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In vitro Regeneration and Genetically Transformed Culture of *Artemisia diffusa*

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Abstract

Introduction: The present study has introduced a simple and rapid tissue culture system aimed at *in vitro* regeneration of *Artemisia diffusa* and *in vitro* artemisinin production in its genetically transformed culture.

Materials and Methods: An *in vitro* regeneration of *A. diffusa* was developed using different combinations of plant growth regulators including Naphthalene Acetic Acid (NAA), Indole-3-Acetic Acid (IAA), Thidiazuron (TDZ) and Benzyl Adenine (BA). Also, an efficient genetically transformed root induction system for *A. diffusa* was developed through *Agrobacterium rhizogenes*- mediated transformation using four bacterial strains, A4, ATCC15834, MSU440, and MAFF-02-10266. The stem and leaf of one month old sterile plants of *A. diffusa* were used as explants. Molecular analysis of transformed root lines was confirmed by PCR using primers specific for the *rol*B gene.

Results: The highest regeneration occurrence was obtained using MS medium containing 0.5 mg/L TDZ and 0.1 mg/L IAA (75%). Root induction was obtained on MS medium supplemented with 0.5 mg/L IBA. The results showed a significant increase in transformation frequency when the strain MSU440 was used (80.7%). Approximately 0.05 % artemisinin was detected by High-performance liquid chromatography (HPLC) analysis in root cultures. To the best of our knowledge, this is the first report of *A. diffusa in vitro* organogenesis and transformation.

Conclusions: This study describes an efficient protocol for hairy roots culture of *A. diffusa* which can be used for scaling-up the plant active phytochemicals or for genetic manipulations of the plant.

Keywords: *Artemisia diffusa*, Artemisinin, Genetically Transformed Culture, *In vitro* Regeneration

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Introduction

Artemisia diffusa is one of 34 *Artemisia* species growing wildly in Iran.[1] Artemisinin, a sesquiterpene lactone endoperoxide, is an effective and well known antimalarial drug found in this medicinal plant.[2,3] The primary cost of artemisinin production is very expensive through extraction and purification from *Artemisia annua*.[4] *Artemisia* genus show antimalarial, antitumor and anti-inflammatory properties.[5,6] Due to the presence of peroxide bond in artemisinin, its industrial and chemical synthesis is difficult.[7] Despite the hypothesis that biosynthesis of artemisinin may be an ancestral trait for the genus, previous studies have found that not all *artemisia* species appear to contain artemisinin.[8] Rustaiyan et al. reported a new sesquiterpene lactone (tehranolide) found in *A. diffusa* which could be considered responsible for the antimalarial properties of this species, given the relevance of the peroxide bridge in this medicinal property.[9,10] Tehranolide, a natural sesquiterpene lactone with an endoperoxide group in its structure, purified from *Artemisia diffusa*, can inhibit multi drug resistance of *Plasmodium falciparum* and cell growth in cancer cells.[10-13] Investigations specifically indicated the inhibitory effects of the *A. diffusa* crude extracts and the fraction, which contained sesquiterpene lactones including tehranolide, on the developmental stages of *Plasmodium berghei* by decreasing parasitaemia.[14,15] In recent years, there has been more progress in the molecular regulation of secondary metabolites biosynthesis. So, for successful plant transformation, an efficient protocol for *in vitro* organogenesis is necessary.

Transformation using *Agrobacterium rhizogenes* creates a rapid and simple tool to integrate and express foreign genes in plant cells. The molecular basis of hairy root induction is the possibility to insert the T-DNA from Ri plasmid of *A. rhizogenes* into the plant genome. T-DNA integrated into the genome has *rol* gene loci which have a major role in genetically transformed roots.[16] Hairy root can be cultivated in a simple medium without the addition of plant growth regulators and hairy root can produce valuable secondary metabolites.[17] The present study focused on the development of an efficient protocol for *in vitro* shoot organogenesis, plant regeneration and induction of genetically transformed

roots from *A. diffusa*. In addition, a detection and measurement of artemisinin, an effective antimalarial drug and potent anticancer compound, has been performed in transgenic root cultures of *A. diffusa*.

