Genetic Transformation of *Artemisia* by *rol* genes

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The Arabic word, *Bismillah*, 'In the name of God', created in the shape of an ostrich by Sudanese artist Hassan Musa. Courtesy of Grandir Editions.

In the name of ALLAH who is the Beneficent and The most Merciful, Guardian of faith, The Majestic, The Bestower and The Forgiver, Whose help and guidance I

always importune at every step.

DECLERATION

This thesis submitted by **Bushra Hafeez Kiani** is accepted in its present form by the Department of Biochemistry, Quaid-i-Azam University, Islamabad, as fulfilling the thesis requirement for the degree of Master of Philosophy in Biochemistry / Molecular Biology.

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DEDICATION

This humble effort is sincerely dedicated to

My Abbu whose wishes and devotions

Made me capable of achieving such a Success

and to my sweet, ever-loving Ammi

whose hands always raised for my Success,

My Family,

My Dadi Jee,

And

My sweet nephews

(Words are futile to that

They have done for me)

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Finally, all the errors that remains, are mine, alone.

Bushra Hafeez Kiani

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CFU	Colony forming unit
°C	Degree centigrade
HPLC	High performance liquid chromatography
LSD	Least significant difference
MS	Murashige and Skoog
Ri	Root inducing
RPM	Revolution per minute
T-DNA	Transfer DNA
Ti	Tumour inducing
UV	Ultraviolet
gm	Gram
mm	Millimeter
L	Liter
µ	Micro
µl	Micro liter
nm	Nanometer
rpm	Revolution Per Minute
µl/ml	Micro liter / Milliliter

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ABSTRACT

Artemisinin, a potent antimalarial drug is the major constituent of *Artemisia* species. The aim of present study was to compare artemisinin production in the roots of *Artemisia dubia* transformed with *rol ABC* genes by using *Agrobacterium tumefaciens* with the non transformed plants. As a number of factors have been reported that affect the efficiency of transformation with *Agrobacterium tumefacienes,* different parameters like type of explants, sterilization condition and co-cultivation period. *Agrobacterium tumefacienes* strain LBA4404 containing pRT99 harboring *rol ABC* genes were used for the transformation experiments. Mercuric chloride 0.1% for 2 minutes showed the best results for seed surface sterilization with 95% germination. Leaves and stems were found to be the best explants for transformation. Dipping explants in bacterial suspension for 10 minutes and co-cultivating them on selection medium (MS medium with 200 µM acetosyringone and 50mg/l kanamycin) for 48 hours resulted in the maximum number of transformants. Regeneration of transgenic shoots was obtained from both stem and leaves explants on regeneration medium (MS medium containing 1.0mg /l BAP supplemented with 20mg/ml kanamycin and 500µg/ml cefotaxime). Eighty percent of the transgenic shoots showed rooting response on Half MS medium with 0.025mg/l NAA. Control and the transgenic plants were transferred to small pots containing soil and acclimatized. Morphological differences like increased plant height and broad leaves were observed. Transformed plants were selected on 100ug/ml kanamycin and final selection of transformed plants was made on the basis of PCR for the detection of *rol ABC* genes.

Transformed and control roots were subcultured on solid B5 medium for further root proliferation. Better roots proliferation was observed on solid B5 medium in the case of transformed root while control root showed minimum proliferation. After 30 days of roots proliferation Artemisinin content was analyzed with the help of spectrophotometer. Maximum Artemisinin was observed in transformed roots of *Artemisia dubia* while minimum content of Artemisinin was observed in control plants.

INTRODUCTION

INTRODUCTION

Plants are extremely important in the lives of people throughout the world. For centuries mankind has been totally dependent on plants as source of carbohydrates, proteins, and fats for food and shelter (Shinwari *et al.*, 1989). Furthermore, plants provide materials for many types of pharmaceuticals, as well as coffee, alcohol and other drugs (Ellof, 1995). The compounds that are responsible for medicinal property of the drugs are usually secondary metabolities, which are used as pharmaceuticals as well as agrochemicals flavors, fragrance, colours, biopesticides and food additives (Vormfelde, and Paser, 2000). The use of the medicinal plants for curing disease has been documented in history of all civilizations (Street, 1977). One such plant is *Artemisia dubia* L. belonging to genus *Artemisia.*

1.1 History of *Artemisia*

Artemisia is a large, diverse group of plants with between 200-400 species belonging to the daisy family Asteraceae and it is well known since ages through the study of taxonomy. It comprises hardy herbs and shrubs known for their volatile oils (Watson, 2002).

According to Cullen's (1975) view, *Artemisia* is a late flowering and well pollinated genus. *Artemisia* is an important traditional medicinal plant, which has been used over thousand years in medicine to reduce fever as malarico therapy (Klayman, 1985; Charles and Simon, 1990).

1.1.1 Morphology

Artemisia is annual, biennial or perennial, usually bitter-aromatic or non-aromatic glabrous, hairy or punctuate-glandulase herb or small to large shrub with tap root or horizontal root stock. Leaves alternate, basal large and petiolate, upper often subsessile to

sessile, undivided or toothed-shallowly to deeply incised or lobed, palmati or pinnatipartite or 2-4 pinnatisect.

Synflorescence a pancicle, sometime racemoid or spicate, occasionally globoid or fascieled capitulla generally numerous, small, rarely mediocre, nodding or erect, oblong-cylindrical or ovate to almost globose, hetorogamacy. Involucre ovoid to campanulate or hemispherical, phyllaries in 2-4 (-6)-sereis imbricate, hairy (or) glabrous inner one mostly scarious-margined or scarious. Receptable plane, convex, conical or hemi-spherical, glabrous or pilose.

Flowers brownish, reddish or yellowish, all tubular. Ray-florets: pistilate and fertile, corolla narrowly tubular, gewrally tapering upwards, 2-3(-4) toothed 1 ± oblique, eligulate, style exerted, 2-cleft, branches recurved, linear filiform and terete-oblong, ± flattened. Discflorets bisexual, fertile or sterile, corolla tubular to ± companulate or funnel shaped, 5 toothed, anthers oblong, larger than the filaments, obtuse or ± cordate at the base, apical appendages lanceolate or subulate, style exserted or included, either 2-cleft with flat, truncate and apically erose or fimbriate, ± recurved branches or columnar with an erose or fimbriate disc or cup at the apex. Cypselas of both marginal and disc (when fertile) terete, oblong-ellipsoid or obvoid to nearly fusiform-prismatic, faintly many striate or smooth, glabrouse, apecially rounded or trunate. Pappus absent or sometimes cypselas with a minute annulus or scarious corolliform ring (Ghafoor, 2002).

1.1.2 Distribution

Artemisia species are widely distributed in north temperate regions of both new and old world, extreme west and South Africa, South America (Ghafoor, 2002), Asia, west America, Canada, New Zealand (Stewart, 1972), Afghanistan, and extends westwards to the Atlantic (Said, 1969).

Artemisia dubia L. grows wild in northern India, China, Xizang, Nepal and northern hilly areas of Pakistan specially Swat, Parachinar, Abbottabad, Hazara, Bagh

(Haq, 1983), Chitral, Srinagar, Pahlgam, Tsrar Sharif, Skardu, Ladakh, Kurram valley, Kishtwarpass (Ghafoor, 2002). *Artemisia* is highly rich in many areas of Kashmir between altitudes of 5000-7000 ft. (Basu and Kirtikar, 1988; Dasture, 1952). It has also been reported in Margalla hills of Islamabad (Rizwana *et al.*, 2002).

According to Rizwana *et al.*, (2002) 30 species of *Artemisia* are growing naturally in Pakistan. Another publication indicates the presence of 32 species (Stewart, 1972). However ten-species found in Pakistan are available from Japan, China and Russia (Junshen *et al.*, 1996). *Artemisia* is also found in Saudi Arabia (Ghafoor and Al-Turki, 2000). Hajra illustrated 33 species of *Artemisia* from India (Hajra *et al.*, 1995; Dasture, 1952; Palumin and Stainton, 1992).

1.1.3 Morphology of *Artemisia dubia*

Artemisia dubia is a peremial herb with several erect, 1-1.8m tall, sulcate, purpureus glabrescent, staut stems from woody, upright root stock as shown in figure 1.1. Leaves shortly petiolate to almost sessile, oblong-elliptic to broadly ovate, 8-12 x 6-9 cm, green and white dotted above along with or without sparse T-shaped hairs, grayish-green arachnoiad hairy beneath to almost glabrous, bipinnatisect, primary segments, elliptic-lanceolate, 3-4.5 x 1.5-2.5 cm, acute mueronate, secondary segments ± elliptic lanceolate, 1-1.5 x 2.5-4.5 mm, acute or ± obtuse revolute; uppermost leaves linear lanceolate, with or without basal auricles. Capitula numerous, heterogamous, oblong-companulate, 3-3.5 x C.2 mm, ± approximate, almost sessile, in a narrow to broad, 15-30 x 10-20 cm panicle with ascending to ± patent (upper), upto 25 x 2-3 cm primary braneles (Ghafoor, 2002). Involcure 4-seriate, phyllaries loosely imbricate, all sparsely arachnoid hairy outside, outermost narrowly ovate, green, acute, median elliptic to narrowly obovate, 2.5 x 1 mm, obtuse, widely scarious hyaline on margins and apex. Receptacle ± flat, glabrous.

Figure 1.1: ***Artemisia dubia***

Involcure 4-seriate, phyllaries loosely imbricate, all sparsely arachnoid hairy outside, outermost narrowly ovate, green, acute, median elliptic to narrowly obovate, 2.5 x 1 mm, obtuse, widely scarious hyaline on margins and apex. Receptacle ± flat, glabrous. Florets 16-20 purplish, all fertile; marginal florets 6-8 with C. 1mm large, basally broadened, bidentate, glandulose corolla tube and long exerted style branches, disc florets 10-12, with C.1.75mm long, 5 toothed, glandulose apically very sparsely hairy corolla tube. Cypselas brown, 1.25-15 mm large, with terminal carolliform scar (Ghafoor, 2002).

Artemisia dubia is a common weed in open localities, fallovo fields, waste places, and roadsides, rare in regularly cultivated fields. Propagate mainly by underground stolens. Native of Europe, cartimental Asia, China, Pakistan, Northern India, and Nepal, used as a medicine in various ways. Host plant for meloid, gyne root knot nematodes. Blooming period is August to October (Ghafoor and Al-Tyrki, 2002).

1.1.4 Medicinal Constituents

This genus is famous due to its well known therapeutically significant constituents and has undergone extensive phytochemical investigations over the past two decades. Till now a large number of sesquiterpines have been isolated (Sy and Brown, 2001). Most important one is artemisinin (Till *et al.*, 1982), a compound that shows premises as an antimalarial agent (Klayman, 1985). The widely used derivatives of Artemisinin are artemether, arteether, and artesuate which are the methyl ether, ethyl ether and hemisuccinate ester of dihydroartemisinin respectively (Li, *et al.*, 1982; Anon, 1982). Artemisinin derivatives have been studied for their efficacy as antimalarial agent.

A large number of constituents have been isolated from its volatile oils (0.5 to 1.6% oil/fresh weight). That include α and β thujone (0.042%), phellandrine (12.3%), thygylalcohal (4.5%), azulene (2%), glycoside (0.003%), resin (5.2%), starch (1.45%), alpha-pinene (0.032%), camphene (0.047%), β-pinene (0.882%), myrecene (3.8%),1,8-cineole (5.5%), *Artemisia* ketone (66.7%), linalool (3.4%), camphor (0.6%), barneole (0.2%), and β-caryophylene (1.2%) (Srivastava, 1999).

Other constituents isolated include flavonoids (Hoffmann and Hermann, 1982), ascorbic acid (Slepetys, 1975), carotenoids (Sergeeva and Zakharova, 1977), Tannins (Slepety, 1975), lignans (Greger and Hofer, 1980), Pinitol, artemin, ridentin, santolineal, stigmasterol, daucosteral, sesanin, beta steral, alpha amyrin, Judaicin (cardio tanic), cirsimaritin and glaucolide like sesquiterpine lactones (Tan *et al.*, 1991; Khafagy and Tosson, 1968; Khafagy *et al.*, 1988; Galal *et al.*, 1974). *Artemisia* plants represent still a large number of untapped savree of structurally novel compounds that might serve as lead for the development of novel drugs (Hostettmam, 1987).

