration media (SRM) on different types of explants on transformation efficiency.

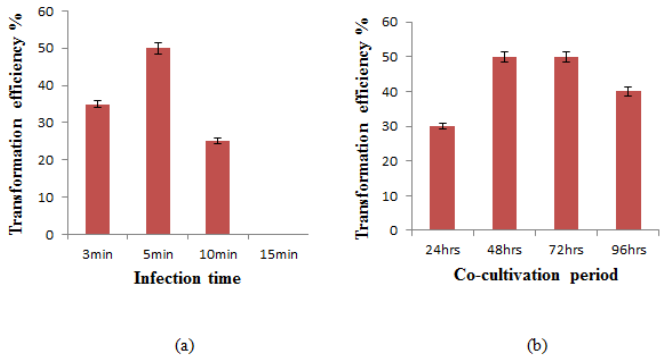

Figure 5: Effect of infection time and co-cultivation period on percentage regeneration of ex-plants.

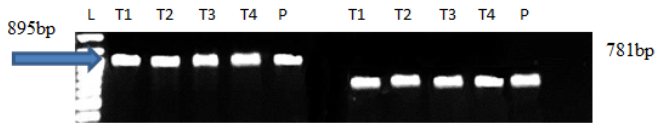

Figure 6: PCR amplified product of gus (895bp) and nptII gene (781bp): T1-T4 represent the transgenic lines, "P" indicate the Plasmid DNA used as template, WT stands for wild type plant of A. carvifolia.

explants with bacteria in the presence of acetosyringone increases transformation efficiency up to 20% [49]. However, we observed that co-cultivation period of 48 hours was found optimum for maximum transformation efficiency (Figure 5b), as previously reported that longer co-cultivation time only results in overgrowth of bacteria on explants and does not increase the transformation efficiency [18].

Histochemical gus assay and molecular analysis

Transformed explants after staining with GUS solution showed positive GUS expression in the form of blue dots scattered over the stem and leaf tissue (Figure 4f). On the contrary, the untransformed control explants were unable to show the GUS expression. The GUS expression was found to be maximum for co-cultivation of 48-72 hours after that it started to decline. Similar findings were obtained by Mannan et al. [19] who got the GUS+expression in the leaf, root and hypocotyl explants of *A. absinthium* L. after infection and co-cultivation of these explants with *Agrobacterium tumefaciens* C58C1 harboring the harboring p35SGUSINT vector.

PCR reaction performed to confirm the stable transgene integration showed the amplified products of 895bp for *gus* and 781 bp for *nptII* gene. Plasmid DNA also showed the similar amplified products, on the contrary wild type plant was unable to show these amplified PCR products (Figure 6).

Conclusion

Above results allow us to conclude that *Artemisia carvifolia* can be transformed with *Agrobacterium tumefaciens* strain C58C1 at optical density 1.0 with infection time of 15 minutes and co-cultivation period of 48 hours. The best regeneration response and transformation efficiency can be obtained by using stem explants with nodes at shoot regeneration medium containing 2.5 mg/l BAP, 0.25 mg/l NAA. Rooting can be obtained at NAA concentration of 0.1mg/l in half MS. These conditions would be helpful in conducting the experiments of *in vitro* propagation and transformation of *A. carvifolia* Buch in future.

Acknowledgement

We are thankful to the Higher Education Commission of Pakistan for providing the scholarship to author Erum Dilshad during her PhD studies.