Materials and Methods

Plant Material

Seeds of *A. diffusa* were obtained from Pakan bazr Company, Isfahan, Iran. Seeds of *A. diffusa* were surface-sterilized with 70% (v/v) ethanol for 1 min and 3% (v/v) sodium hypochlorite for 12 min and then rinsed five times with sterilized water. The seeds were placed on agar solidified MS medium. The medium was adjusted to pH 5.8 before adding agar and then sterilized by autoclaving. The seeds were incubated in a growth chamber under a 16/8-h (light/dark) photoperiod at 25 °C. Three-week-old seedling leaves were cut and used as explants (Figure 1a).

Tissue Culture and Regeneration Media

The leaves from four-week-old plants were cut from the leafstalk and cultured on solidified MS medium supplemented with different combination of NAA, IAA, BA and TDZ, as follows: BA (0.5, 1, 1.5 and 2 mg/L), TDZ (0.1, 0.5 and 1 mg/L); NAA (0, 0.05 and 0.1 mg/L), IAA (0.1 and 0.5 mg/L).

Preparation of A. rhizogenes Strains

Four strains of *A. rhizogenes* were used in the study: A4, ATCC15834, MSU440 and A13 (MAFF-02-10266). The *A. rhizogenes* strains were cultured in liquid LB medium. The bacteria were pelleted by centrifugation for 12 minutes at 3,500 rpm and re-suspended in inoculation medium (½ MS liquid medium [pH 5.5] containing 50 mg/L sucrose and 150 µM acetosyringone) at a cell density (OD_{600}) of 0.7.

Establishment of Transgenic Roots

Leaf explants from four-week-old *A. diffusa* seedlings were used for co-cultivation with different strains of *A. rhizogenes*. The explants were inoculated with different bacterial strains for five minutes using the immersion method. The explants were briefly dried on sterile filter paper and transferred to solidified co-cultivation medium (the same inoculation medium solidified with 0.7% [w/v] agar),[16] after two days of co-cultivation, the explants were transferred to MS media supplemented with 350 mg/L cefotaxime to eliminate the bacteria. Control explants were treated similarly without exposure to bacteria. In this study, the effect of factors such as *Agrobacterium* strain and, pre-culture period (0, 24 and 48 h), co-cultivation period (24, 48 and 72 h), absence and presence of acetosyringon (150 µM) during incubation and co-cultivation on induction rate of hairy root were evaluated.

PCR Analysis

Total genomic DNA was isolated using the Cetyl-Trimethyl Ammonium Bbromide (CTAB) method from hairy root samples and control roots. To investigate the presence of the *rol*B gene transferred from Ri plasmid, the PCR was performed using the following primers: 5´-GCTCTTGCAG TGCTAGATTT-3´ (forward primer) and 5´-GAAGGTGCA AGCTACCTCTC-3´ (reverse primer) and the primers 5´-ATGCCCGATCGAGCTCAAGT-3´ and 5´-CCTGACCCA AACATCTCGGCTGCCCA-3´ were considered for *virD* gene. The primer sequences of rolB were used for the validation of transformed roots and the *virD* gene was taken for recognizing the contradiction of infection by *Agrobacterium*.

HPLC Analysis

Hairy roots and control samples were dried in oven at 60 °C for 24 h. The dried leaves were refluxed by 30 ml ethanol at 70 °C for 12 h. The soup was filtrated and evaporated; the obtained sediment materials were dissolved in 5 ml HPLC grade ethanol (Merck Co.). The HPLC analysis was performed based on Simth's method (Smith et al., 1997). The mobile phase consisted of acetonitrile and water 60:40 (% v/v) and was delivered at a flow rate of 1.0 ml/min using a double-reciprocating pump and the analysis wave length was at 210 nm (Waters, MA, USA, model Breeze). The sample was injected through a 20 µL sample loop. A C8 analytical column (150 mm Technik, Germany) equipped with a guard column of the same packing was used.