1.1.5 Medicinal Uses

Phytopharmacological evaluation of *Artemisia* species shows the presence of anti-inflammatory (Sommer *et al.*,1965), antipyretic (Ikram *et al.*,1987), antifertility (Rao *et al.*, 1988), antibacterial (Kaul *et al.*,1976), antifungal (Manuzzella *et al.*, 1960), anthelmintic (Caius and Mahasber, 1920), antiamoebic (Tahir *et al.*, 1991), antimalarial (Hernandez *et al.*, 1990; Zafar *et al.*, 1990), antihepatitic (Gellani and Jambaz, 1995), hepatoprotective (Kiso *et al.*, 1984; Handa et al., 1986; Oshima *et al.*, 1984) and antidiabetic activities (Tan *et al.*, 1999).

Studies show that some species of *Artemisia* (*A. judacia* L.) have cardiotonic effect (due to Jundaicin) that does not deviate from the general frame work of dagitoxin actin (Galal *et al.*, 1974). Antiviral and antibacterial activities are due to the cirsimaritin, which also has inhibitory effect on several mammalian enzymes (Abdalla and Abu-Zarga, 1987). There may be synergism of components too as shown by methoxylated flavenoids of *Artemisia dubia*, which enhance the activity of artemisinin (Elford *et al.*, 1987).

Artemisia dubia leaves as well as flowering tops are used medicinally but leaves are preferred. The fresh plant is always more effective than the dry plant. The green tops of this herb are excellent remedy for disorders of the stomach (Dasture, 1952). Traditionally it is used in different cultures as insecticide (Said, 1996) and vermifuge (Caius, 1986). The whole plant is an aromatic tonic and formerly enjoys a high reputation in debility of the digestive organs (Caius, 1986; Basu and Kirtikar, 1988).

This species also act as anemma gogue in amenorrhoea, caused by uterine disorders or genel debility. It is also prescribed in nocturnal pollutions, chlorosis, anaemia, wasting disease (Dasture, 1952), headaches, migraine, paralysis, facial paralysis, spasmodic affections (such as epiplepsy), and hysteria in nervous irritability nervous disorders and piles. It is an effect diuretic also causes perspiration (Said, 1996). A week decoction of the plant is given to children in measles (Dasture, 1952). Many of

its constitutents are used as flavouring agents (Lee and Geismam, 1970; Marco and Barbera, 1990; Heinrich *et al.*, 1998; Sy and Brown, 2001).

It is considered effective for jaundice and dropsy. It is used as a tonic either alone or in combinations in atonic diseases of the digestive system (Dasture, 1952). Infusion of top of fresh plants has excellent effect for all disorders of the stomach, creating appetite, promoting digestion and preventing sickness after meals (Caius, 1996). In suitable combinations it is applied as paste over the corresponding area of the abdomen to reduce liver and spleen inflammations (Said, 1996). The herb is commonly prescribed in the form of a poultice or fermentation as an antiseptic and discutiend (Caisus, 1986; Basu and Kirtikar, 1988). Wood tea, or powdered herb in small doses mixed in a little soup is used to relieve bilians melancholia. It is also used in intermittent fever (Caius, 1986).

Indigenous system of medicine use its leaves and flowering tops in crude form as anthelmintic, antiseptic, febrifuge and stomachic. It has been employed successfully to alleviate chronic fever, dyspepsia and hepatobilary ailments (Said, 1982). *Artemisia* plant has great potential, much work need to uncover its other activities. That is why; this plant is called "the plant of future".

1.1.6 Artemisinin Synthesis and Storage

Artemisinin an endoperoxide sesquiterpene lactone produced by aerial parts of *Artemisia annua* L. is effective even against multi-drug resistant strains of the malarial parasite. The isolation and characterisation of artemisinin from *Arremisia annua* is considered as one of the most novel discoveries in recent medicinal plant research. It was isolated from the plant in 1972 (Roth and Acton, 1987) and in 1979, its structure was determined by X-ray analysis (Brown, 1993). It has an empirical formula of C, H, O. Artemisinin has a peroxide bridge to which its antimalarial properties are attributed. It has a unique structure and lacks nitrogen containing heterocyclic ring, which is found in most anti-malarial compounds as shown in figure 1.2. Artemisinin is an odourless, colourless compound and forms crystals with melting point of 156 - 157 O C. The

molecular weight as determined by high resolution mass spectroscopy is m/e 282.1742 m+ (6).

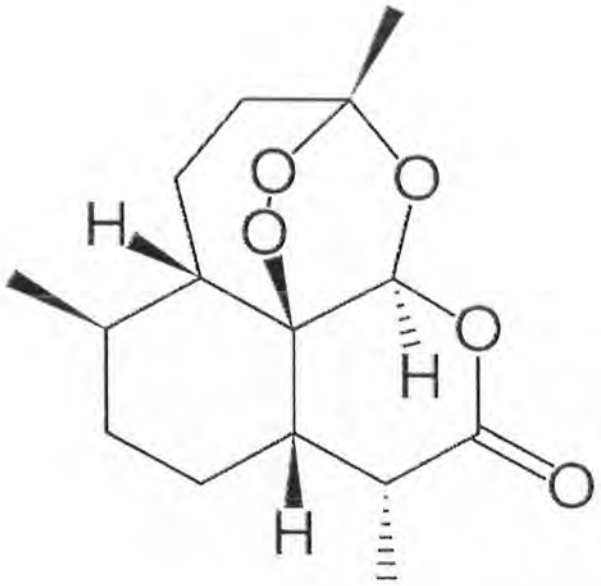


Fig.1.2: Structure of Artemisinin

Artemisinin has proved to be one of the most promising drugs. It has also shown to possess considerable antimicrobial and antifungal activities (Dhingra *et al.*, 2000b). Although the complete organic synthesis has been established, but chemical synthesis of artemisinin is not yet economically feasible because of its complexity and low yield. Currently the leaves, roots and flowers of of *Artemisia* species form the only source of this drug. Artemisinin is found in very low quantities (0.05%-1.1%) in different cultivars of *Artemisia dubia.* High artemisinin yielding clones are being isolated by selection and other non-conventional approaches; however, these have their own limitations. Therefore, in the recent past, in vitro culture system of *A.annua* has been exploited for the production of artemisinin (Delabays *et al.*, 1993).

Artemisinin is the active ingredient found in the *Artemisia* plant and it is used as potent antimalarial agents. Artemisinin is called as sesquiterpene lactone that contains an endoperoxide moiety or internal oxygen bridge, which forms carbon, based free radicals

when exposed to uni-iron. This reaction is selectively toxic to cells with uncontrolled or elevated growth such as cancer cells and the proliferating cells found in diverse conditions, including psoriasis, hyperplasia and proliferative, eye and binding diseases (Acton *et al.*, 2008).

Artemisinin accumulates in glandular trichomes, which are present in leaves, stems and flowers of the plants (Ferreira *et al.*, 1997). As a whole, artemisinin has been detected in leaves; small green stems bud flowers and seeds of *Artemisia* (Martineg and Staba, 1988; Ferreira *et al.*, 1995). Biosynthesis of artemisinin seems to be restricted to the green parts of the plants.So it is not synthesized in roots of field-grown plants (Ferreira *et al.*, 1995) or pollens. ELISA analysis showed that normal green roots accumulate artemisinin (0.001% dry weight). The presence of artemisinin in the extract was confirmed by GC-MS analysis (Jaziri *et al.*, 1995).

Detection of artemisinin from seeds appears to be due to the presence of floral debris (Ferriera *et al.*, 1995). The highest concentration of artemisinin is found in the inflorescence, which may certain more than ten times as much artemisinin as leaves (Ferriela, *et al.*, 1996).

1.1.7 Toxicity

Many species of *Artemisia* are toxic. Habitual uses and large dose of *Artemisia* causes restlessness, vomiting, vertigo, tremors and canvulsions (Said, 1996). Its tendency to produce headaches and other nervous disorders is well known by travelers in Kashmir and Ladak (Caius, 1986; Basu and Kirtikar, 1988). The juice of the large leaves developed from roots is highly nauseous (Dasture, 1952). Many *Artemisia* species are sources of allergies in humans (Lewis, *et al.*, 1983). Large doses of volatile oils are effective narcotic poison (Caius, 1986).

1.2: Improvement of Artemisinin Content

1.2.1: Selection of best cultivar

Artemisia is a perennial and older, well-established plants and often woody in nature. Different cultivars of Artemisia are available like *Artemisia lactiflora, Artemisia vulgaris, Artemisia frigida, Artemisia absinthium*. The only known source of the artemisinin, which is uneconomical to synthesize chemically, is the plant *Artemisia annua*. The *A. annua* plant of the family Asteraceae, indigenous to south east Asia, is an annual herb/shrub, which has become naturalized or is in cultivation as a horticultural or medicinal plant in many parts of Asia, Africa, Europe, America and Australia. The natural populations and genetic resources of *A. annua* from different areas are known to demonstrate considerable variability in the accumulation of artemisinin in the leaves and the capitula of the plant (Charles and Simon, 1990). The foliage and inflorescence of groups have identified and used genotypes of *A.annua* with high artemisinin content for developing agrotechnologies suitable for cultivating this industrial crop under a variety of soil and agroclimatic conditions (Woerdenbag *et al.*, 1994; Kumaret *et al.*, 1999). Artemisinin yields ranging from 3.2 to20 kg/h have been reported (Singh *et al.*, 1988; Woerdenbag *et al.*, 1994).

1.2.2: Selection of different stages

In the vegetatively growing 20–30 weeks old plants of *Artemisia,* leaves are the principle organs for the synthesis and accumulation of artemisinin; stems have artemisinin in about 10-fold lower amount. Usually, the younger leaves have more artemisinin than the older leaves. The leaves of young rosette plants during their growth in the winter season (December through March) have very low concentrations of artemisinin. The expression of artemisinin synthesis and accumulation in the leaves progressively increases with the onset of summer in March/April and becomes high by May/June (summer) and peaks during rainy season (July–September).The flowering plants accumulate bulk of their total artemisinin in leaves (30%) and capitula (40%).

Since the presence of lipids (oil) in the achenes of capitula makes artemisinin extraction cumbersome, the plants harvested in their vegetative state, when ≥ 90% of the artemisinin is in the leaves and fine stem, offer the best economy in chemical extraction of artemisinin. (Sushil *et al.*, 2004) Roots lack artemisinin at all stages of plant growth, therefore tissue culture method is adopted to increase the artemisisnin content in roots. Weathers et al. (1994) reported high levels (0.4%) of artemisinin in hairy root cultures of *A. annua* transformed with *Agrobacterium rhizogenes*.

1.2.3: Tissue Culture of *Artemisia*

Several tissue culture studies have been carried out on various species of *Artemisia*, findings of some of these are as follows.

Optimum proliferation of French tarragon (*Artemisia dracumulus* L. var. sativa) short tips was obtained on MS medium supplemented with 1.8μM NAA and 3% sucrose. After 4 weeks of culture, Maximum proliferation was obtained with unpinched shoot tips placed horizontally on the medium. Maximum rooting was observed with cutting>10mm in length. A 5-sec dip of the basal portion of the cuttings in either NAA or IBA increased rooting percentage and root numbers (Mackay and Kitto, 1998).

Liu *et al.*, (2002) regenerated new shoots from hypocotyis in *Artemisia judaica* L. via callus on medium supplemented with TDZ (thiadiazuron). Upto 16 shoots formed per seedling for an exposure of 20 days. Regenerated shoots formed roots when subcultured onto a medium containing indole-3-butyric acid. The regeneration protocol developed in this study provides a basic knowledge for germplasm preservation and for further investigation of medicinally active constituents of *A. judaica*.

Round wormwood (*Artemisia sphaerocephala* Krasch) seeds were germinated on MS medium without plant growth regulators. The hypocotyls of seedlings were sliced and cultured on medium with 2, 4-D to induce callus (Xu and Jia, 1996).