References

1. Heble MR, Chadha MS (1986) Plant cell culture technology perspective and application. In: Nimbalkar NA (Eds). Fragrance and Flavor Industry: A leap into the future PAFAI, India.
2. Mulder-Krieger TH, Verpoorte R, Baerheim Svendren A, Scheffer JJC (1988) Production of essential oils and flavours in plant cell and tissue culture: A review. Plant Cell Tiss Org Cult 13: 85-154.
3. Ravishankar GA, Bhyalakshmi N, RamachandraRao S (1999) Production of food additives. In: Ramawat KG, Merillon JM (eds.) Biotechnology: secondary metabolites. New Delhi.
4. Dornenburg H, Knorr D (1997) Challenges and opportunities for metabolite production from plant cell and tissue cultures. Food Technol 51: 47-54.
5. Scragg AH (1997) The production of aromas by plant cell cultures. In: Schepier T (eds.) Advances in Biochem Enginee Biotech 55: 239-263.
6. Rout GR, Samantaray S, Das P (2000) In vitro manipulation and propagation of medicinal plants. Biotech adv 18: 91-120.
7. Khan MNA (1977) Cultivation of Davana+ In: CK Atal, BM Mathur (eds.) Cultivation and Utilisation of Medicinal and Aromatic Plants. Regional Research Laboratory Jammu-Tawi, India.
8. Liu CZ, Murch SJ, El-Demerdash M, Saxena PK, Artemisia judaica L (2004) micropropagation and antioxidant activity. J Biotech 110: 63-71.
9. Stojanowska A (2010) In vitro cultures of some plants from the Asteraceae family as a source of bioactive molecules. International Conference of Perspectives Cytostatic Compounds Production Using Biotechnological Methods Warsaw 152.
10. Kazemi M, Dakhili M, Dadkhah A, Yasrebifar Z, Larijani K (2011) Composition, antimicrobial and antioxidant activities of the essential oil of Artemisia kermanensis Podl., an endemic species from Iran. L J Med Plant Res 5: 4481-4486.
11. Vergauwe A, Geldre EV, Inze D, Van Montagu M, Eeckhout VD (1998) Factors influencing Agrobacterium tumefaciens-mediated transformation of Artemisia annua L. Plant Cell Reports 18: 105-110.
12. Biswajit G, Swapna M, Sumita F, Gosh B, Jha S (2000) Genetic transformation of Artemisia annua by A. tumefacienes and artemisinin synthesis in transformed cultures. Plant Scio. Linerich 122: 193-199.
13. Vergauwe A, Cammart R, Vandenberghe D, Genetello C, Inze D (2002) Agrobacterium tumefacienes mediated transformation of A. annua L. plants and the regeneration of transgonic plants. Plant cell Rep 15: 929-933.
14. Vergauwe A, Van Geldre E, Inze D, Van Montagu M, Vanden Eeckhout E (2005) Factors influencing A. tumefacienes mediated transformation of Artemisia annua L. Plant Cell Rep 18: 105-110.
15. Giri A, Narasu M (2000) Transgenic hairy roots. Recent trends and applications. Biotechnol Adv 18:1-22.
16. Rizwana AQ, Mushtaq A, Muhammad A (2002) Taxonomic study and medicinal importance of three selected species of the genus Artemisia Linn. Asian J Plant Sci 1(6): 712-714.
17. Jun-li H, Hong W, He-chun Y, Yan L, Zhen-qiu L (2005) High efficiency of genetic transformation and regeneration of Artemisia annua L. via Agrobacterium tumefaciens mediated procedure. Plant Sci 168: 73-80.
18. Kiani BH, Safdar N, Mannan A, Mirza B (2012) Comparative Artemisinin analysis in Artemisia dubia transformed with two different Agrobacteria harbouring rol ABC genes. Plant Omics J 5: 386-391.
19. Mannan A, Syed TN, Mirza B (2009) Factors affecting Agrobacterium tumefaciens mediated transformation of Artemisia absinthium. Pak J Bot 41: 3239-3246.
20. Janarthanam B, Rashmi P, Sumathi E (2012) Rapid and Efficient Plant Regeneration from Nodal Explants of Artemisia annua L. Plant Tiss Cult and Biotech 22: 33-39.
21. Jamaleddine ZO, Lyam P, Fajimi O, Giwa A, Aina A (2013) In vitro growth response of Artemisia annua seeds to different concentrations of plant growth regulators. Afri J Biotech 10: 17841-17844.

22. Tahir SM, Usman IS, Katung MD, Ishiyaku MF (2013) In Vitro Regenarationof Artemisia Annua (Wormwood) Using Seed Explants. The Internat J Biotech 2: 171-181.

23. Kiani BH, Suberu J, Barker GC, Mirza B (2014) Development of efficient miniprep transformation methods for Artemisia annua using Agrobacterium tumefaciens and Agrobacterium rhizogenes. In Vitro Cellular Devel Biology-Plant 50: 590-600.

24. Aslam N, Zia M, Chaudchary MF (2006) Callogenesis and direct organogenesis of Artemisia scoparia. Pak J Biol Sci 9: 1783-1786.

25. Govindaraj S, Kumary BD, Gioni PL, Flamini G (2008) Mass Propagation and Essential Oil Analysis of Artemisia vulgaris. J Biosci Bioenginee 105: 176-183.

26. Mazzetti C, Donata M (1998) Micropropagation of Artemisia mutellina, ISHS Acta Horticulturae Symposium on Plant Biotechnology as a tool for the exploitation of Mountain Lands 457.