Statistical Analysis

The experiment was carried out with a completely randomized design with three replicates. The data were expressed as the mean ± standard deviation. To compare the treatment groups, ANOVA analysis followed by Duncan's test was used. All statistical analyses were performed by SPSS 16.0.

Results

Tissue Culture and Regeneration Media

Figure 1a shows three-week seedling leaves which were used as explants. After 2-3 sub cultures, the callus appeared on most of the explants (Figure 1b, c). The explants were transferred to MS medium (Figure 1d-n). Induction of callus was observed at all hormonal treatments. The highest regeneration frequency was obtained using MS medium containing 0.5 mg/L TDZ and 0.1 mg/L IAA (75%) (Figure 2). The elongated shoots were transferred to root induction medium for two weeks (Figure 1o). The MS medium was considered for more growth of plantlets. Finally, the plantlets were acclimatized in the greenhouse (Figure 1p, q). In the case of TDZ + NAA and TDZ + IAA hormones, direct regeneration was observed. The use of TDZ treatments led to adventitious shoot organogenesis within 3 weeks.

Figure 1. a) 3 weeks seedlings; b-c) callus induction from *Artemisia diffusa* leaf explants on MS medium containing BA and NAA; d-g) regeneration on MS medium containing BA and NAA; h-n) regeneration on MS medium containing TDZ; o) Root formation on 1/2 MS medium supplemented 5.0 mg/L IBA; P-q) regenerated and Adaptation of *A. diffusa.*

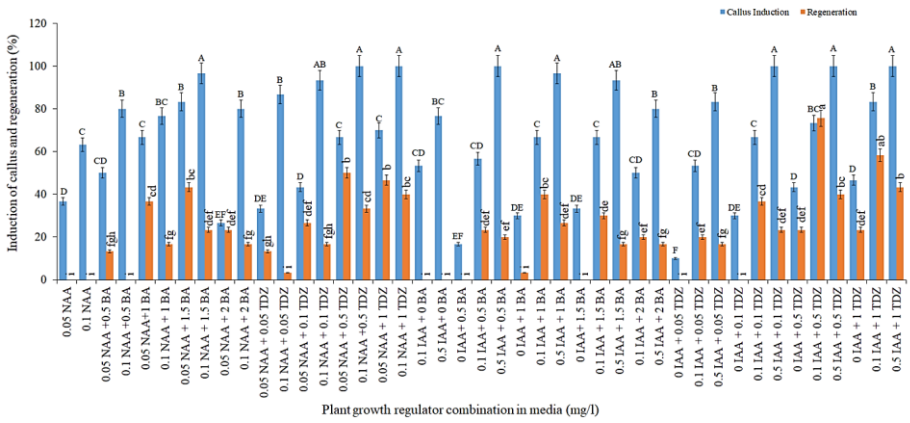

Figure 2. Effect of Different Plant Growth Regulator Combination on Callus Induction and Regeneration of *Artemisia diffusa*.

Figure 3. *A. rhizogenes* Mediated Transformation of *Artemisia diffusa*. a) Hairy root induction after 4 weeks of inoculation in leaf; b) Hairy root culture after 6 weeks of inoculation using *A. rhizogenes* A4; c) Hairy root culture after 6 weeks of inoculation using *A. rhisogenes* A13; d, e) Hairy root culture after 6 weeks of inoculation using ATCC15834; f-h) Hairy root culture after 6 weeks of inoculation using *A. rhisogenes* MSU440.