Much work has been done on *Artemisia dubia* because of its antimalarial activity. Callus can be obtained on media supplemented with combinations of auxins and cytokinins, but non-friable callus is usually obtained. Ferreira and Janick (1996) obtained the highest yield of friable callus with a combination of BA at 4.44 μM and 2, 4-D at 4.52 μM. Vitamin C reduced browning, and GA3 improved friability. However, after 17 months in culture, only 3 out of 24 clones kept as shoot cultures generated calli. Cell cultures were established using the same callus-inducing medium, without agar. However, artemisinin was not detected from callus or cell cultures or from the liquid medium.

Artemisinin is a potential antimalarial drug produced by transformed hairy roots of *Artemisia dubia*. Studies were done to determine the effect of varying the strength of B5 culture media, source and level of nitrogen in the media (nitrate and ammonium), phosphate and the phytohormone, gibberellic acid (GA3), on both biomass and artemisinin production in hairy root cultures. Preliminary results show that a 33% increase in B5 medium, as increase in nitrate, an elimination of ammonia, and addition of GA3 increase biomass yields. Artemisinin production was stimulated by regular strength B5 (with lower levels of nitrate than for maximum biomass), low levels of phosphate, and GA3 (Weathers *et al.*, 1996).

Wang and Tan in (2002) modified MS medium for enhancing Artemisinin production in *Artemisia dubia* hairy root cultures. They altered the ratio of NO_3^-/NH_4^+ and the total amount of initial nitrogen. Increasing ammonium to 60mM decreased both growth and Artemisinin accumulation. With NO_3^-/NH_4^+ at 5:1 (w/w), the optimum concentration of total initial nitrogen for artemisinin production was 20mM. After 24 days of cultivation with 16.7mM nitrate and 3.3mM ammonium, the maximum artemisinin production of hairy roots was about 14mg/L, a 57% increase over that in the standard MS medium.

Nin *et al* (1996) initiated micropropagated *A. annua* L. plantlets on MS basal medium supplemented with different concentrations of BA, Kin, NAA, IAA, and 2,4-D

alone or in combination. Supplementing the medium with low doses of both BAP in combination with NAA, and Kinetin in combination with NAA enhanced the growth rate of callus cultures. Initiation of root and shoot primordial directly from leaf explants cultured on 1.81 μM 2,4-D, while adventitious shoot formation from callus was observed occasionally when BA was added to the medium in combination with IAA. Furthermore, medium containing 2.22 μM BA and 2.69 μM NAA stimulated both callus growth and organogenesis on some callus cultures derived from leaves and stems of young stock material. The best results were obtained with leaf explants. Cytological analysis of root meristems revealed that all regenerants were diploid (2n=18), as expected.

1.2.4 Transformation

Recombinant DNA technology can be defined as a technology through which a segment of DNA responsible for desirable character is transferred from any source (plant, animal, and microbe) to a desired organism. This technology is free of sexual barriers, fast and independent of variability, rather it adds variability to the breeding population. Recombinant DNA technology involves:

1- identification and isolation of desirable gene,

2- modifying the gene to express in the target plant system,

3- cloning in a suitable vector,

4- transformation (transfer of cloned gene of interest into the target cell).

Through transformation we can enhance the production of artemisinin in *Artemisia dubia* by inserting our desired genes as explained below.

1.2.4.1. Agrobacterium-mediated Transformation

Agrobacterum tumefaciens has played a major role in the development of plant genetic engineering and the basic research in molecular biology. It accounts for about 80% transgenic plants produced so far. Initially, it was believed that only dicots, gymnosperms and a few monocot species could be transformed by this bacterium, but

recent achievements totally changed this view by showing that many "recalcitrant" species not included in its natural host-range such as monocots and fungi can now be transformed (Chan *et al.*, 2004; Bundock *et al.*, 2005). In addition, the transformed cells usually carry single or low copy number T-DNA integrated in their genome with less rearrangement, and very large DNA segments can be transformed into the plants (Hamilton *et al.*, 1996; Liu *et al.*, 1999).

1.2.4.1.1: Classification of *Abrobacterium*

The genus *Agrobacterium* has been divided into a number of species. However, this division has reflected, for the most part, disease symptomology and host range. The two most commonly used species for transformation purposes are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Stanton and Gelvin, 2003).

1.2.4.1.2: *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is the soil bacterium which, when containing the Ti plasmid, is able to form crown galls on a number of dicotyledonous plant species (Brown, 2001). Transformation is brought about by the transfer of a large segment of the Ti plasmid called T-DNA to the nuclear genome of a susceptible plant (Chilton *et al.*, 2003). T-DNA contains genes for growth regulator autonomy (Garfinkel *et al.*, 2006 and Joss *et al.*, 1989), and for the synthesis of a wide variety of opines (Murai and Kemp, 1988) which are noble metabolites able to catabolishd by the inciting *Agrobacterium.*

1.24.1.3: *Agrobacterium rhizogenes*

Agrobacterium rhizogenes is a natural plant pathogen responsible for adventitious root formation at the site of infection (Hooykaas, 2004). It incites a disease, hairy root disease, in dicotyledonous plants in a manner very similar to *A. tumefaciens* (Old and Primrose, 1995). This morphogenic effect of *A. rhizogenes* is due to the integration and expression of T-DNA of the Ri (root inducing) plasmid in the plant cell genome

(Willmitzer *et al.*, 2006). It is also responsible for pathogenicity and the induction of opine synthesis (White and Nester, 2003; Chilton *et al.*, 1982). The neoplastic roots are characterized by a high growth rate and are able to synthesize some secondary metabolites (Flores and Filner, 2007).

The Ri plasmid share little homology with Ti-plasmids. The main difference between Ri and Ti plasmid is that transfer of the T-DNA from a Ri plasmid to a plant results not in a crown gall but in hairy root disease, typified by a massive proliferation of a highly branched root system (Brown, 2001). They are of interest because tissue transformed by *A. rhizogenes* readily regenerates into plantlets, which continue to synthesize opine. For these reasons transformed roots of many medicinal and aromatic plants have been widely studied for the *in vitro* production of secondary metabolites (Hamill *et al.*, 1986; Benjamin *et al.*, 1993; Jung and Tepfer, 2004; Mano *et al.*, 2005).

Great progress has been made in recent years in studies on the mechanism of *Agrobacterium*-mediated transformation and its application. Many details of the key molecular events within the bacterial cells involved in T-DNA transfer have been elucidated, and it is notable that some plant factors which were elusive before are purified and characterized (Wei *et al.*, 2000).

1.3: Molecular mechanisms of *Agrobacteirum*-mediated transformation

The mechanism of T-DNA processing and transfer during *Agrobacteirum* infection has been subjected to a number of excellent reviews (Zambryski, 2004; Zupan and Zambryski *et al.*, 2006). Principally, the bacterium can transfer a piece of its plasmid DNA into the infected plant cells, where it integrates into the nuclear genome and expresses its own genes, whose products disrupt the hormonal balance within the plant cells and induce their proliferation to form tumors. In addition, it also produces enzymes to synthesize opines, which the bacteria can use for their own nutrition. The T-DNA is located on a large plasmid called Ti (tumor-inducing)-plasmid, which also contains other functional parts for virulence (*vir*), conjugation (*con*) and the origin of its own replication

(*ori*). In the natural infection by wild type bacteria, the T-DNA and the *vir* genes are essential for inducing plant tumors.

The *vir* region is about 30 kb and encodes at least 10 operons (*virA-virJ*) whose products are vital to T-DNA processing and transfer. Any genes located in the T-DNA region in principle can be transferred, but they themselves are dispensable for this process. Only the 25-bp direct repeats at the right and the left borders are necessary, of which 14 base pairs are completely conserved and cluster as two separate groups.

The infection begins at the wounded sites, and the injured plant cells release some compounds such as the phenolic acetosyringone (AS) acting as specific signals to bind and activate *virA*, a membrane protein, by autophosphorylation, which subsequently activates *Vir*G by phosphorylating one of its asparate residue. The active form of *vir*G binds specifically to the upstream of other *vir* genes called *vir* box, inducing their expression. The *vir*D1 and *vir*D2 are responsible for the T-strand generation, a single-stranded copy of the T-DNA, by specifically recognizing and cutting the bottom strand at the two borders, of which the right one is the start site and thus more important.

After cutting, *vir*D2 remains covalently attached to the 5' end of the T-strand, forming a complex with, which is then targeted into the nucleus by the nuclear target signals (NLSs) of its associated *vir*D2 and *vir*E2, where the T-DNA randomly integrates into the plant genome as single or multiple copies. *Vir*D2 has an active role in the precise integration of T-strand in the plant chromosome.

Novel strategies have to be developed for inserting new DNA into the plasmid. Two are in general use:

(1) The binary vector strategy is based on the observation that T-DNA does not need to be physically attached to the rest of the Ti plasmid. A two-plasmid system, with a T-DNA on a relatively small molecule, and the rest of the plasmid in normal form, is just as effective at transforming plant cells. In fact some strains of *A. tumefaciens*, and related

Agrobacteria, have naturally binary plasmid systems. The T-DNA plasmid is small enough to have a unique restriction site and to be manipulated using standard techniques (White and Nester, 2003).

(2) The cointegration strategy uses as entirely new plasmid based on pBR322 or a similar *E. coli* vector, but carrying a small portion of the T-DNA. The homology between the new molecule and the Ti plasmid means that if both are present in the same *A. tumefaciens* cell, recombination can integrate the pBR plasmid into the T-DNA region. The gene to be cloned is therefore inserted into a unique restriction site on the small pBR plasmid, introduced into *A. tumefaciens* cells carrying a Ti plasmid, and the natural recombination process integrate the new gene into the T-DNA. Infection of the plant leads to insertion of the new gene, along with the rest of the T-DNA, into the plant chromosome (Brown, 2001).

1.3.1: Production of transformed plants with Ti plasmid

As introduction of new genes into every cell in the plant is needed. For this reason a culture of plant cells and protoplasts in liquid medium are infected. A mature plant regenerated from transformed cells will contain the control gene in every cell and will pass the control gene to its offspring.

However, regeneration of a transformed plant can occur only if the Ti vector has been "disarmed" so that the transformed cells do not display cancerous properties. Disarming is required, as infectivity is mainly controlled by the virulence region of the Ti plasmid i.e., two 25bp repeat sequences found at the left and right borders of the region integrated into the plant DNA. Any DNA placed between these two repeat sequences will be treated as T-DNA and transferred to the plant. A number of disarmed Ti cloning vectors are now available; a typical example is the binary vector pBIN19. The left and right T-DNA borders present in this vector flank a copy of the lac Z gene, containing a number of cloning sites, and a kanamycin resistance gene that functions after integration of the vector sequences into the plant chromosome. As with a yeast shuttle vector, the initial manipulations that result in insertion of the gene to be cloned into the pBIN19 are

carried out in *E. coli*, the correct recombinant pBIN19 molecule then being transferred to *A. tumefaciens* and then into the plant. Transformed plant cells are selected by plating on agar medium containing kanamycin (Brown, 2001).

Several *Agrobacterium* strains and binary vectors have been used for the production of transgenic *Artemisia dubia* (Vergauwe *et al.*, 1998; Nin *et al.*, 2004).

1.3.2: Mechanism of *A. rhizogenes* infection

A.rhizogenes agropine type strains carry two T-DNAs, the TL-and the TR-DNA (Jouanin, 1988). At least two pathogenic pathways, both leading to root formation by the host, are realized by genes of the TL- and TR-DNA. The TR-DNA harbors two auxin synthesizing genes, *aux*1 and *aux*2 (Camilleri and Jouanin, 1999).
The auxin biosynthetic pathway catalyzed by the *aux*1 and *aux*2 gene products is similar to that in *A. tumefaciens* and *Pseudomonas savastanoi* and comprises two steps. First, oxidative decarboxylation of tryptophan leading to indole-3-acetamide (IAM) is catalyzed by tryptophan 2-monooxygenase (t2m), encoded by the *tms1, aux1,* and *iaaM* genes of *A. tumefaciens, A. rhizogenes,* and *P.savastanoi*, respectively (Camilleri and Jouanin 1999). The second step, the conversion of IAM to indole-3-acetic acid (IAA), is catalyzed by IAM hydrolase, the product of the *tms*2, *aux*2, and *iaaH* genes in the above bacteria (Jouanin, 1988).

