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PRODUCTION OF ARTEMISININ AND ITS DERIVATIVES IN HAIRY ROOTS OF *ARTEMISIA DUBIA* INDUCED BY *rol*A GENE TRANSFORMATION

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Abstract

Artemisinin and its derivatives are phytochemical constituents of genus Artemisia. Demand of these plant secondary metabolitesis increasing due to their immense therapeutic significance. Besides their established antimalarial role, recent studies have also disclosed their anticancer potentials. It has made imperative to develop new and efficient sources of these compounds. Inherent synthetic challenges give biological sources preference over chemical synthesis of artemisinin and its derivatives. Therefore, genetic improvement of plants and, rather less preferentially, microbes is focus of current research to gain increase productivity of these valuable drugs. This study has analyzed *A. dubia*as potential source of artemisinin and its derivatives. Transformation of *Artemisia dubia* was carried out using *A. tumefaciens* strain LBA 4404 containing *rolA* gene constructed on pRB 29. Healthy and acclimatizable transgenic plants were produced using optimized concentrations of BAP and NAA. Previously acclimatized rol *ABC* transgenic plants were also *In vitro* regenerated for comparative analysis of artemisinin and its derivatives. PCR amplification of *rolA* gene was done to confirm the integration of T-DNA in transgenic plants.TLC analysis was performed to evaluate comparative production of artemisinin and derivatives in *rolA* and *rol ABC* transgenic *A. dubia*. It revealed that *rolA* transgenic plants contain comparable amounts of these metabolites. Both type oftransgenic plants manifested the enhancement of other uncharacterized compounds as well. Besides systematic optimization of *In vitro* regenerative protocol for *Artemisia dubia*, relative regeneration ability of *rol* transgenic and control plants was also assessed at four regenerative stages. It was observed that unlike control, *rol* transgenic plants showed best root induction only on combination of auxins and cytokines. It was concluded that *rol* genes transformation of plants is an efficient tool to enhance their secondary metabolites production. *RolA* transgenic *A. dubia*are cultivable, viable and efficient source of artemisinin and its derivatives to meet their ever-growing demands.

Key words: Genetic transformation, Artemisinin, *Agrobacterium tumefaciens*.

Introduction

Artemisia dubia belongs to family Asteraceae and contains secondary metabolites of medicinal interest. Production of these valuable compounds in large quantities has been focus of recent research to combat lethal diseases like malaria and cancer. Genetic transformation is an effective molecular tool that has been employed to achieve this end. It is long maintained that *rol* genes transgenic plants contain increased secondary metabolites content. These genes are strong activators of secondary metabolism (Shkryl *et al*., 2007). It has been observed that *rolB* and *rolC* (probably *rolA* as well) act antagonistic to each other (Maurel *et al.,* 1991). Therefore, use of one *rol* gene at a time has been described by Christophe *et al*., 1991 to be a worthy choice to increase plant secondary metabolites productivity.Plant transgenic studies involving *rolA* gene have shown that it possesses huge potential to enhance the secondary metabolites production in various plants. For instance, in case of *Tobacco* and *Rubia cordifolia*, it incredibly increased nicotine and anthraquinone production, respectively (Bulgakov *et al*., 2002). Previous studies also support *rol* genes enhancement of secondary metabolites in *Artemisia dubia*. Mannan *et al.* (2008) transformed *Artemisia dubia* with *rol* genes using LBA 9402 and 8196 strains of *Agrobacterium rhizogenes* and observed increased fresh weight of hairy roots and artemisinin contents. Kiani *et al.* (2012) carried out *A. rhizogenes* and *A. tumefaciens*-mediated transformation of *Artemisia dubia* with *rol ABC*genes and observed increased artemisinin contents in transgenic plants and hairy roots. Other studies reveal that *A. dubia*exhibits huge proliferative potential (Kryzeviciene *et al.,* 2010 and manifests anticancer (Huang *et al.,* 2010), Brine-shrimp cytotoxic, antimicrobial (Haq *et al*., 2012) and allelopathic activities (Pudel *et al*., 2005). Hence *rolA* gene transformation of *A. dubia* possessing medicinal values is essentially plausible scientific venture.

Natural plant pathogens have long been exploited to produce genetically superior plants. Studies, especially, using *A. tumefaciens*, have now culminated into remarkable systems. As for example, *A. tumefaciens*-mediated TMV-based viral vectors transfer has enhanced the production of green fluorescent proteins (GFP) upto 10^3 and more than10^6 folds in *Nicotiana benthamiana* and *Nicotiana tabacum*, respectively, literally turn the plants into factory of these secondary metabolites (Marillonnet *et al*., 2005). It suggests that optimization of *A. tumefacienes*-mediated transformation system can help scale up the production of artemisinin derivatives.