Virulence of Agrobacterium Strains

A. rhizogenesis strains ATCC15834, A4, A13 and MSU440 were used for the induction of hairy roots. As shown in Figure 3, all the strains were virulent against the leaf explants. The highest amount of transgenic and root induction were obtained from MSU440 (80.7%) and ATCC15834 (67%), respectively. There were no significant differences ($P \leq 0.05$) between the virulence of MSU440 and ATCC 15834. There was no hairy root formation in control explants. The hairy roots of *A. diffusa* were initiated from leaf explants after 10 to 15 weeks. The results showed a drastic increase in the transformation frequency when *A. rhizogenes* MSU440 in combination with 150 µM acetosyrengone were used (~81%) (Figure 4).

Effect of Pre-Culture Period on Hairy Root Induction

Figure 5 shows that the highest percentage of hairy root induction was observed in MSU440 and (80.7%) and in ATCC15834 (67%) without pre-culture period.

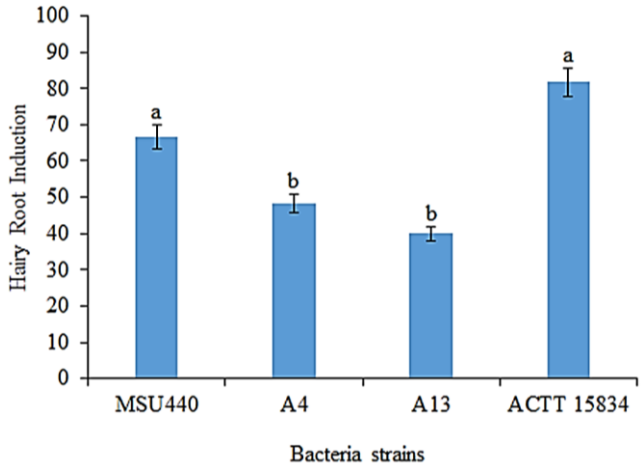

Figure 4. Effect of Different Strains of *Agrobacterium rhizogenesis* on Hairy Root Induction of *Artemisia diffusa*. Data represent mean values of three replicates ± SE.

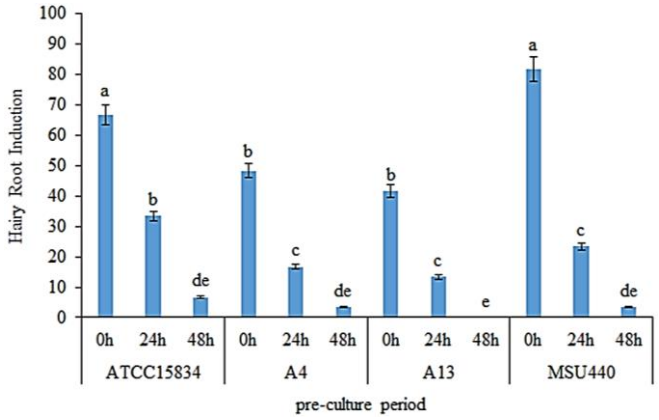

Figure 5. Effect of Different Pre-Cultivation Periods on Hairy Root Induction of *Artemisia diffusa*. Data represent mean values of three replicates ± SE.

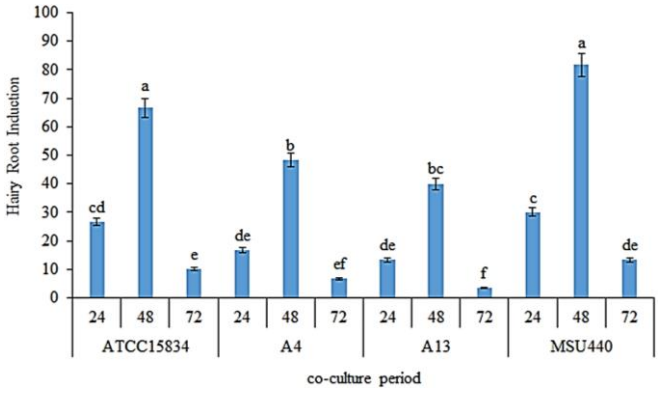

Figure 6. Effect of Different Co-Cultivation Periods on Hairy Root Induction of *Artemisia diffusa*. Data represent mean values of three replicates ± SE.