On the TL-DNA of agropine type *A. rhizogenes,* the *rol ABCD* genes are primarily responsible for root induction (White *et al.*, 2008), possibly by enhancing the auxin sensitivity of cells (Maurel *et al.*, 2007; spano *et al.*, 2006). In some instances, rolB and additional auxin act in concert to induce root formation; the factors alone are not effective individually (Spena *et al.*, 2002; White *et al.*, 2008). TR-DNA genes could provide this additional auxin if both T-DNAs are transferred. However, data indicate that TL-DNA located genes in some hosts increase the auxin content, independent of the presence of the TR-DNA. For example, it was shown that TL-DNA transformed pea roots contain increased IAM and IAA concentrations (Prinsen *et al.*, 1992). Interestingly, iaam/tms1, gene expressing, transgenic asparagus and petunia accumulate both IAM and

IAA, while *tms1*-transformed tobacco accumulates only IAM (Prinsen *et al.*, 1998; Van Onkelen *et al.*, 1985). This indicates that conversion of IAM to IAA in cells that express only t2m depends on a host-specific factor such as an endogenous hydrolase (Prinsen *et al.*, 1990).

A candidate gene responsible for the increase IAM content in TL-DNA transgenic tissue is the open reading frame 8 (*ORF8*). Transcripts possibly corresponding to *ORF8* have been reported for TL-DNA transformed tissues (Durand- Tardif *et al.*, 2006; Ooms *et al.*, 2008), indicating that the gene is transcribed in infected cells.

1.4: Production of secondary metabolites by hairy root culture

For some years, there has been great interest in the exploitation of plant cells cultures to produce fine chemicals. With a few exceptions, progress in commercialization has been slow, largely due to the low and/or unstable productivity of many undifferentiated cultures. Developments leading to the production of rapidly growing, organized, 'hairy' root cultures following the genetic transformation of plant with *Agrobacterium rhizogenes* may revolutionize certain areas of plant cell biotechnology (John *et al.*, 1987).

Hairy roots, the result of genetic transformation by *Agrobacterium rhizogenes* often grow as fast as or faster than plant cell cultures (Flores *et al.*, 2007) and do not require hormones in the medium. The greatest advantage of hairy roots is that hairy root cultures often exhibit about the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Banerjee *et al.*, 1998). Many valuable secondary metabolites are synthesized in roots in vivo, and often synthesis is linked to root differentiation (Robins *et al.*, 2003; Flores *et al.*, 2007). Even in cases where secondary metabolites accumulate only in the aerial part of an intact plant, hairy root cultures have been shown to accumulate the metabolites. For example, lawsone normally accumulates only in the aerial part of the plant, but hairy roots of *Lawsonia inermis* grown in half or full strength MS medium (Murashige and Skoog, 1962) can produce lawsone under dark conditions (Bakkali *et al.*, 2005).

Genetic stability is another characteristic of hairy roots. For example, cultures of *Hyoscyamus muticus* hairy roots showed equal or higher levels of hyoscyamine synthesis compared to the roots of a whole plant (Flores and Filner, 2007) and have maintained the same biosynthetic capacity for more than 15 years (Flores *et al.*, 2007) even after cultures are shifted back and forth between the more productive differentiated roots and the less productive undifferentiated cells state (Flores, 2004).

1.4.1: Production of artemisinin by hairy root culture of *Artemisin* sp.

Although artemisinin was thought to accumulate only in the aerial part of *Artemisia dubia* plant (Wallaart *et al.*, 1999), several laboratories have shown that hairy roots can produce artemisinin (Weathers *et al.*, 2004; Jaziri *et al.*, 2004; Liu *et al.*, 1999). Four Asteraceae species were tested: *Artemisia dubia, Callendula officinalis, Mikania glomerala* and *Helianthuus annuus*. The explants of all species were inoculated in *Agrobacterium rhizogenes* strains 8196 and 15834. *Artemisia dubia* showed a positive hairy root response. These roots were excised and cultured in *vitro*. In order to confirm the transgenic character of the hairy roots Southern blot hybridization was carried out (Pellegrino *et al.*, 2008).

Transformed cultures of *Artemisia dubia* L. (*Asteraceae*) were established by the co-culture method using leaf segments of *A. annua* and *Agrobacterium rhizogenes* NCIB 8196 or MAFF 03-0172 by Jaziri *et al.*, (2004), the hairy root clones grew vigorously on hormone free medium. The genetic transformation of the root was proved by the opine assay. A highly specific and sensitive enzyme-linked immuno-sorbent assay (ELISA) method was used for the detection and semi-quantitative determination of artemisinin and structurally related compounds in these cultures. Transformed root cultures of several strains of *Artemisia annua* were also obtained by infection with *Agrobacterium rhizogenes* ATCC 15834 and found to be positive for accumulation for artemisinin (Weathers *et al.*, 2004).

Accoridng to Nin *et al.*, (1997), hairy roots were obtained after infection of *Artemisia annua* shoots with *Agrobacterium rhizogenes* strains 1855 and LBA 9462. The susceptibility to hairy root transformation varied between plant genotypes and bacterial strains. Hairy roots showed macroscopic differences from control root cultures. Southern blot hybridization confirmed the integration of T-DNA from both p1855 and pBin19, while polymerase chain reaction analysis indicated the presence of the NptII gene in the hairy root genome. Sub-cultured transformed root lines grew well in selective B5 agar-solidified medium containing kanamycin or rifampicin and without hormones. Great differences were found in the profiles of the essential oils isolated from normal and hairy roots.

1.5: *Agrobacterium* mediated transformation in *Artemisia*

Biswajit *et al.* (2000) established transformed organ (petiole, lamina, node and internode) cultures of the medicinally important *Artemisia annua* following infection with two wild type nopaline strains of *Agrobacterium tumefaciens*. Parameters such as explant type, strain type and age of the explant source significiantly affected tumorigenesis frequency. Crown galls were formed both on *in vivo* and *in vitro* plants: 2-3% of the *in vitro* galls regenerated spontaneously to produce shooty teratoma of altered phenotype. Artemisinin contents were measured in all transformed as well as non-transformed clones. While shooty teratomas synthesized 0.063 g/100 g DW artemisinin, non-transformed shoots synthesized only 0.0179 g/100 g DW of the compound.

A transformation system was developed by Vergauwe *et al.* (2002a), for *Artemisia annua* L. plants. Leaf explants from *in vitro* grown plants developed callus and shoots on medium with 0.05 mg.L NAA and 0.5 mg/L Ba after transormation with the C58C1 Rif^R (pGV2260) (pTJK136) *Agrobacterium tumefaciens* strain. A concentation of 20mg/L kanamycin was added in order to select transofmed tissue. Kanamycin resistant shoots were rooted on NAA 1.0 mg/L. Polymerase chain reactions and DNA sequencing of the amplification products revealed that 75% of the regenerants contained the foreign genes. 94% of the transgenic plants showed a beta glucuronidase-positive response.

Vergauwe *et al.* (2005), with a view to optimizing a previously described *Agrobacterium tumefaciens*-mediated transformation procedure for *Artemisia annua*, studied the importance of some parameters such as explants type, age of explants source, *A. tumefaciens* strains and type of binary vector. Several binary vectors were useful for the production of transgenic callus on explants of different ages. In transformed calli, a good correlation between integration and expression of foreign DNA was observed: Different assays showed expression of beta-glucuronidase, neomycin phosphotransferase II, superoxide dismutase and bleomycin acetyltransferase. The regeneration of transgenic plants required more restricted conditions. Only with the pTJK136 vector could transgenic plants be obtained from leaf and stem explants of 12-18 weeks old plants. Cocultivation of 48 h seemed favourable for the regeneration of transgenic plants. Stable integration and expression of the transgenes were also shown in the progeny.

Transformed root cultures of several strains of *Artemisia annua* were obtained by infection with *Agrobacterium rhizogenes* ATCC 15834 (Weathers *et al.*, 2004).

1.6: Role of *ROL genes* in Plants

Agrobacterium rhizogenes and *Agrobacterium tumefaciens* infect wounds of several dicotyledonous and some monocotyledonous species and thereby generate tumorous outgrowths at the site of infection (Nilsson and Olsson, 2005). The genes required for tumorigenesis are found on extra-chromosomal elements, Ti (tumor inducing) plasmids for *A. tumefaciens* and R i (root inducing) plasmids for *A. rhizogenes*, of which a T-DNA (transferred DNA) portion is integrated into the plant genome (Zupan *et al.*, 1995; Zhu *et al.*, 2000). Both pathogens are able to re-specify differentiated cells to gain meristematic functions and, in case of *A. rhizogenes*, infections are characterized by a massive production of adventious roots (Meyer *et al.*, 2002).

Transformation with the *rol A* gene results in plants with a highly aberrant phenotype, characterized by wrinkled, intensely green leaves, long internodes, dwarfism or semi dwarfism and retarded senescence (Altvorst *et al.*, 1992; Schmulling *et al.*, 2006).

The pleiotropic alterations observed in tobacco and potato has led to the hypothesis of a functional imbalance in the auxin/cytokinin ratio in favour of cytokinins (Schmulling *et al.*, 2006). In transgenic tobacco plants a reduction of gibberellic acid content has also been reported, suggesting the involvement of *rol A* in gibberellin metabolism (Dehio *et al.*, 1998).

The *rol B* protein on the other hand, has been shown to have a tyrosine phosphatase activity and therefore a possible role in the auxin signal transduction pathway (Filippini *et al.*, 2005). *Artemisia* plants transgenic for *rol B* have wider leaves and a reduced apical dominance than the wild type (Altvorst *et al.*, 1992). The *rol B* gene introduced in *Nicotiana tabacum* was responsible for an auxin like activity, such as enhanced adventitious root formation, which is correlated with increased auxin sensitivity (Maurel *el al.*, 1991; Filippini *et al.*, 1994; Maurel *et al.*, 1994). Similar effect of *rol B* was also observed in tobacco leaf explants and thin cell layers by Altamura *et al.* (1998). Hairy roots from different species (Shen *et al.*, 2001; Shen *et al.*, 2003) as well as leaf fragments from hairy root tobacco regenerants (Spano *et al.*, 2005) were shown to be more sensitive to auxin than their normal counterparts. This feature suggests that Ri T-DNA genes induce the proliferation of transformed cells by a unique mechanism, as compared to *A. tumefaciens* oncogenes, which cause disease by encoding enzymes for hormone biosynthesis (Zambryski *et al.*, 2006).

Estruch *et al.* (1999) have demonstrated that *rol C* can be involved in the released of active cytokinins from their inactive glucosides due to its cytokinin glucosidase activity. This role is consistent with the observed phenotype of *rol C* transgenic plants, characterized mainly by a reduction of apical dominance and plant height (Estruck *et al.*, 1991; Nilsson *et al.*, 2002 Schmulling *et al.*, 2006). The integration and expression of the T-DNA of Ti plasmids causes metabolic changes mainly determined by the *iaa M* and *iaa H* genes which code for enzymes involved in indoleacetic acid synthesis and the *ipt* gene whose product catalyses the first step in cytokinin biosynthesis (Akiyoshi *et al.*, 1984; Buchmann *et al.*, 2003; Yamada *et al.*, 2006). In ornamental plants such as carnation and *Petunia* the insertion of *rol C* leads to the expression of advantageous traits, i.e.,

increased axillary bud break and development, better rooting ability of stem cuttings (Zuker *et al.*, 2001), increased branching and reduction in time to flowering (Winefield *et al.*, 1999). Some data reveals that the *rol C* gene could affect activity of defense proteins, such as 1, 3-D-glucanases. Expression of the *rol C* gene in ginseng cells caused significant increase of 1, 3-D-glucanase activity by production of a new enzyme isoform. Activities of other glucanases tested such as 1, 4-D-glucanases and 1, 6-D-glucanase, which are known to be unrelated to defence proteins, were unchanged (Bulgakov *et al.*, 2002).