Artemisinin and its derivatives manifest diverse potentials of immense value.Lateclinical trials showed that antimalarial artemisinin is also effective for treatment of a variety of cancers, including leukemia, breast, colon, prostate and brain cancers (Maria *et al*., 2003). It selectively kills cancer cells by making use of their rapacious appetite for iron (Narendra & Henry, 2001). Semisynthetic derivatives of artemisinin exhibit great anticancer potentials (Thomas *et al.,* 2005). WHO has recommended their use in ACTsas well to combat malaria (Anon., 2006). Artemether/lumefantrine, artesunate-

amodiaquine and dihydroartemisinin-piperaquine are artemisinin derivatives-based drugs commonly in vogue today (Issaka *et al*., 2007). Artemether, arteether, artesunate and dihydroartemisinin are notable artemisinin derivatives of therapeutic importance.Cytotoxic, anti-proliferative, anti-angiogenic(Maria *et al*., 2003), anti-cytomegalo (Suzanne *et al*., 2006) and hepatitis B virus(Marta *et al*., 2005)and anti-tuberculosis (Marvin *et al.*, 2011) are remarkable activitiesshown by these compounds.These drugs inhibit proliferation of human lymphoblastic, xenografted Kaposi's sarcoma and cancer cells(Efferth & Thomas, 2006). Artemisinin conjugates induce apoptosis in breast cancer cell lines (Woong *et al*., 2007). Additionally, these compounds inhibit brain glioma growth and arrest thecell cycle at G2phase in ovarian and human pancreatic cancer cell lines (Yang *et al*., 2007). Nuclear receptor modulation, interference with cell migration (Gary & Shyam 2009) and disruption of iron hemostasis (Narendra and Henry, 2001) are oxidative damage independent routes of action for artemisinin and its derivatives.

Research has been accelerated to discover and improve sources of these valuable compounds. Inherent challenges associated with synthetic means of production, limit the generation of artemisinin diversity. Therefore, Njuguna *et al.* (2012) emphasized on developing biotransformation systems for artemisinin derivatives. Azerad and Robert in 2012 attempted on microbes to generate molecular diversity and overexpression of these drugs. However, *in planta* production of these compounds from transgenic plants with improved secondary metabolism are intriguing for various reasons (Kirsi *et al*., 2004). Moreover, development of improved and stable transgenic systems can be useful for their further agrotechnological exploitations.

The aim of this project was to develop *Agrobacterium tumefaciens*-mediated genetic transformation of *Artemisia dubia* with *rolA* gene and evaluation of different *rol* genes transgenic plants for artemisinin and derivatives. Micropropagation and transformation conditions of *A. dubia* were reported by Kiani *et al.* (2012) during transformation of the same plant with *rol ABC* genes. The same method was followed to regenerate explants and induction of roots and shoots except where conditions were optimized to conform to present study.

Materials and Methods

Plant material: Plants used in this research work were growing in green house of Plant Molecular Biology Laboratory, Quaid-i-Azam University, Islamabad, under natural conditions for last six months before work. They were at juvenile lush green stage, bearing profuse leaves and fast growing tips.

Explant sterilization and culturing: Sterilization of explants taken from *A. dubia* was achieved by following the protocol described by Kiani *et al.,* 2012. Stem explants of *A. dubia* containing node (s) were washed under running tape water for 10 minutes. Five subsequent washes with double distilled water were given before blotting. Explants treated with 15% bleach, 80% ethanol and 0.1% mercuric chloride for 6 minutes, 3 minutes and 10 seconds, respectively, showed maximum regeneration and reduced contamination.

Bacterial strains: *Agrobacterium tumefaciens* strain LBA 4404 carrying *rolA* gene constructed on pLBR29 was used for transformation. Bacterial cultures (50µl) were grown in 50ml LB medium (Tryptone; 1 g/l, Yeast extract; 0.5 g/l, Sodium chloride; 1 g/l) and incubated at 28^{o}C at 120 rpm/minute overnight in orbital shaker incubator. Cultures were streaked on Luria Agar andsingle colony was added in fresh LB medium containing 0.05 mg/l kanamycin. Double selection was also given to assert the growth of genuine cultures. These cultures of*A. tumefaciens* were used after PCR confirmation.

***Agrobacterium tumefaciens*- mediated transformation of *A. dubia*:** Stem explants were regenerated on MS medium containing 0.05 mg/l BAP. Plantlets were precultured on MS medium supplemented with 1 mg/l BAP. Co-cultivation medium was comprised of MS added with 0.1 mg/l BAP and 0.02 mg/l acetosyringone. Explants were washed with liquid half MS containing 0.05 mg/l cefotaxime and shifted on selection medium. Selection medium was same as mature shoot induction containing 0.1 mg/l cefotaxime and 0.25 mg/l kanamycin respectively. Shoots were subcultured on either mature shoot or multiple shoot induction medium. Roots were induced by different hormonal concentrationsfor transgenic and control plants in half strength MS medium.

Molecular Analysis

Polymerase chain reaction: Genomic DNA from *rolA*, *rol ABC* transgenic and control *A. dubia*was isolated by using method described by (Ahmed *et al*., 2009) to perform molecular analysis.Plasmid (pLBR29) DNA was also isolated using alkaline lysis method from LBA 4404 stain of *A. tumefaciens*.A programmed DNA thermal cycler (Biometra, USA) was used for PCR analysis. Forward (5'-AGAATGGAATTAGCCGGACTA-3') and reverse primer (5'-GTATTAATCCCGTAGG TTTGTT-3') of*rolA* gene were used. PCR reaction was carried out in 25 µl final reaction volume containing 50 ng DNA templates.Thermal cycling conditions were adjusted to 35 cycles for 5 minutes at 94ºC, 1 minute at 53- 55°C and 1 minute at 72ºC. An aliquot of 10 ul PCR product was run on 2% w/v agarose gel for molecular analysis of plants.

Thin layer chromatographic analysis of transgenic and control *A. dubia*

Extraction of artemisinin and derivatives: Dried leaves and stems of *rolA, rol ABC* transgenic and control *A. dubia* were micronized separately and mixed with HPLC grade toluene, acetonitrile and methanol in a ratio of 6: 7: 7 or 1: 1 acetonitrile and methanol solvent mixture. Further uniformity was achieved with the help of homogenizer. Cell walls disruption percentage was supposed to increase in homogenizer. Mixture was sonicated and vigorously shaken with intervals of 5 minutes for 1 hour. Disrupted cell debris was centrifuged for 10 minutes at 13000 rpm. Supernatant was taken and used to mark spot on TLC plate to run against standards.