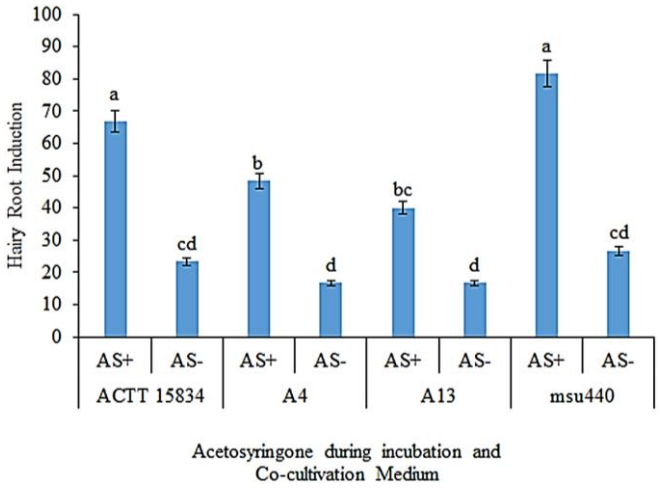

Figure 7. Effect of Acetosyringone in Incubation and Co-Cultivation Medium on Transgenic Root Induction of *Artemisia diffusa*. AS+ indicates presence and AS- indicates absence of acetosyringone.

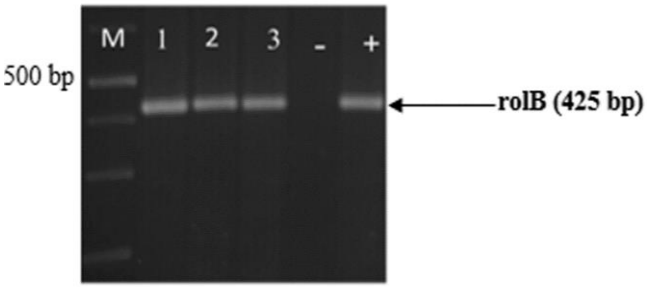

Figure 8. PCR Analysis for Detection of the *rolB* Gene in 3 Hairy Root Lines of *A. diffusa*. M: Molecular size marker (1 kb); 1-3: hairy root lines, C: negative control; +: positive control.

Effect of Co-Cultivation Period on Hairy Root Induction

The co-cultivation period is a significant factor that influences transformation efficiency. There was no hairy root from controls (0-day co-cultivation). As shown in Figure 6, the effect of co-cultivation period on the transformation frequency was remarkable. The highest transformation frequencies were obtained after 48 h of co-cultivation on ½ MS medium with 150 µM acetosyringone in the dark (Figure 6).

The Effect of Acetosyringone during Incubation and Cocultivation on Hairy Root Induction

The elements present in the inoculation and co-cultivation media have effects on *A. rhizogenes* mediated transformation, as it has been shown that the transformation efficiency was drastically increased by removing some major mineral components such as phosphate, nitrate.[16] Therefore, in the present study, ½ MS medium was used as inoculation and co-cultivation medium instead of full strength MS. In this investigation, the effect of acetosyringone on the transformation frequency was evaluated in inoculation and co-culture media, (absence (AS-) and presence of acetosyringone (AS+)) (Figure 7).

Molecular Confirmation of Transformation

Transgenic lines of hairy roots were confirmed using the presence of *rol*B gene. The PCR led to amplification of *rol*B gene in all transgenic hairy root lines (Figure 8). The *rol B* genes were not detected in PCR product of wild roots (negative control). The PCR products size was 425 bp for the *rol*B gene (Figure 8). To confirm that the obtained roots were not contaminated with bacteria, PCR using *vir*D specific primers was performed and the lack of *vir*D gene demonstrated that the obtained roots were bacteria-free (data not shown).

HPLC Analysis

Artemisinin in hairy root culture was detected by HPLC analysis (0.05%) (Figure 9).