Concerning the *rol D* gene, its function as an ornithine cyclodeaminase enzyme, catalyzing, catalyzing the conversion of ornithine to proline, has recently been elucidated (Trovato *et al.*, 2001). In transgenic tobacco plants, *rol D* induces a striking earliness in the induction of the flowering process and an increase in the number of flowers (Mauro *et al.*, 2006) that have been related to an accumulation of proline or to a depletion of ornithine. In *Artemisia* plants *rol D* has a pleiotropic effect, affecting traits of economic interest such as plant productivity, as well as characters generally correlated with the defence response to pathogens (Bettini *et al.*, 2003). In this study, the analysis of transgenic plants did not show any morphological modifications. First generation of transgenic plants was found to flower earlier, and showed an increased number of inflorescence and higher fruit yield. They have also shown that the plants harbouring *rol D* gene were more tolerant to the toxin produced by the fungus *fusarium oxysporum* in ion leakage experiments, with respect to the untransformed regenerated controls.

The organogenetic competence of roots and *Agrobacterium rhizogenes* induced hairy roots of twelve *Artemisia* genotypes has been investigated (Peres *et al.*, 2001). Both roots and hairy roots of *Artemisia dubia* L. derived genotypes regenerated shoots after 24 weeks of incubation on zeatin contained medium. Anatomical analysis showed that shoot regeneration in roots could be direct or indirect, depending on the genotype considered. Hairy roots showed considerable differences in their morphogenetic responses, when compared to the corresponding non-transgenic roots. The differences observed might reflect the influence of the introduced *rol* genes on hormonal metabolism/sensitivity. Data pointed to the possibility of the use of *A. rhizogenes*, combined with regenerating

Artemisia genotypes, in a very simple protocol, based on genetic capacity instead of special procedures for regeneration, to produce transgenic *Artemisia* plants expressing *rol* genes, as well as, genes present in binary vectors.

Root proliferation is not due to diffusible cell division factors and a direct interaction of the proteins encoded on the T-DNA with plant hormonal metabolism could not be shown. The T-DNA encodes up to 18 open reading frames, depending on the bacterial strain (Slightom *et al.*, 2003). Insertional mutagenesis showed that insertions in only 4 of the potential 18 loci noticeably affected the morphology of the hairy roots that were produced (White *et al.*, 2008). These loci were named root locus *A-D (rol A-d)*. The *rol A, B, C* and *D* genes have been identified as the main determinants of the hairy root disease caused on dicotyledonous plants by the soil bacterium *Agrobacterium rhizogenes*. The *rol A, B, C* and *D* loci correspond, most likely, to open reading frames (ORFs) 10, 11, 12 and 15 of the TL-DNA (Slightom *et al.*, 2003). When individual *rol* genes are inserted in plants, they have different phenotypic effects that can be at least in part ascribed to modifications in the endogenous hormone equilibrium and in some cases have shown to be potentially interesting also for economic purposes.

The roots induced by the integration of T-DNA of various *Agrobacterium rhizogenes* wild type strains are characterized by an extensive growth, associated with lateral branching, leading to an important mass of adventitious roots exhibiting a typical phenotype. Furthermore, these roots has been found to produce a high yield of secondary metabolites as compared to that of undifferentiated plant cell suspensions, such as indole alkaloid production in *Catharantus roseus* cultures (Palazon *et al.*, 2003), ginsenoside production in cultures of *Catharatus roseus* cultures (Palazon *et al.*, 2003), ginsenoside production in cultures of *Panax ginseng* (Bulgakov *et al.*, 2002), and anthraquinone production in callus cultures of *Rubia cordifolia* (Bulgakov *et al.*, 2002). The hairy roots obtained from the transformation of *Atropa belladonna* with *A. rhizogenes* 15834 produced various amounts of tropane alkaloids, and in most cases higher than in normal field grown plants (Kamada *et al.*, 2008; Saito *et al.*, 2001; Aoki *et al.*, 2008). The *rol A*.

rol B, and *rol C* genes together induced root formation and tropane alkaloid biosynthesis in tobacco and in *Datura stramonium* (Spena *et al.*, 2002; Palazon *et al.*, 1997). The *rol A*, *B* and *C* genes could play a major role in hairy root induction and metabolite production, also leading to an increase in the production of secondary products in *A. belladonna* hairy roots as is the case with tobacco root cultures (Palazon *et al.*, 1997). The capacity to induce root formation in the host is greatly increased when the *rol A* and/or *C* loci are combined with the *rol B* locus. Root induction is shown to be correlated with the expression of the *rol loci*. Transgenic plants exhibit all the characteristics of the hairy root syndrome only when all three loci are present and expressed. Although the activity of the *rol* genes encoded functions is synergistic, each of them appears to independently influence 3 host functions involved in the determination of root differentiation (Spena *et al.*, 2002).

A number of synergistic effects of *rol* genes have been reported. The presence of *rol A*, *rol B* and *rol C* together dramatically enhances the growth rate and nicotine production, which shows that the effect of these three *rol* genes was synergistic (Plazon *et al.*, 1997). Several reports suggest that *rol A* and *rol C* alter Polyamine (PA) metabolism and plant phenotype (Michael *et al.*, 1998; Sun *et al.*, 1999; Martin-Tanguy *et al.*, 2006). In another study, transgenic plants containing the *rol A* constructs showed an increase in the level of PA biosynthetic enzymes and PA titres (Altabella *et al.*, 2007). It appears that the degree of expression of *rol A* and/or *rol C* phenotype depends on the level of their penetration, type of tissue used, promoter strength and site of insertion of the foreign gene into the plant genome. Transgenic roots, especially those transformed either by a combination of the three *rol* genes (*A*+*B*+*C*) or the *rol C* alone, grew faster than the untransformed roots (Altabella *et al.*, 2007). Putrescine, spermidine and traces of spermine were present in all samples, both in free and bound forms, while *rol A* roots showed increased levels of free and bound polyamines (Altabella *et al.*, 2007). The higher polyamine contents found in roots transformed by *rol A* paralleled with higher ornithine and arginine decarboxylase activities as well as higher nicotine production (Altabella *et al.*, 2007).

Little is known about mechanisms by which the *rol* genes interact secondary metabolism. Studying the transgenic for the *rol B* and *rol C* genes callus cultures of *R. cordifolia*, it has been found that anthraquinone (AQ) production was greatly increased in both transformed cultures compared with the non-transformed cultures (Bulgakov *et al.*, 2002). The induction of AQ biosynthesis by the *rol* genes did not proceed through the activation of the common Ca^{+2} –dependent NADPH oxidase pathway that mediates signal transduction between an elicitor receptor complex via transcriptional activation of defence genes (Gulgakov *et al.*, 2003). Okadaic acid and cantharidin, inhibitors of protein phosphatases 1 and 2A, caused an increase of AQ production in transgenic cultures. Okadaic acid stimulated AQ accumulation in the non transformed cultures, whereas cantharidin had no effect. These results have shown that different phsophatases are involved in AQ synthesis in normal and transgenic cultures of *R. cordifolia* (Bulgakov *et al.*, 2003).

1.7: Aims and Objectives

The aim of the present study was to transform the *Artemisia dubia* plant with *rol* ABC genes using *Agrobacterium tumefaciens* and to compare artemisinin production in the transformed roots of *Artemisia dubia* with the non transformed plants.

MATERIALS AND METHODS

MATERIALS AND METHODS

The aim of present study was to compare artemisinin production in the transformed roots of *Artemisia* species (*Artemisia dubia*) transformed with *Agrobacterium tumefacienes* and their comparison with the non transformed roots of the *Artemisia species*. The present research work was carried out in Plant Molecular Biology laboratory, Department of Biological Sciences, Quaid-i-Azam University, Islamabad. The present research work was started on Thursday 13 February, 2008. A brief account of the materials and methods used and the procedures adopted is given below.

2.1: Source of seed

The plants and seeds of *A. dubia* L. were collected from Donga gali Ayyubia pipeline (NWFP), which were identified by Taxonomy Lab, Department of Biological Sciences, Quaid-i-Azam University, Islamabad, and were stored in refrigerator at 4°C. These seeds keep their vigor for at least 3 years if stored under dry and cool conditions.

2.2: Chemicals

All the chemicals used in the experimental work were of the highest grade of purity. Chemicals were purchased from Sigma Chemical Co: USA and E. Merck of Germany.

2.3: Culture procedure

The glassware used in the preparation of media was made of borosilicate glass. All the glassware was washed using commercial detergent. Cleaned glassware was dried at 150 °C for 30 minutes in an oven and wrapped with aluminum foil.

2.4 : Medium

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Appendix-III), (MS; Sigma cat# M5519) was used for germination of seeds in the present investigation. MYA (Appendix-II) and YMB (Appendix-I) media were used for the growth of bacteria.

2.5: Inoculation area and manipulation tools

Transfer room was cleaned on monthly basis and sprayed with 95% ethyl alcohol. Surgical instruments, Petri dishes, flasks containing distilled water were sterilized in an autoclave at 121 °C and 15psi for 20 minutes. Surgical instruments and Petri dishes were autoclaved in aluminum foil or newspaper

2.6: Culturing of tissues

Aseptic transfer of tissues was carried out in a Laminar Flow Cabinet fitted with a HEPA filter. Before using the Laminar Flow Cabinet, working surfaces were swabbed down with 95 % ethyl alcohol.

Surgical instruments, Petri dishes, distilled water and culture vessels were brought into the cabinet. Then the working area and instruments were exposed to UV light for 20 minutes to ensure sterility. Transfer work was started about half an hour after the UV light was switched off. Surgical instruments were dipped in 95 % ethyl alcohol. After each manipulation, the instruments were again dipped in ethyl alcohol, reflamed and reused.

2.7: Seed Germination

Stored seeds of *Artemisia dubia* were surface sterilized with 0.1 % (w/v) Mercuric chloride ($HgCl_2$) solution for varying durations of 2, 3, 4 and 6 minutes. Then seeds were rinsed several times with sterile distilled water under laminar flow hood. Seeds were sown under sterile conditions in Petri dishes containing half strength MS salts, supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH was adjusted to 5.8 with 1 N NaOH / HCl before the addition of agar. The medium was autoclaved at 15 psi, 121°C for 20 minutes. This medium is referred to as the Germination Medium (GM). Then these plates were incubated in growth chamber [at 25°C 16 h of photoperiod, illumination of 45 $\mu E\ m^{-2}\ s^{-1}$ and 60 % relative humidity] under aseptic conditions. The seeds were germinated at Monday 3rd March, 2008.

2.8: Culture environment

Tissue cultures were incubated under conditions of well-controlled temperature, light intensity and photoperiod. Temperature of culture room for the present study was maintained at 25 ± 2 ^{0}C with 16 / 8 hours light dark cycle and the light intensity was maintained at 1000 lux throughout the experiments.

2.9: TRANSFORMATION OF *ARTEMISIA* WITH *ROL ABC* GENES

The transformation procedure involving tissue culturing was carried out with two different types of explants i.e. Stem and leaves with one strain of *Agrobacterium tumefacienes* i.e. LBA4404 containing pRT99 harbouring rol ABC genes.

2.9.1: PLANT MATERIAL

Stem and leaves (0.5-1 cm in length) were cut transversely from 20 days –old in vitro grown seedlings and used for genetic transformation. In transformation experiment, 20-40 control explants were used without cocultivation with *Agrobacterium.*

2.9.2: BACTERIAL STRAIN AND PLASMID CONSTRUCTION

Agrobacterium tumefacienes strain LBA4404 harbouring *rol* ABC genes was kindly provided by Dr. David Tepfer, institute National de la Recherche (INRA), Vesailles, France. T-DNA region of pRT99 carries *rol* A, rol B and rol C coding sequences, each of which is under the control of CaMV 70S promoter and CaMV 35S terminator. T-DNA of pRT99 also contain NPTII gene with NOS promoter and NOS terminator.