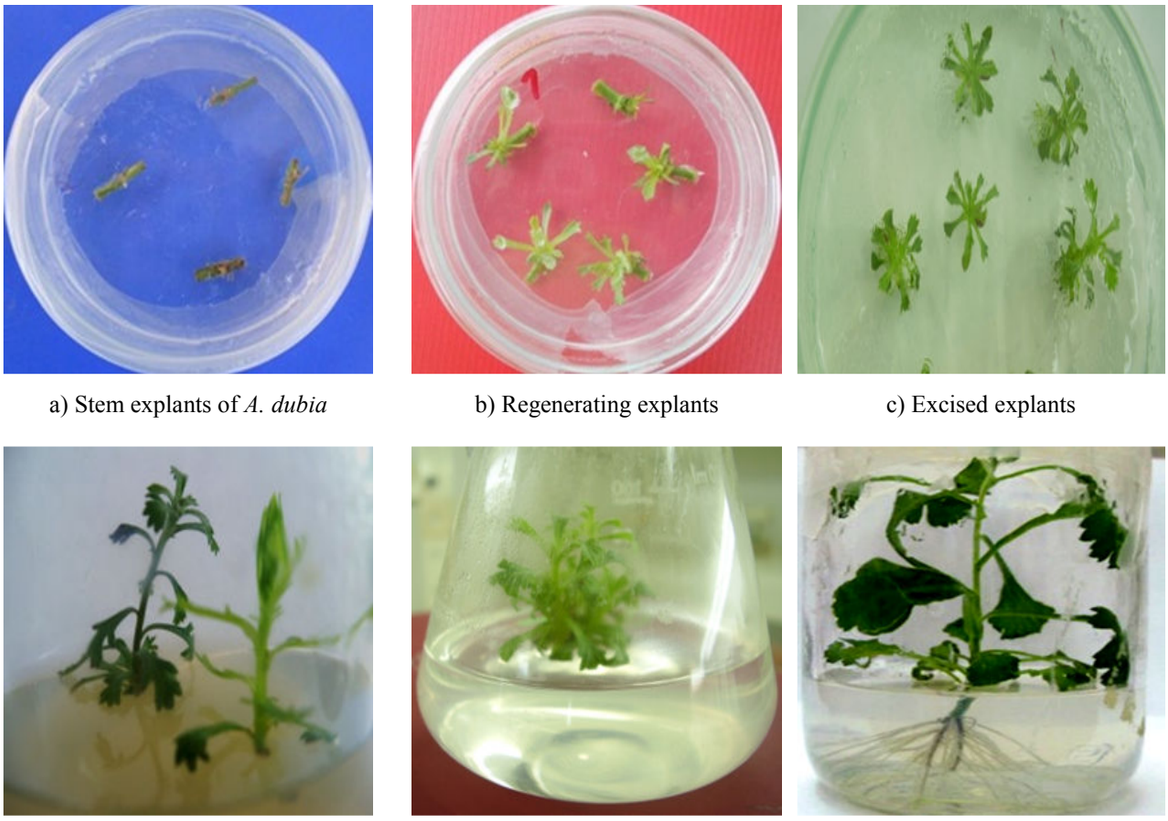
Preparation of standard solutions: Initial stock solutions of artemisinin, artemether and artesunate were prepared at the concentration of 10mg/ml. Final concentration of 1mg/ml of standards was achieved to be used for TLC analysis. Samples were sonicated a little for complete dissolution and kept at -20^{o}C immediately for further use.

Mobile phase for TLC analysis: Methanol and acetonitrile were used in 2: 1 ratio as mobile phase. Mobile phase was optimized on the basis of spot separation.

TLC analysis of artemisinin, artemether and artesunate: Reverse phase TLC (2.5× 6.5cm was cut and spots were marked on the line drawn 1cm above the edge with lead pencil equidistance apart. Standards (0.7 µl) were marked alongside the plants samples. Extracts of*rolA*, *rol ABC* transgenic and control *A. dubia*were employed to analyze the presence of artemisinin, artemether and artesunate against the standards.

Results

***In vitro* regeneration of *Artemisia dubia*:** Stem explants of *Artemisia dubia* bearing node(s) were cultured. A small bunch of tender leaves, sharing a common origin, appeared at the node that was excised off and shifted to for mature shoot induction. Mature shoots were cultured for induction of multiple shoot. Shoots were grown on half MS containing NAA for roots induction. Unaided NAA produced thin and a few number of roots in transgenic plants. Use of BAP in combination with NAA improved the roots induction (Fig. 1).



a) Stem explants of *A. dubia* b) Regenerating explants c) Excised explants

d) Mature shoot induction e) Multiple shoot induction f) Root induction

Fig. 1. *In vitro* regeneration scheme of *Artemisia dubia*.