27. Murashige T, Skoog E (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15: 472-493.

28. Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: ß-glucuronidase as a sensitive and versatile gene marker in higher plants. EMBO J 6: 3901-3907.

29. Ahmed I, Islam M, Arshad W, Mannan A, Ahmad W (2009) High-quality plant DNA extraction for PCR: an easy approach. J appl genetics 50: 105-107.

30. Wang YY, Jing FY, Yu SY, Chen YF, Wang T (2011) Co-overexpression of the HMGR and FPS genes enhances artemisinin content in Artemisia annua L. J Med Plants Res 5: 3396-3403.

31. Palama TL, Menard P, Fock I, Choi YH, Bourdon E (2010) Shoot differentiation from protocorm callus cultures of Vanilla planifolia (Orchidaceae): proteomic and metabolic responses at early stage. BMC plant biol 10: 82.

32. Nair MS, Acton N, Klayman DL (2001) Production of artemisinin in tissue cultures of Artemisia annua L. J Nat Prod 49: 504-507.

33. Davioud JV, Shank KM, Montagu MJC, Zambryski R (1988) Efficient and genotype independent Agrobacterium mediated transformation of Artemisia annua. J Plant Physiol 160: 1253-1257.

34. Maurel C, Barbier-Brygoo H, Spena A, Tempe J, Guern J (1991) Single rol genes from the A. rhizogenes TL-DNA alter some of the cellular responses to auxin in Nicotiana tobacum. Plant Physiol 97: 212-216.

35. Morgan AJ, Cox PN, Turner DA, Peel E, Davey MR (2004) Transformation of Artemisia annua using Ti plasmid vector. Plant Sci 49: 37-49.

36. Vatsya B, Bhaskaran S, Thorpe TA (2002) Transformation of Artemisia with Agrobacterium tumefaciens. Plant Tiss Cult Man 6: 1144.

37. Jorsobe KL, Wullems GJ, West J, Artiz D (2003) Agrobacterium mediated transformation and its further application to plant biology. Annu Rev Plant Physiol 38: 467-486.

38. Bettini P, Michelotti S, Bindi D, Capuana M, buiatti M (2003) Pleotropic effect of the insertion of the A. rhizogenes rol D gene in tomato. Theor Appl Genet 107: 831-836.

39. Michalczuk B, Danuta W (2004) Effect of medium composition and date of explant drawing on effectiveness of Agrobacterium-mediated transformation in the Petunia (Petunia hybrida pendula). J fruit ornament plant rese 12: 5-16.

40. Gamborg OL, Miller RA, Ojima K (1968) Transfer of T-DNA from Agrobacterium to the plant cell. Plant Physiol 107: 1041.

41. Bidney DL, Shepard JF, Kaleikau E (1991) Regeneration of plants from different explants of Artemisia species. Plant Cell Tiss 117: 89-92.

42. Skoog F, Miller CO (1997) A revised medium for rapid growth and bioassay with Artemisia tissue culture. Plant Physiol 102:363-372.

43. Murata M (1998) Effect of genotype and explant type in regeneration frequency of Artemisia plant in vitro. Acta Horti 231-234.

44. Robertson D, Earle ED (2000) Plant regeneration from leaf explant of Artemisia annua. Plant Cell Rep 5: 61-64.

45. Vergauwe A, Ronny C, Dirk, Christiane G, Dirk I (1996) Agrobacterium tumefacienes-mediated transformation of Artemisia annua L. and regeneration of transgenic plants. Plant Cell Rep 15: 929-933.

46. Nin A, Bennici G, Roselli D, Mariotti S, Schiff RM (1997) Agrobacterium-mediated transformation of Artemisia absinthium L. (wormwood) and production of secondary metabolites. Plant Cell Rep 16: 725-730.

47. Jaiwal PK, Kumari R, Ignacimuthu S, Potrykus I, Sautter C (2001) Agrobacterium tumefaciens-mediated genetic transformation of mung bean (Vigna radiata (L.) Wilczek) a recalcitrant grain legume. Plant Sci 161: 239-247.

48. Jaiwal PK, Sautter C, Potrykus I (1998) Agrobacterium rhizogenes mediated gene transfer in mung bean (Vigna radiata (L.) Wilczek). Current Sci 75: 41-45.

49. Schmid G, Hofheinz W, Hart P, Duke SO (2005) Efficient and genotype independent Agrobacterium mediated transformation of Artemisia annua. J Plant Physiol 160: 1253-1257.