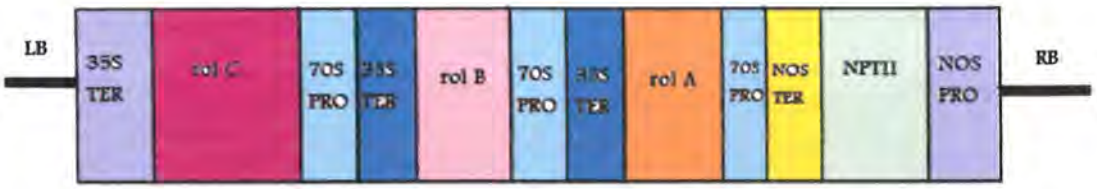


Fig 2.1: Schematic diagram of the T-DNA region of the plasmid pRT99

RB: Right border; LB: Left border; NOS PRO: Nopaline synthase promoter; NOS TER: Nopaline synthase terminator; 35SPRO: Cauliflower mosaic virus 35S promoter; 35S TER: Cauliflower mosaic virus 35S terminator; 70S PRO: Cauliflower mosaic virus 70S promoter; NPTII: Neomycin phosphortransferase coding region.

2.10: TRANSFORMATION PROCEDURE

2.10.1: Preparation of Explants

Leaves and stem segments, were excised from 20 days old seedlings and cut into 0.5-1 cm pieces under laminar flow hood and transferred to MS medium containing 50µl acetosyringone in petriplates. These pieces were used as explants for further manipulation.

2.11: Co cultivation

2.11.1: Co cultivation medium

Co-cultivation medium was prepared by adding 50 ul acetosyringone and 50mg/l kanamycin to MS, pH was adjusted at 5.8. The medium was prepared in 500ml flasks which were plugged with cotton, covered with aluminium foil and autoclaved at 15psi, 121°C for 20 minutes. The autoclaved medium was poured under laminar flow hood, into Petri plates (75mm) containing 15ml of medium in each. The media in the

Petri plates was allowed to solidify under the sterilized conditions of laminar flow hood.

2.11.2: Co cultivation

1. *Agrobacterium tumefacienes* strain LBA4404 containing pRT99 was grown overnight in 59 ml of liquid MYB medium (0.5% (w/v) yeast extract, 0.05%(w/v) casein hydrolysate , 0.8% (w/v) mannitol, 0.2% (w/v) ammonium sulfate, 0.5%(w/v) sodium chloride, pH 6.6). Medium was supplemented with 50mg/l kanamycin sulphate, added to the cold media, after autoclaving under the laminar flow hood. After inoculation, bacterial cultures were maintained at 28°C and 130 rev/min in shaking incubator.

2. Bacterial cells from strain were then collected by centrifugation at 3,500 rpm for 10 minutes at 4°C in 15ml Falcon tubes.

3. The pellets were resuspended in a hormone free MS (Murashige and Skoog, 1962) medium.

4. The density of bacterial strain was adjusted at approximately 5×10^{10} CFU/ml.

5. After one day of preculturing explants were immersed in the bacterial suspension for 10-15 minutes.

6. Thereafter the explants were blotted on sterilized filter paper and placed on cocultivation medium containing MS medium with 50ul acetosyringone. In each plate about 25 explants were cocultivated.

7. These plates with explants were kept in growth chamber at 27 °C, 16h of photoperiod, illumination of 45 uEm^{-2} s^{-1} and 60% relative humidity, for two days.

2.12: SELECTION AND REGENERATION

After three days of cocultivation, the explants were washed with washing medium (WM) that consisted of sterilized MS medium containing 500ug/ml cefotaxime to kill bacteria and blotted on sterilized filter paper. All antibiotics were filter sterilized and added to the autoclaved medium. The explants were then transferred to the flasks containing selection medium SM containing MS medium with 0.1mg/l BAP (Benzyl amino purine) before autoclaving, and 20mg/l Kanamycin, 500mg/l cefotaxime was added with the help of micropipette under the laminar flow hood, when the autoclaved media cooled down at 45°C.

These flasks with explants were maintained at 27 °C, 16h of photoperiod, illumination of 45 uEm^{-2} s^{-1} and 60% relative humidity. The explants were then transferred to fresh medium (SM) weekly during the fist month. Afterwards subcultures were made every two weeks. After eight weeks, the concentration of cefotaxime was reduced to 50 mg/L. For rooting, the developed shoots cut off segments were cultured on Half MS medium containing 0.025mg/L NAA and solidified with 0.1% gelrite.

When roots appeared then sub culturing of parent plants were done in order to increase the number of transformed plants. For sub culturing same medium is used which was used for rooting containing Half MS medium with 0.025mg/L and 0.1% gelrite for solidification.

2.13: TRANSPLANTATION TO POTS AND ACCLIMATIZATION

After selection of transformed seedlings, the controlled and kanamycin-resistant plants were transferred individually to small pots containing soil. The soil was made by mixing equal amounts of clay, sand and peat. Small pores were made in their bottoms so that seepage of excessive water may occur at the bottom and may not injure the roots.

Plants after transferring to pots were kept enclosed with transparent polythene bags to retain moisture. Acclimatization was done for 15 days in growth room 25±2°C, 16h of photoperiod, illumination of 45 uEm^{-2} s^{-1} and 60% relative humidity. Once being hardened, the plants were shifted to green house. Extensive care was taken till they reached maturity. Number of differences was found in morphological features of the

control and transgenic antibiotic resistant plants. The morphological parameters were observed and recorded.

Seeds were collected from these green house grown flowers. This resulted in the achievement of first generation of seeds from the transformed plants.

2.14: MOLECULAR ANALYSIS

For confirmation of the transformation and integration of the desired genes. molecular analysis was carried out through PCR. Plants transformed with *Agrobacterium tumefacienes* strain LBA4404 were used for PCR analysis for NPTII and *rol A* genes. For PCR analysis, genomic DNA was isolated from transformed plants, and plasmid DNA was also isolated from the strain of *Agrobacterium tumefacienes.*

2.14.1: ISOLATION OF GENOMIC DNA FROM PLANT LEAVES

For extraction of genomic DNA from the seedlings simplified CTAB (cetyl trimethyl ammonium bromide) method of Doyle and Doyle, (1990) was used. DNA was extracted from both transformed and untransformed control plant by grinding individually frozen seedlings to very fine powder using ice cold pestle and mortar. 5ml CTAB extraction buffer at 65°C was added to the powder leaves and incubated at this temperature for 20 minutes with occasional vigorous shaking. 2ml of chloroform was then added and shaked thoroughly and placed on inverter at room temperature for 20 minutes, then centrifuged at 3000 rpm for 5 minutes to resolve phases. After which aqueous phase was transferred to fresh tube and 2ml of isopropanol was added, mixed well and stored on ice for 10 minutes. Centrifuged again at 3000 rpm for 5 minutes to collect ppt. The liquid was drained away and sides of tube were dried using blotting paper. To dissolve ppt. 200ul of TE was added. Then 200ul of 4 M LiAc was added and incubated on ice for 20 minutes. Centrifuged again for 10 minutes at 3000 rpm and to supernatant in a fresh tube 1 ml of absolute ethanol was added and placed on ice for 20 minutes. To collect ppt the

tube was centrifuged again for 10 minutes at 3000 rpm. The liquid was drained away and DNA was dissolved in 200 µl TE by gentle pipeting. 100 µl of 3 M NaOAc was added, and then equal volume of chloroform was added. After which 2 volume of absolute ethanol was added and stored on ice for 5 minutes to precipitate DNA. It was then centrifuged for 10 minutes to collect ppt. Ethanol was evaporated and DNA was dissolved in 200 µl of TE and stored in refrigerator at -20°C.

COMPOSITION OF CTAB BUFFER

Sorbitol	140 mM
Tris, Ph 8	220 mM
EDTA	22 mM
NaCl	800 mM
Sarkosyl	1%
CTAB	0.8 %

These were combined; pH was adjusted to 8 and autoclaved.

2.14.2: EXTRACTION AND PURIFICATION OF PLASMID DNA

Minipreparations of plasmid DNA were obtained by alkaline lyses method described below.

- *Agrobacterium tumefacienes* strain LBA 4404 containing pRT99 was grown overnight in 50ml of liquid MYB medium. Medium was supplemented with 50 mg/L kanamycin sulphate, added to the cold media, after autoclaving under the laminar flow hood. After inoculation, bacterial culture was maintained at 28°C and 130 rev/min in shaking incubator.

- 1.5 ml of bacterial culture was poured into two microfuge tubes and centrifuged at 14,000 rpm for 15 minutes at 4°C to collect the cells. The supernatant was removed by micropipette and the cells were left to dry.

- The pellet was resuspended in 100 µl of solution 1 by vortexing and was left at room temperature for 10 minutes.

- 200 µl of solution 2 was added and the contents were mixed by inverting the tubes for 4-5 minutes and left at room temperature for 5 minutes.

- Then 100 µl of solution 3 was added and the contents were mixed gently for 4-5 times, the tubes were stored on ice for 20-30 minutes. Low temperature and pH precipitates the denatured DNA which was pelleted by centrifugation at 14,000 rpm for 10 minutes and the supernatant was transferred into fresh tube.

- To purify DNA equal volume of chloroform was added and centrifuged again for 5 minutes at 14,000 rpm. The supernatant was transferred to a fresh tube and double volume of ethanol was added to supernatant and stored at -20°C for 1 hour to precipitate DNA and centrifuged for 10 minutes at 14,000 rpm to collect the ppt.

- The pellet was washed twice with 70% ethanol to remove any remaining salt. After drying, the pellet was resuspended in 30 µl of TE buffer and was then stored at -20°C in refrigerator.

SOLUTIONS

Solution 1	Glucose	50 mM
	Tris	25 mM (pH 8.0)
	EDTA	10 mM (pH 8.0)
Solution 2	SDS	1%

	NaOH	0.2 N
Solution 3	Sodium Acetate	3 M
TE	Tris pH 8.0	10 mM
	EDTA	1 mM

2.15: POLYMERASE CHAIN REACTION

Polymerase chain reaction for the detection of *rol A* gene was performed following the standard method of Taylor, (1991). PCR reaction was performed in 0.2 ml tubes containing 50 µl total reaction mixture. DNA (0.3 µg) was incubated in a final volume of 50 µl with 0.25 µg of reverse and forward primers, 0.2 mM each of d ATP, d GTP, d CTP, and d TTP and 2 mM MgCl2 and 2 units of Taq polymerase and 5 µl of 10X PCR buffer. The reaction mixture was centrifuged for few seconds thorough mixing.

The reaction mixture was taken through thermo cycling conditions as: 5 minutes of 95°C for template denaturation followed by 25 cycles of amplification each consisting of 3 steps; 30 seconds at 95°C for DNA denaturation into single strands; one minute at 53-55°C for rol A primers to hybridize or "anneal" to their complementary sequences on either side of the target sequence; and one minute at 70°C for extension of complementary DNA strand from each primer. Final 10 minutes at 70°C for Taq polymerase to synthesize any unextended strands left. PCR was performed using gene Amp PCR system 2400 and gene Amp PCR system 9600 thermocyclers (Perkin Elmer, USA).

2.15.1: Primers used during PCR

During PCR following forward and reverse primers for *rol A* gene were used.

Forward primer 5`-GAAGACGACCTGTGTTCTC-3 and Reverse Primer 5`-CGTTCAAACGTTAGCCGATT- 3` amplifying a fragment of 308 bp.

2.16: AGAROSE GEL ELECTRPHORESIS

Agarose gel electrophoresis was carried out to analyze the amplified DNA samples. 1.5 percent w/v agarose gel was prepared by melting 1.5 gm of agarose in 100 ml of 1 X TBE buffer in microwave oven. 10 µl ethidium bromide solution (10mg/ml) was added to stain DNA. DNA samples were mixed with DNA loading dye containing 0.25% bromophenol blue prepared in 40% sucrose solution. Electrophoresis was performed at 100 volts (50 mA) for 45 minutes in 1 X TBE running buffer. After electrophoresis amplified product was detected by placing the gel on UV-Tran illuminator (Life Technology, USA).

COMPOSITION OF 10 X TBE

Tris	890 mM
Boric acid	25 mM
EDTA	0.1 mM (pH 8.3)

2.17: Determination of transformed root cultures

To determine growth rate of transformed root cultures, 3-4 cm root tips were cut and placed on solid B5 medium. Growth rate was determined by visual examination for increase in fresh weight and root hairs for thirty days at the interval of 10 days. Untransformed control roots obtained from shoots were also grown similarly. These

roots were then harvested and placed in Vacuum oven for 3-days and then examined for artemisinin contents after 30 days.