***Agrobacterium tumefaciens- mediated rolA* gene transformation of *A. dubia*:** Plantlets growing at nodes of stem explants were excised and directly used as explants for transformation of *Artemisia dubia*. Average size of the explants ranged from 2-3cm. Explants were pre-cultured for three days on mature shoot induction medium. Petri plates were kept at growth room at 26°C ± 2 with 16/ 8 hours light dark cycle and light intensity maintained at 1000 lux. Pre-cultured explants were co-cultivated for two days. Co-cultivation involved the infection of explants regaining their lost vigor and manifesting swollen appearance prepared with *A. tumefactions* strain LBA 4404 for 3 minutes. Optical density of culture of *A. tumefactions* used for infection was found optimum in the range of 0.6-0.8. After co-cultivation, explants were washed and shifted on selection medium. They were transferred on new medium every week with reduced concentration of antibiotics each time. Transferto fresh selection medium was made weekly during the first month as described by Kiani *et al.,* 2012. Explants growing into mature shoots were cultured on multiple shoot induction medium.

Mature shoots were cultured on rooting medium. Percentage induction of roots, shoots and multiple shoots was calculated for *rolA* and *rol* ABC transgenic *A. dubia* along with control plants.

Effect of infection time on explants survival and regeneration: Time of infection was optimized on the basis of explants survival rate and bacterial overgrowth during co-cultivation. Explants were subjected to infection for 2, 4, 6, and 8 minutes and put on co-cultivation medium for 2 days. Greatest survival and regeneration response was observed among the explants that were incubated for 2 minutes (Fig. 2). Further increase in infection timing decreased the efficiency of regeneration. Infection of explants for 2 minutes produced 64% explants survival efficiency whereas explants infected for 4 minutes with *A. tumefaciens*, only produced 52% efficiency.

Molecular analysis of transgenic plants: Molecular analysis of putative transformed plants was performed by amplifying *rolA* gene by polymerase chain reaction to confirm the transformation of *Artemisia dubia*. PCR products of both plasmid and genomic DNAs were resolved on agarose gel. Amplified products of five transgenic lines of putative *rolA* along with *rol ABC* genes transformants and positive control were systematically loaded in the wells. PCR product of pLBR29 plasmid served as positive control. Gel electrophoresis resolved a band of 308 bp in each well, which is the PCR product size of *rolA* gene (Fig. 3). It confirmed the presence of *rolA* gene in all the samples, endorsing the successful transformation.

Biochemical analysis of transgenic *A. dubia* by thin layer chromatography: TLC analysis of artemisinin and its derivatives was optimized using two different extraction systems and mobile phases. Plants transformed with *rolA*, *rol ABC* genes construct and untransformed plants were crushed to extract artemisinin, artesunate and artemether. Samples were spotted against standards for qualitative analysis. It was evident from the TLC analysis that untransformed plants show lighter spots than *rol* genes transgenic *A. dubia* (Fig. 4). Transgenic plant spots appeared invariably denser under same conditions and concentrations. *A. dubia* transformed with *rolA* and *rol ABC* genes manifested comparable spots. Moreover, there were other spots that were observed prominently expressed in transgenic plants.

Percentage efficiency of *rolA* gene transformation of *A. dubia*: Transformation of *Artemisia dubia* with *rolA* gene appeared sensitive to many factors. Time of infection, optical density of cultures and texture of explants had great impact. Percentage transformation efficiency was calculated to be 64% by using formula:

$$\frac{\text{Number of PCR – Positive plants survived}}{\text{Total number of explants}} \times 100$$

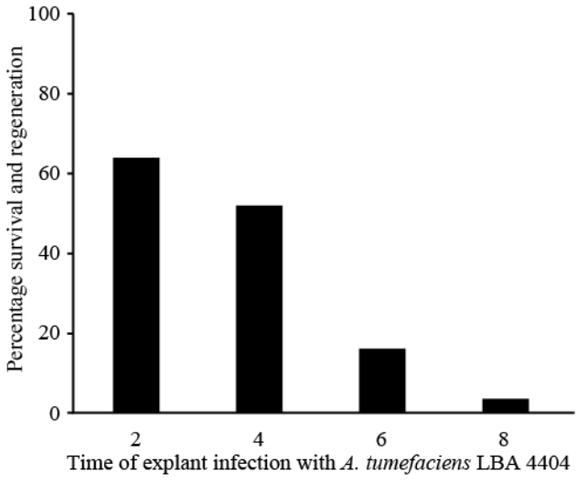


Fig. 2. Effect of incubation period on survival and regeneration of explants.

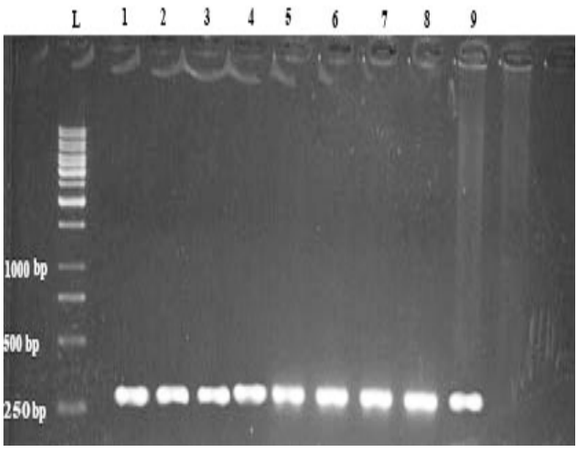


Fig. 3. Polymerase Chain Reaction (PCR) amplified *rolA* transgene of 308bp PCR product size (against 1kb ladder, L). First five bands (1, 2, 3, 4 and 5) represent five *rolA* transgenic lines of *A. dubia* (T_1, T_2, T_3, T_4, T_5); 6th and 7th band represent *rol ABC* transgenic *A. dubia*; 8th and 9th bands show positive control.