Discussion

Artemisia genus has important antimalarial compound artemisinin. An *in vitro* regeneration of *A. diffusa* was developed using different combinations of hormones including NAA, IAA, TDZ and BA. In the case of TDZ + NAA and TDZ + IAA hormones, direct regeneration was observed. Zayova et al. established a protocol for *A. annua* and they reported that optimal shoot multiplication and rooting were obtained on 1.0 mg/L BAP and 0.1 mg/L IBA, respectively.[18] Sharafi et al. showed that the shoot regeneration from *A. sieberi* on MS medium contained BA + NAA from induced callus.[19] Numerous studies had previously reported that an optimal concentration of cytokinins, alone or in combination with auxin, is necessary for in vitro shoot regeneration of plants.[20-22] Among plant growth regulators, cytokinins have proven to be the most important factor affecting shoot regeneration.[23] Kazeroonian et al. reported that the callogenesis rates and organogenesis types showed some differences among the

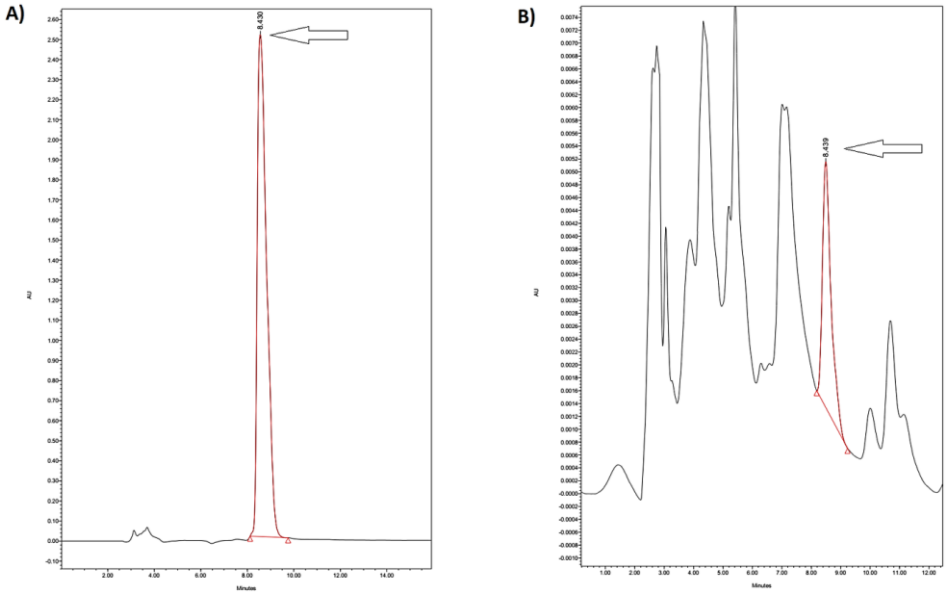

Figure 9. HPLC Analysis. a) Standard; b) Hairy root sample.

studied explants of *Chrysanthemum morifolium*. The type of cytokinin can affect the type of regeneration[22] which is depends on plant species. The present investigation showed that low concentrations of TDZ can be effective for direct regeneration in *A. diffusa* within three weeks. In the present study, it was observed that explants grown under TDZ treatments produce more branches. This finding is in agreement with Faisal and Anis's investigation showing that efficient *in vitro* plant regeneration occurred in low concentrations of TDZ.[24] It can be concluded that TDZ, a diphenyl urea derivative, is the optimal plant growth regulator for *A. diffusa*. The TDZ is the most active cytokinin-like substances and it induces greater in vitro shoot proliferation than many other cytokinins in many plant species.[25-27] Like other synthetic cytokinins, TDZ appears less susceptible to enzymatic degradation *in vivo* than other naturally occurring amino purine cytokinins, and therefore at low concentrations it can be more efficient than other cytokinins. However, it can have undesirable side effects, such as inhibited shoot elongation and hyperhydricity but, it has been shown to be suitable for micropropagation and the regeneration of some species.[28,29] Adventitious shoot organogenesis in *Artemisia* genus has been studied in some species.[30-33]