2.18: Extraction of artemisinin

Artemisinin was extracted from roots by using method described by Kim *et al.*, (2001). Extraction procedure was divided in following steps.

Grinding:

Dry roots at different stages were taken mixed with HPLC grade toluene (1g/10ml). Micronised with mortar and pestle until homogenous mixture formed. Small amount of cells lysed here and were transferred to homogenizer.

Homogenization

Further uniformity was achieved with the help of homogenizer (Nissei homogenizer, model-AM-11). Homogenizer speed was 50x100 rpm for 30 minutes. Cell walls of some cells were also lysed. A complete homogenized mixture was then transferred to sonicator.

Sonication

During exposure to sonicator (Branson sonifier, model 450, duty cycle 80% and out put control 8) for 30 minutes, cell wall ruptured and artemisinin released into toluene.

Centrifugation

The next step was removal of liquid phase from debris. Liquid phase contained artemisinin, which was separated with the help of centrifugation machine (Kokusan centrifuge, model H-103 RS). The centrifugation was carried out at -20°C with speed of 3500 rpm for 20 minutes. This step was followed by evaporation.

Evaporation

Toluene was evaporated in open air at 4 °C in three days. Dried plant extract obtained contained artemisinin and was stored at -20°C until spectrophotometric analysis.

Preparation of sample

Dried extract was solubilised with 400µl methanol (HPLC grade, Merck) and 1600µl of NaOH solution (0.2% w/v). The mixture was hydrolyzed for 45 minutes at 50°C. The reaction was stopped by adding 1600µl of acetic acid (0.2M) and placing the test tube in ice water. To make the final volume of 4ml, 400µl methanol (HPLC grade, Merck) was added.

The samples were again dried and then dissolved in 5ml ethanol (HPLC grade) and 0.3ml of 10 % v/v Tri fluoro acetic acid (TFA) was added in each sample.

Preparation of standard solutions

Stock solution (1mg/ml) was prepared by dissolving 10 mg artemisinin (ICN biomedical inc.) in 10 ml of ethanol. Its further dilutions were made as shown in table 2.1.

Table No 2.1. Different dilutions of standard solution

S. No.	Conc. of standard solution (µg / ml)	Stock solution (ml)	Ethanol (ml)	TFA 10% v/v (ml)	Final Volume (ml)
1	100	1.00	8.70	0.300	10
2	50	0.50	9.2	0.300	10
3	10	0.10	9.60	0.300	10
4	2.5	0.025	9.675	0.300	10

The λ max was observed at 218nm by wave length scanning of the standard solution. Absorbance of all the standards was taken at 218nm. The concentration of the artemisinin in the sample was calculated by comparing with absorbance of standard artemisinin. After that artemisinin content was calculated by considering the dry weight of sample.

RESULTS

RESULTS

The aim of present study was to compare artemisinin production in the roots of *Artemisia dubia* transformed with *role ABC* genes by using *Agrobacterium tumefaciens* with the non transformed plants. Numbers of factors have been reported that affect the efficiency of transformation with *Agrobacterium tumefaciens*, different parameters like seed surface sterilization with different time durations, effect of type of explants and co-cultivation period were studied. *Agrobacterium tumefacienes* strain LBA4404 containing pRT99 harboring *rol ABC* genes were used for the transformation experiment.

3.1 Collection of Plant Material

The plants and seeds of *A. dubia* L. were collected from Donga gali Ayyubia pipeline (NWFP), which were identified by Taxonomy Lab, Department of Biological Sciences, Quaid-i-Azam University, Islamabad, and were stored in refrigerator at -70^{0}C. These seeds keep their vigor for at least 3 years if stored under dry and cool conditions.

Agrobacterium tumefaciens mediated transformation of *Artemisia dubia* with *rol ABC* Genes

Agrobacterium tumefacienes strain LBA4404 containing pRT99 harboring *rol ABC* genes were used for the transformation experiments. The *A. tumefacienes* strain LBA4404 grown overnight in MYA medium. Explants were infected with the bacterial strains following the transformation method described in material and methods.

3.2 Seed Surface Sterilization

For surface sterilization of seeds, Mercuric chloride ($HgCl_2$) 0.1% (w/v) was used. Seeds were exposed to sterilizing agents for varying durations. No contamination was observed in seeds of all treatments. The number of seeds germinated varied with different duration of exposure to the sterilizing agent i.e. Mercuric chloride ($HgCl_2$) 0.1% (w/v). The germinated seed are shown in Fig 3.1.

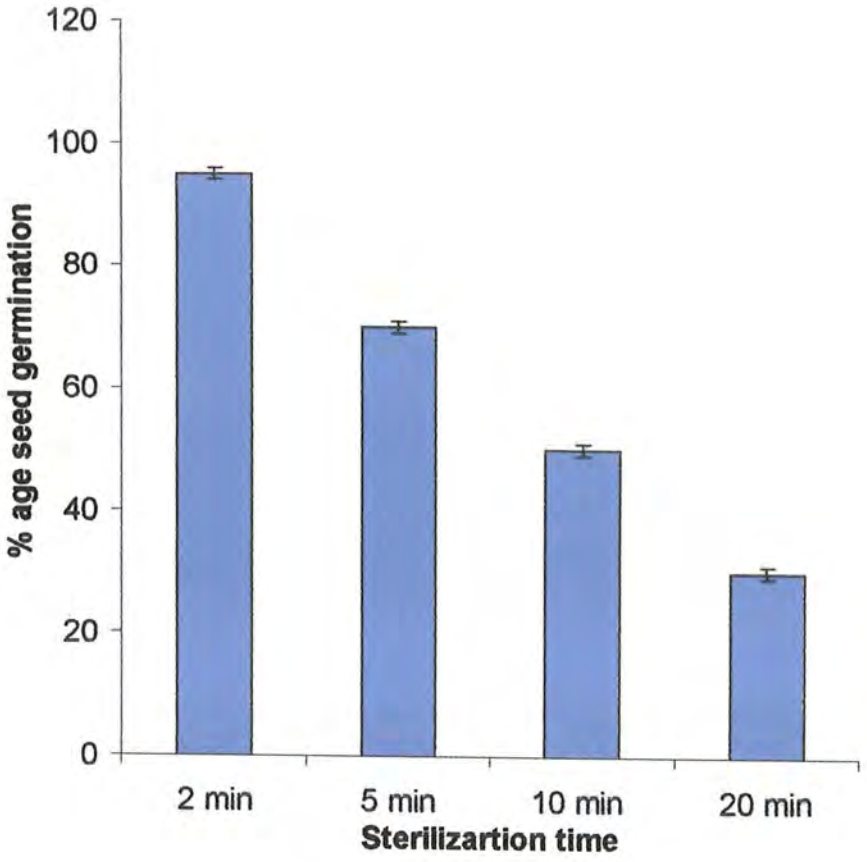


Fig 3.1: Percentage seed germination with different duration of exposure to 0.1% (w/v) Mercuric Chloride

Fig 3.2: *In vitro* grown seedlings of *Artemisia dubia* on MS.

3.3 Medium for Seed Germination

Seeds sown on half strength MS medium showed 100% germination within 3 days. Whereas on plain agar although 80% of the seeds germinated but the duration was almost 6 days (as shown in the Table 3.1). Moreover, the seeds germinated on half MS medium were healthier and fresh as compared to the ones on plain agar.

Table 3.1: Effect of medium on germination of seeds.

Medium	Duration of germination	% age of seeds germinating
½ MS	3 days	100%
Plain agar	6 days	80%

3.4: TYPES OF EXPLANTS

The *Agro bacterium tumefaciens* mediated transformation procedure involving tissue culturing was carried out with two different types of explants i.e. Stem and leaves with one strain of *Agrobacterium tumefaciens* i.e. LBA4404 containing pRT99 harboring *rol ABC* genes. Explants were prepared by cutting Leaves and stem segments from 20 days old seedlings (as shown in figure 3.3) into 0.5-1 cm pieces and transferred to MS medium containing 200µM acetosyringone. These pieces were used as explants for further manipulation.

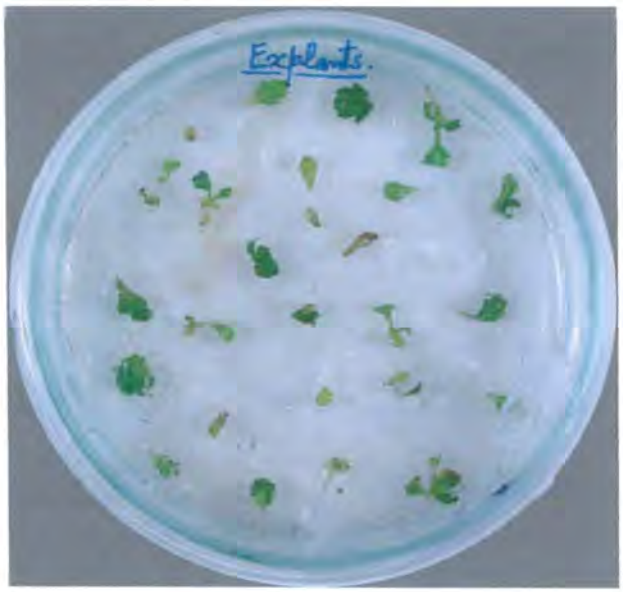
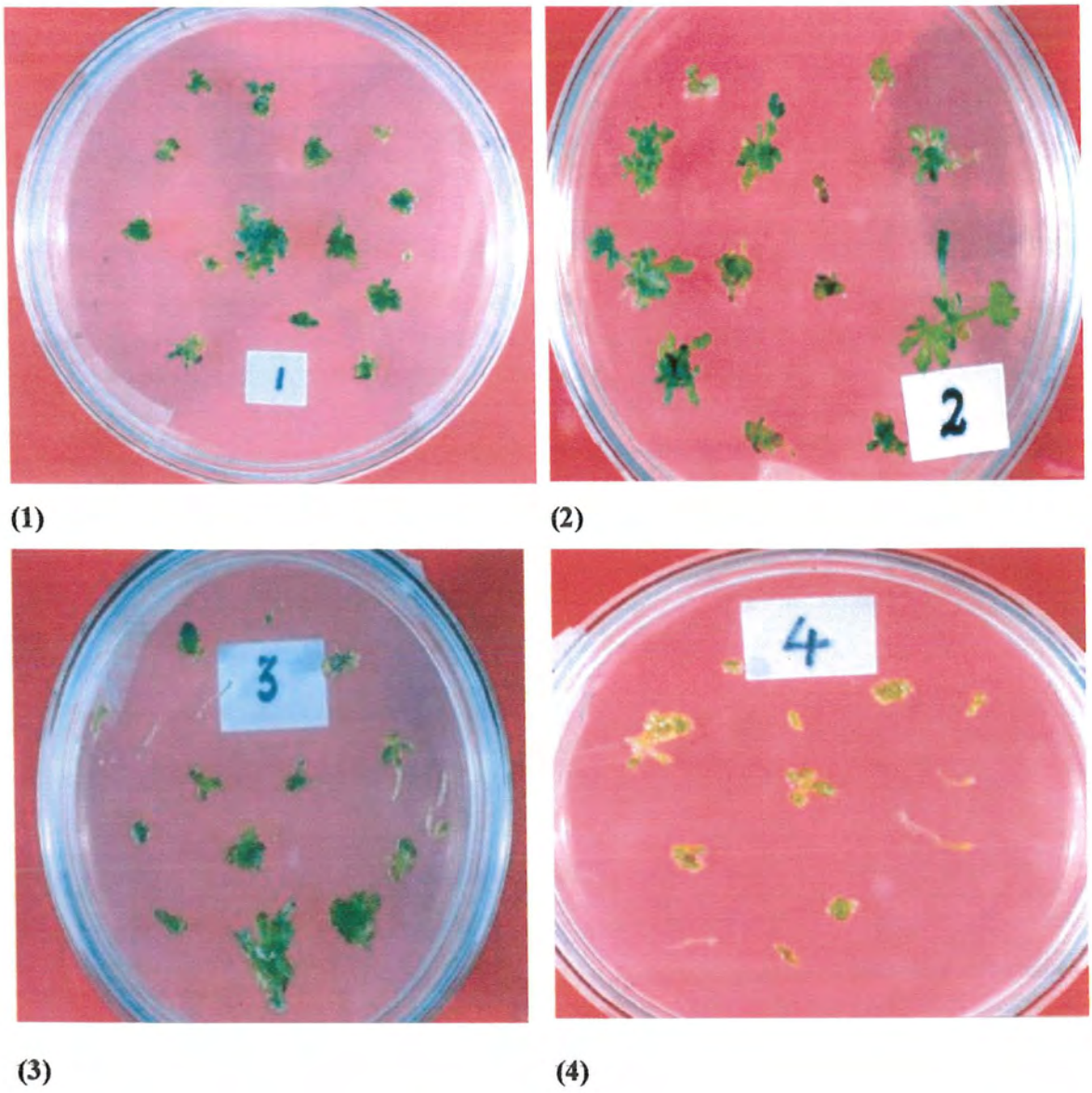