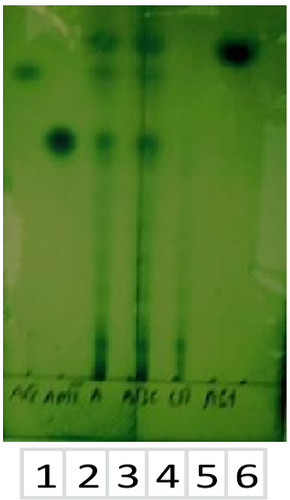


Fig. 4. TLC analysis of *rol* transgenic and control *A. dubia*.

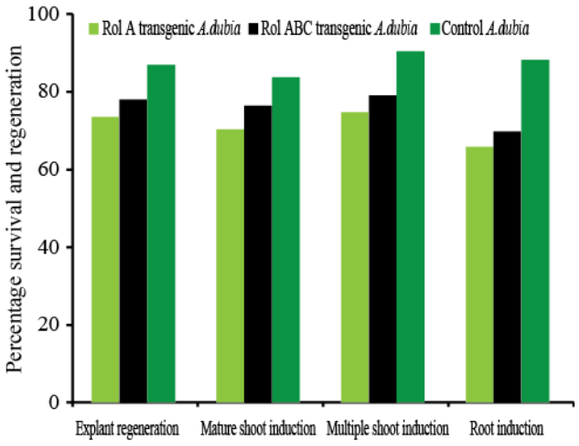


Fig. 5. Graphical representation of *In vitro* regeneration of rolA, rol ABC and control *A. dubia*

Two spots on extreme left (1 and 2) and first spot on extreme right (6) represent standards i.e. artemisinin, artemether and artesunate, respectively. 3, 4 and 5 show TLC separation of extracts of *rol A*, *rol ABC* transgenic and untransformed *A. dubia*, respectively.

***In vitro* regenerative analysis of transgenic and untransformed *A. dubia*:** Four parameters i.e. explants regeneration, mature shoot, multiple shoot and root induction were observed during the micropropagation and regeneration of transgenic and untransformed *A. dubia.RolA, Rol ABC* genes transformed and control*A. dubia* showed different percentage inductions for these parameters (Fig. 5). It can be seen that untransformed plants show better regeneration than transgenic plants. Comparative analysis of *rolA* and *rol ABC* transgenic plants showed that acclimatized *rol ABC* transgenic plants regenerate rather better compared to *In vitro* regenerated *rolA* transgenic plants.

Roots induction of *rol* transgenic *Artemisia dubia*: For root induction, 7-8 cm long mature shoots of *rolA* transgenic *A. dubia* were cultured on rooting medium i.e. half strength MS (Murashige & Skoog, 1962) medium containing combination of NAA and BAP. Like acclimatized *rol ABC* transgenic plants, *rolA* transformed plants showed better rooting on 0.5 mg/l NAA along with the supplementation of 0.1 mg/l BAP. Comparatively, roots induction in afresh *rolA* transgenic mature shoots was rather less efficient and irregular than micropropagated acclimatized *rol ABC* transgenic plants.

Morphological analysis of *rolA* transgenic and untransformed plants: *RolA* gene transgenic *A. dubia* showed characteristic wrinkled leaves which were curled towards the stem. Surface area of the leaves was reduced compared to control plants. At mature shoot induction stage, the distance between the two nodes was much shorter in transgenic plants. *RolA* transgenic *A. dubia* exhibited irregular pattern of root induction. Underdeveloped delayed, relatively stunted and thin roots were observed in case of transgenic plants when grown on NAA alone whereas untransformed plants invariably produced long and abundant roots. On contrary to controls, *rolA* and acclimatized *rol ABC* genes transgenic *A. dubia* produced improved roots on combination of NAA and BAP. At early stages of development, the color of the leaves of the transgenic plants was observed light green with delicate texture which reverted to dark green color during successive progression. It was observed that while control plants developed inflorescence, *rolA* transgenic plants were still short of maturity. Inflorescence was tightly packed in case of transgenic plants. In the beginning, overall size of transgenic plants always lacked behind the controls with gradual improvement in dwarf morphology. Acclimatized *rol ABC* transgenic *A. dubia* showed *rol* gene transgenic characteristics but they were hardly distinguishable at the later stages of development from control plants.

Discussion

Plants produce secondary metabolites numerous of which carry medicinal values (Verpoorte *et al.,* 2002 and Mannan *et al.,* 2007). Their production can be escalated by genetic transformation and rapid propagation of transgenic plants (Vasil, 1998). Transgenic studies have described that single *rol* gene transformation may be powerful tool to manipulate the expression of plant secondary metabolism(Christophe *et al*., 1991) because of antagonistic effects of these genes (Maurel *et al.,* 1991).This study reports an increase in artemisinin and derivatives by *rolA* gene transformation in *Artemisia dubia*. Mannan *et al*. (2008) and Kiani *et al*. (2012), in their independent studies, have already informed about increased secondary metabolite synthesis in *rol* transgenic *A. dubia*.