To the best of our knowledge, this is the first report of *in vitro* adventitious of *A. diffusa*. This protocol provides a basis for future studies on genetic improvement and could be applied to large-scale multiplication systems for commercial nurseries of *A. diffusa*. In *Hyssopus officinalis* and *Brassica oleracea* species, it has been previously reported that the highest regeneration percentage were achieved on MS medium supplemented with TDZ and IAA.[25,34] The MS medium containing 0.5 mg/L TDZ and 0.1 mg/L IAA (75%) were effective media. This approach as a simple *in vitro* tissue culture method for obtaining a high frequency of *A. diffusa* regeneration in a short period, is very effective. *In vitro* direct shoot organogenesis considered as an effective procedure for producing of genetically alike plants without somaclonal variation which occurred after callus induction.

Also, an efficient genetically transformed root induction system for *A. diffusa* was developed through *Agrobacterium rhizogenes*- mediated transformation using four bacterial strains, A4, ATCC15834, MSU440, and MAFF-02-10266. All the strains were virulent against the leaf explants. The highest amount of transgenic and root induction were obtained from MSU440 (80.7%) and ATCC15834 (67%), respectively. There were no significant differences ($P \leq 0.05$) between the virulence of MSU440 and ACTT 15834. The strain MSU440 has been successfully used for hairy root induction in *Citrullus colocynthis* and *Plumbago europaea*.[35,36] Overall, it seems that pre-culturing does not only enhance hairy root induction in *A. diffusa*, but also has a negative influence on it. The highest transformation frequencies were obtained after 48 h of co-cultivation on ½ MS medium with 150 µM acetosyringone in the dark. Similarly, co-cultivation period of 48 h was also found effective for *Momordica charantia*, *Linum mucronatum* and *Cucumis sativus* cultures.[37-39] It can be concluded that short co-cultivation period does not ensure integration of transferred DNA, while long in co-cultivation period decrease root induction because of overgrowth of the bacterium. However, there was significant differences between AS+ groups and AS- groups hairy root induction ($P \leq 0.05$). This result proves that this compound also increased the virulence of the *Agrobacterium* strains. It can actually be explained by an insufficient amount of *vir*-inducing phenolic compounds produced by this species when wounded and infected with

bacteria. In fair agreement with our findings, the enhancement effect of acetosyringone on transformation efficiency of *Eustoma grandiflorum*, *Passiflora edulis*, *Arachis hypogaea* and *Astragalus membranaceus* species was previously indicated.[40-43] In early studies, production of artemisinin in hairy root cultures of *A. dubia* and *A. annua* have also been investigated and they found a higher growth rate and biosynthesis of artemisinin content as compared to control untransformed roots.[44,45] Some studies reported that different strains of *Agrobacterium rhizogenesis*, different pre-cultivation periods, different co-cultivation periods, presence and absence of Acetosyringone in incubation and co-cultivation medium play an important role in the success of transformations.[35,46]

Conclusion

This study has developed an efficient protocol for *in vitro* regeneration and *in vitro* artemisinin production in the genetically transformed culture of *Artemisia diffusa*. In this investigation, treatments containing TDZ produced direct regeneration. Direct shoot regeneration is important as it can be used for breeding work of *Artemisia*, since effective shoot regeneration will improve the recovering shoots from transformed cells. In addition, genetically transformed root is the most appropriate technique for producing secondary metabolites. To conclude, the present study believes that hairy roots have the potential for elicitation, biotransformation and metabolic engineering. These results can help to develop promising strategies to the mass propagation of *A. diffusa* for gene transformation goals, production of large amounts of this valuable therapeutic compounds and commercial cultivation.

Authors' Contributions

MB and S Performed the part of *in vitro* regeneration and S R and EM performed the transformation. AS proposed the idea of the current study and analyzed the data.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Acknowledgment

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