Stem explants of *A. dubia*were used for micropropagation of plant material. Meristematic tissues at the nodes make them plausible choice as explants. Uncertainty of availability and germination of seedsfrequently urges to develop explant based *In vitro* regeneration systems. Besides, direct plantlets regeneration gives a faster and easier method of tissue culturing compared to the protocols involving callus induction. Explants were precultured that allows cells to elongate and divide actively and renders them prone to infection and withstand following stresses (Christian *et al*., 2006). Healthy and rejuvenated explants were co-cultivated for 2 days. Factors like explant texture and optical density of bacterial culture affect the incubation period. An explants infection period of 2 minutes produced 64% explants survival efficiency whereas those infected for 4 minutes with *A. tumefaciens*, only produced 52% efficiency and showed delayed rejuvenation. But former could not survive on medium containing kanamycin whereas the latter resulted in survival of almost 85% explants on selection. This fact is also supported by the results of Kiani *et al*. (2012) who infected explants of *A. dubia* for 5 minutes.Various factors reduce the transformation efficiency and transgenic lines generated. Of the surviving putative transgenic plants, many were not able to make it through subsequent shifting and subculturing. Polarity and necrosis affect the survival rate of explants. Upright position of explants brings about better regeneration

frequencies (Garcia Luis *et al.,* 1999). Orientation also defines the nutrient and oxygen provision to explant (Garcia Luis *et al.,* 2006) and determines morphogenic pathways as well. Explants necrosis can be related to delicate texture and virulence of *Agrobacterium*. Many studies have used antioxidant agents to overcome this problem (Marc, 1990).

Our work analyzed artemisinin and derivatives in leaves and stem extracts of *A. dubia*. Highest artemisinin content has been reported by Xiang *et al.* (2012) in leaves followed by stems, flowers and roots. In case of artemisinin and derivatives, it is suspected that wavelength absorbed by these metabolites is also absorbed by other compounds in the extracts. Therefore, TLC method of analysis was optimized that overrules any such confusion. It may further be used for quantification of spots by densitometer. Enhancement of secondary metabolites under consideration was observed as good for *rolA* gene as*rol ABC* construct. However, latter was revealed exertingal though antagonistic, still somehow compensatory effects. Nonetheless, onlya marginal difference in increase of secondary metabolites is reported for single and combined *rol* genes. In addition to *rol* genes, variable synthesis of secondary metabolites in transgenic plants can also be attributed to various components of medium and tissue culture conditions. Temperature and pH determine the activity of plant enzymes and exert significant blow on their biosynthesis. Carbon source, different auxins and cytokines (Chattopadhyay *et al*., 2002), phosphate to nitrogen ratio in medium (Ramachandra & Ravishankar, 2002), photoperiod (Wenbin *et al.,* 2002) and humidity (Chiachung Chen, 2004 and Zia *et al.,* 2007a&b) greatly impact the production of these metabolites. Besides artemisinin, artemether and artesunate our results have also shown many other denser spots in case of *rol* transgenic *A. dubia* that were observed just about detectable in controls. These compounds can be other derivatives of artemisinin and characterized using different standards.

Characteristic morphology of *rol* genes transgenic plants can be ascribed to ethylene levels. Transgenic plants produce 40 times more ethylene than wild type. Studies have proved that increased ethylene production affects the proportions of morphology and phenotype of plants (Salisbury and Ross, 1991). During *In vitro* regeneration of transgenic plants, growth medium contents (Jin *et al.,* 1995) and *rol* genes affect the cellular differentiation (Kiselev *et al*., 2007). Differential growth of leaf and root tissues caused by *rol* genes can also be the reason of different morphology (Casanova *et al.,* 2005; Christensen & Muller 2009). These traits have also been observed in *rol* genes transgenic tobacco, potato and tomato plants (Michael *et al.,* 1998).

Christophe *et al*. (1991) suggested that some cellular responses to auxins could be selectively altered by even a single *rol* gene. Transgenic plants were observed sensitive to auxins by factors up to 10^4.Now it is well established that *rol* genes induce auxins production (Maurel *et al*., 1991). These genes also cause hormonal imbalance and modulation of cell growth. It is also the reason why *rol* transgenic plants would require different PGRs concentrations for roots induction as compared to controls. Comparative *In vitro* regenerative and morphological analyses can help further improve the features of *rol* transgenic plants for agronomic scale cultivation. It may be transcended to develop same systems for geographically and genotypically related species (Block, 1988; Sung *et al*., 2003). Our results show that not only acclimatized *rol* transgenic plants manifest almost as good regeneration as controls but also better micropropagation. Hence, it suggests that *rol* transgenic *A. dubia* can be used to harness artemisinin and derivatives.

Conclusion

In *Artemisia dubia rolA* gene significantly enhanced the production of artemisinin and its derivatives.*RolA* gene transformation of *A. dubia* proves single *rol* gene transformation to be exceedingly effective that can help meet the increasing demand of artemisinin and derivatives. It can be safely predicted that transgenic system optimized in this study canproduce same results for other plant secondary metabolites of different plant origin.

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References

Ahmed, I., M. Islam, W. Arshad, A. Mannan, W. Ahmad and B. Mirza. 2009. High-quality plant DNA extraction for PCR: an easy approach. *Journal of Applied Genetics*, 50(2):105-107.

Anonymous. 2006. WHO calls for an immediate halt to provision of single artemisinin malaria pills.

Azerad and Robert. 2012. Microbial transformations of artemisinin and artemisinin derivatives: an example of the microbial generation of molecular diversity. *Current Bioactive Compounds*, 8 (2): 151-158.

Block, D.M. 1988. Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*, *Theoretical and Applied Genetics*, 76 (5): 767-774.

Bulgakov, V.P., G.K. Tchernoded, N.P. Mischenko, M.V. Khodakovskaya, V.P. Glazunov and E.V. Zvereva. 2002. Effects of salicylic acid, methyl jasmonate, etephone and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with *rol B* and *rolC* genes. *J. Biotechnol.,* 97: 213-221.

Casanova, E., M.I. Trillas, L. Moysset and A. Vainstein. 2005. Influence of *rol* genes in floriculture. *Biotechnol., Adv.,* 23: 3-39.

Chattopadhyay, S., A.K. Srivastava, S.S. Bhojwani and V.S. Bisaria. 2002. Production of podophyllotoxin by plant cell cultures of podophyllum hexandrum in bioreactor. *Journal of Bioscience and Bioengineering*, 93: 215-220.

Chiachung Chen. 2004. Humidity in plant tissue culture vessels. *Biosystems Engineering*, 88(2): 231-241

Christensen, B. and Muller. 2009.The use of *Agrobacterium rhizogenes* and its *rol* -genes for quality improvement in ornamentals. *Europ. J. Hort. Sci*., 74(6): 275-287.

Christian, M.B., D. Steffens, S. Schenck, M. Burmester, Bottger and H. Lüthen. 2006. How does auxin enhance cell elongation? Roles of auxin-binding proteins and potassium channels in growth control. *Plant Biol (Stuttg)*. 8 (3): 346-52.

Christophe, M., B.B. Helene, S. Angelo, T. Jacques and G. Jean. 1991. Single *rol* genes from the *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to Auxin in *Nicotiana tabacum*. *Plant Physiol*., 97: 212-216.

Efferth and Thomas. 2006.Molecular pharmacology and pharmacogenomics of artemisinin and its Derivatives in Cancer Cells.*Bentham Science Publishers*, 7(4): 407-421.

Garcia Luis, A., Y. Bordon, J.M. Moreiradias, R.V. Molina and J.L. Guardiola. 1999. Explant orientation and polarity determine the morphogenic response of epicotyl segments of T*royer citrange*. *Ann Bot*., 84(6): 715-723.

Garcia-Luis, A., R.V. Molina, V. Varona, O.S. Castell and J.L. Guardiola. 2006. The influence of explant orientation and contact with the medium on the pathway of shoot regeneration in *In vitro* epicotyl cuttings of *Troyer citrange*. *Plant Cell, Tissue and Organ Culture*, 85(2): 137-144.

Gary, L.F. and N.S. Shyam. 2009. Anticancer activities of artemisinin and its bioactive derivatives. *Expert Reviews in Molecular Medicine*, 11: 32-35.

Haq, I., A. Mannan, I. Ahmed, I. Hussain, M. Jamil and B. Mirza. 2012. Antibacterial activity and brine shrimp toxicity of *Artemisia dubia* extract. *Pak. J. Bot*., 44(4): 1487-1490.

Huang, Z.S., Y.H. Pei, C.M. Liu, S. Lin, J. Tang, D.S. Huang, T.F. Song, L.H .Lu, Y.P. Gao and W.D. Zhang. 2010. Highly oxygenated guaianolides from *Artemisia dubia*. *Planta Med*., 76(15): 1710-16.

Issaka, Z., D. Grant, R. Noel, D. Christian, S. Yves, J. Philip and B.O. Jean. 2007. Randomized comparison of amodiaquine plus sulfadoxine-pyrimethamine, artemether-lumefantrine, and dihydroartemisinin-piperaquine for the treatment of uncomplicated *plasmodium falciparum* malaria in Burkina Faso. *Clin Infect Dis*., 45(11): 1453-1461.

Jin, H.K., H.Y. Jeong, S.H. Yong, Y.B. Sang and I.K. Dong 1995. Production of taxol and related taxanes in *Taxusbrevifolia* cell cultures: Effect of sugar. *Biotechnology Letters*, 17 (1): 101-106.

Kiani, B.H., N. Safdar, A. Mannan and B. Mirza. 2012. Comparative artemisinin analysis in *Artemisia dubia* transformed with two different *Agrobacteria* harboring *rol ABC* genes. *Plant Omics Journal,* 5(4): 386-391.

Kirsi, M., C. Oksman and I. Dirk. 2004. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. *Cell*, 9 (9): 433-440.

Kiselev, K.V., A.S. Dubrovina, M.V. Veselova, V.P. Bulgakov, S.A. Fedoreyev and Y.N. Zhuravlev. 2007. The *rol B* gene induced overproduction of resveratrol in *Vitisamurensis* transformed cells. *J. Biotechnol*., 128: 681-92.

Kryzeviciene, A., L. Sarunaite, V. Stukonis, Z. Dabkevicius and Kadziuliene. 2010. Assessment of perennial mugwort (*Artemisia vulgaris* L. and *Artemisia dubia* Wall. potential for biofuel production. *Zemesukio Mokslai,* 17: 32-40.

Mannan, A., N. Shaheen, W. Arshad, R.A. Qureshi, M. Zia and B. Mirza 2008. Hairy roots induction and Artemisinin analysis in *Artemisia dubia* and *Artemisia indica*. *Afr. J. Biotech*., 7(18): 3288-3292.

Mannan, A., S. Nazia, A. Waheed, Q. Rizwana Aleem, Muhammad Zia and B. Mirza. 2008. Hairy root induction and artemisinin analysis in *Artemisia dubia* and *Artemisia indica*. *African Journal of Biotechnology*, 7(18): 3288-3292.

Mannan, A., S. Nazia, Attiq-ur-Rehman, A. Waheed, Muhammad Zia and B. Mirza. 2007. Production and release of allelopathic compounds by Artemisia hairy root culture. *Allelopathy Journal*, 20(2): 379-386.

Marc, D.B. 1990. Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediated transformation of hybrid Aspen and Poplar Clones. *Plant Physiology*, 93 (3): 1110-1116.

Maria, W., W. Sandra, B. Paula, G. Frank, A. Helmut, H. Jürgen, W. Gerda and Heinrich Sauer. 2003. The antimalarial agent artemisinin exerts antiangiogenic effects in mouse embryonic stem cell-derived embryoid bodies, *Lab Invest*, 83: 1647-1655.

Marillonnet, S, C. Thoeringer, R. Kandzia, V. Klimyuk and Gleba. 2005. Viral mediated plant transformation gets a boost. *Nature Biotechnology*, 23(6): 684-685.

Marta, R., E. Thomas, S. Maria, C. Beatriz, M. Rocio, B. Oscar and M. Jose. 2005. Effect of artemisinin/artesunate as inhibitors of hepatitis B virus production in an "*In vitro*" replicative system. *Antiviral Research*, 68(2): 75-83.

Marvin, M., W. Andrew, Z. Helen, W. Chunrui, M. Garrett, M. Ute, M. Esther, S. Tristani, C.S. Alvin, T.F. Michael, C. Lisa, E. Rachel and B. Helena. 2011. Design, synthesis, and study of a Mycobactin−artemisinin conjugate that has selective and potent activity against tuberculosis and malaria. *Journal of the American Chemical Society*, 133(7): 2076-2079.

Maurel, S., H. Barbier-Bryqoo, A. Spena, G. Tempe and G. Guern. 1991. Single *rol* genes from *Agrobacterium rhizogenes* T (L) DNA alter some of the cellular responses to auxin in *Nicotiana tabacum*. *Plant Physiol*., 97: 212-16.

Michael, A, D. Burtin and D. Tepfer. 1998. Effects of *rolC* locus from the Ri TL-DNA of *A. rhizogenes* on development and polyamine metabolism in tobacco. *GIM 90 Proceddings*, 2: 863-868.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant*, 15: 473-497.

Narendra, P.S. and L. Henry. 2001.Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells. *Life Sciences,* 70: 49-56.

Njuguna, M.N., S.B. Dennis, C. Ongarora and Kelly. 2012. Artemisinin derivatives: a patent review (2006–present) 22(10): 1179-1203.

Pudel, P., Jha, P.K., Gewali, M.B. 2005. Artemisia dubia Wall Ex Besser (mugrowth): a weed to control weed. Scient World 3 (3):32-38Ramachandra, R.S. and G.A. Ravishankar. 2002. Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnol Adv*, 20: 101-153.

Salisbury and Ross. 1991 Système Internationale: The use of SI units in plant physiology. *Plant Physiology*, p. 652.

Shkryl, Y.N., G.N. Veremeichik, V.P. Bulgakov, G.K .Tchernoded, N.P. Mischenko and S.A. Fedoreyev. 2007. Individual and combined effects of the *rol A*, *B* and *C* genes on anthraquinone production in *Rubia cordifolia* transformed calli. *Biotechnol. Bioeng.,* 100(1): 118-125.

Sung, H.P., L.M. Jay, E.P. Jung, D.H. Kendal and H.S. Roberta. 2003.Efficient and genotype-independent *Agrobacterium*-mediated tomato transformation. *Journal of Plant Physiology*, 160(10): 1253-1257.

Suzanne, K., E. Thomas, L. Martina, R. Sabine, A. Sabrina, K. Martina, B. Cathrien, V. Cornelis, S. Thomas and M. Manfred. 2006. The anti-malarial drug artesunate inhibits replication of cytomegalovirus *In vitro* and *In vivo*. *Antiviral Research*, 69(2): 60-69.

Thomas, G.B., D. Detlef, E. Thomas, S.S. Erwin, O.F. Jens, B. Andreas and S. Gerold. 2005. Artesunate in the treatment of metastatic uveal melanoma- first experiences, *Oncology Reports,* 14: 1599-1603.

Vasil, K.I. 1988. Progress in the regeneration and genetic manipulation of cereal crops. *Nature Biotechnology,* 6: 397-402.

Verpoorte, R., A. Contin and J. Memelink. 2002. Biotechnology for the production of plant secondary metabolites, Find out how to access preview-only content. *Phytochemistry Reviews*, 1(1): 13-25.

Wenbin, L., Pathmalojiny Masilamany, J. Ken and P. Peter. 2002. Developmental, tissue culture, and genotypic factors affecting plant regeneration from shoot apical meristems of germinated *Zea mays* L. seedlings. *In vitro Cell. Dev. Biol. Plant,* 38: 285-292.

Woong, N., T. Jungae, K. Ju, J. Mankil, Y. Jong and K. Hyung. 2007. Effects of artemisinin and its derivatives on growth inhibition and apoptosis of oral cancer cells. *Head & Neck*, 29(4): 335-340.

Xiang, L., Z. Yan, G. Wang, W. Liu, K. Tang and Z. Liao. 2012. Relative expression of genes involved in artemisinin biosynthesis and artemisinin accumulation in different tissues of Artemisia annua. *Zhongguo Zhong Yao Za Zhi,* 37(9):1169-73.

Yang, J., G. Chun, M. Qing, C. Jian, T. Jian and F. Sai. 2007. Dihydroartemisinin is an inhibitor of ovarian cancer cell growth. *Acta Pharmacologica Sinica*, 28(7): 1045-1056.

Zia, M., A. Mannan and M. Fayyaz Chaudhary. 2007b. Effect of growth regulators and amino acids on artemisinin production in the callus of *Artemisia absinthium*. *Pak. J. Bot*., 39(2): 199-805.

Zia, M., Riaz-ur-Rehman and M. Fayyaz Chaduhary. 2007a. Hormonal regulation for callogenesis and organogenesis of Artemisia absinthium. *African Journal of Biotechnology*, 6(16): 1874-1878.

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