Fig 3.3: Different types of explants (leaves and stems) used for transformation

3.5: Transformation

3.5.1: Effect of Co-cultivation Period on Transformation

Explants from *in vitro* grown seedlings of 20 days (Fig. 3.3) were pre-cultured for one day on co-cultivation medium containing MS with 200μM acetosyringone and 50mg/l kanamycin. After pre-culturing, explants were co-cultivated with bacterial culture and were put on the co-cultivation medium. After two days on co-cultivation medium, explants were shifted to the selection regeneration media. Leaf and stem explants were incubated for 3, 5, 10 and 15 minutes in *Agrobacterium* solution as shown in Fig 3.4. (70%) regeneration response was observed in explants incubated for 5 minutes. Further increase in the length of incubation decreased the transformation frequency as shown in Figure 3.5.



(1) (2)

(3) (4)

Fig 3.4: Regeneration response of explants on selection media after incubation in Agrobacterium solution for various durations.

1- 3 min
2- 5 min
3- 10 min
4- 15 min

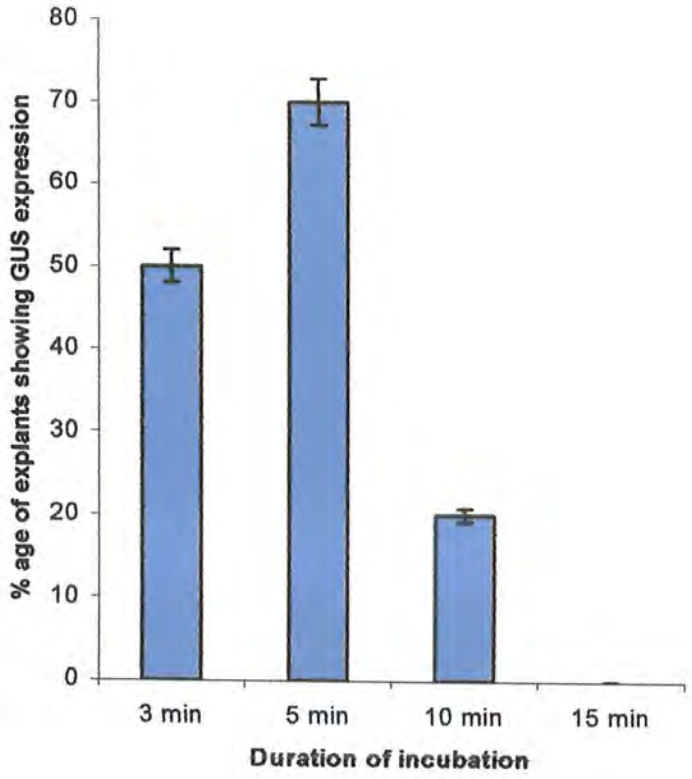


Fig 3.5: Effect of incubation period in *Agrobacterium* solution on regeneration efficiency.

3.5.2: REGENERATION

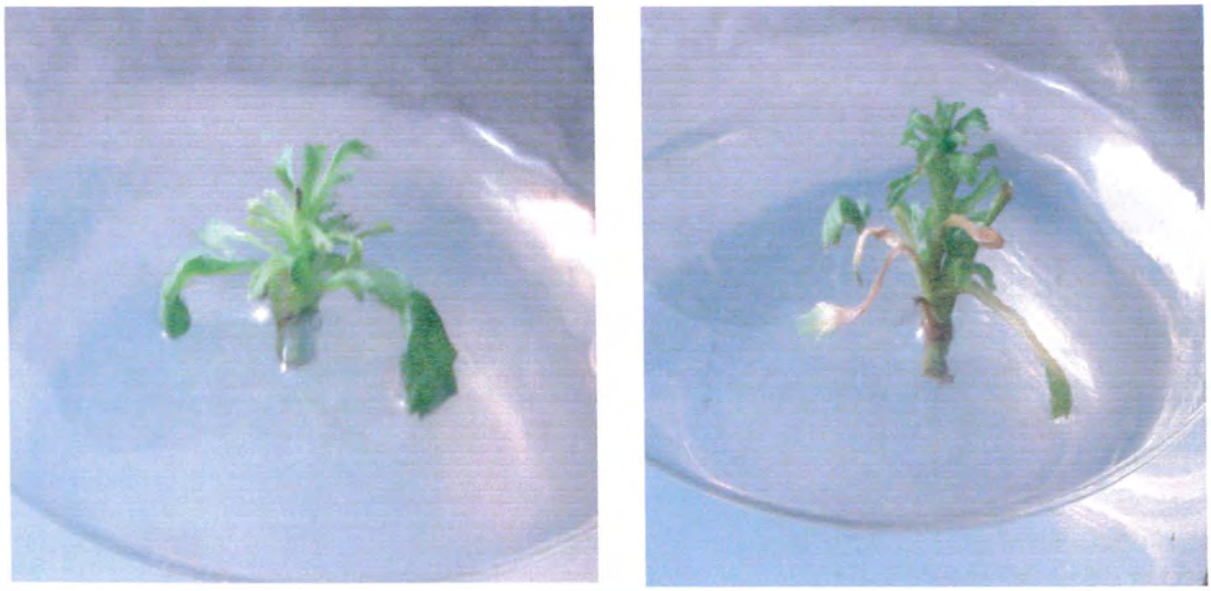
3.5.2.1: Effect of Antibiotics on *Agrobacterium* in Regeneration Medium

To control *Agrobacterium* growth in selective regeneration medium after transformation cefotaxime 500 mg/L was used. Cefotaxime (Claforan®, Hoechst AG. and Frankfurt, Germany) shows good antimicrobial activity against *Agrobacteria* strains and exhibits an auxin like activity on plant material Vergauwe *et al.* (1996b).

In some experiments *Agrobacterium* growth occurred again when regenerated transformed shoots were transferred to fresh regeneration medium with out cefotaxime after 4 weeks and these regenerated shoots died due to over growth of *Agrobacterium*. Therefore, in other experiments cefotaxime was used for two months after their transfer to regeneration medium.

3.5.2.2: Regeneration of Transgenic Plants

Leaf apices, root and hypocotyl explants excised from one week old seedlings were cocultured with *Agrobacterium* on cocultivation medium. After three days these explants were washed with sterile distilled water and then with cefotaxime to remove excess of *Agrobacterium*. Leaf apices were cultured for direct shooting on SRM 2 containing 0.1mg/L BAP supplied with Kanamycin 20mg/L and cefotaxime 500mg/L. After two weeks transgenic shoots were obtained (Fig. 3.6).



(a) (b)

Fig. 3.6: Shooting of transformants from different explants

(a) Leaves (b) Stems

3.5.3: Rooting of Transformed Plants

For rooting, when the transgenic shoots were elongated to 2-4cm, the developed shoots cut off segments were cultured on rooting medium (RM) i.e. half strength MS (Murashige and Skoog, 1962) medium containing 0.025 mg/L NAA. Rooting was quantified on the basis of percentage of shoots showing response for rooting. The rooting response in transformed plants was found to be 50% and has been shown in figure 3.7.

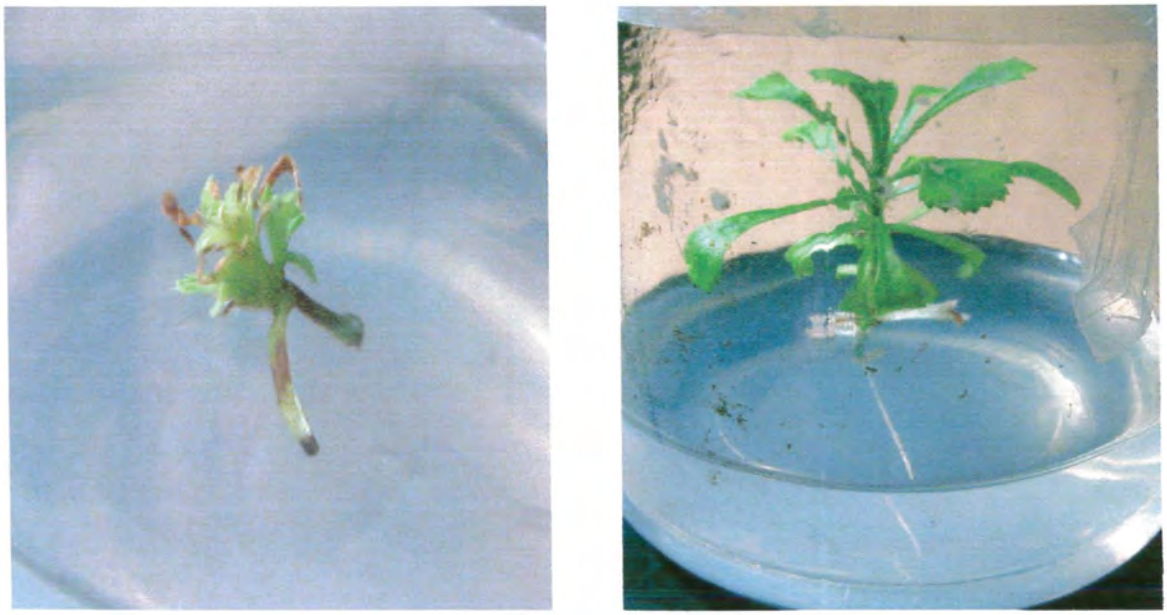
Fig 3.7: Rooting response of transformed plants

3.6: Morphological Analysis of Transformed Plants

Clear morphological differences were found in some of the green house grown transgenic plants as compared to control plants. The morphological differences studied had been listed in table 3.2 and represented in figure 3.8. Transgenic plants were healthier and survived longer as compared to control non-transgenic plants. Marked differences exist in height between control and transgenic plants growing in a greenhouse.

Table 3.2: Morphological differences observed among Transgenic and Control Plants

Morphological characters	Control Plants	Transgenic Plants
Plant height	70cm	87cm
Stem	Straight, unbranched and soft in texture	Branched and hard in texture
Leaves	Small size and narrow	Large size and broad
Inflorescence	Axial, without hairs	Terminal, excessive hairy



(a) (b)

Fig. 3.8: Comparison of Transformed and Non-transformed plants

(a) Non-Transformed plant of *A. dubia* **(b) Transformed plant of A. *dubia***

3.7: Molecular Analysis of Transformed Plants

Molecular analysis of the seedlings transformed by *rol ABC* genes, obtained from tissue culturing was performed.

3.7.1: Genomic DNA Extraction

For PCR analysis, genomic DNA was extracted using CTAB method (cetyl trimethyl ammonium bromide) of Doyle and Doyle, (1990) from the leaves of the transformed plants. DNA extracted through this procedure was run through the Gel (1% agarose) and visualized under U.V transilluminator.

3.7.2: Polymerase Chain Reaction (PCR)

For molecular analysis of transformed plants, the PCR products, after running through gel electrophoresis, were analyzed under U.V illumination. For *rol ABC* genes, the PCR analysis of the extracted DNA was performed for *rol A* (308bp) genes and their amplified product was observed. Amplified product was also obtained from the plasmid DNA of *Agrobacterium* strain. The figure 3.9 shows the amplified product of *rol A* genes.

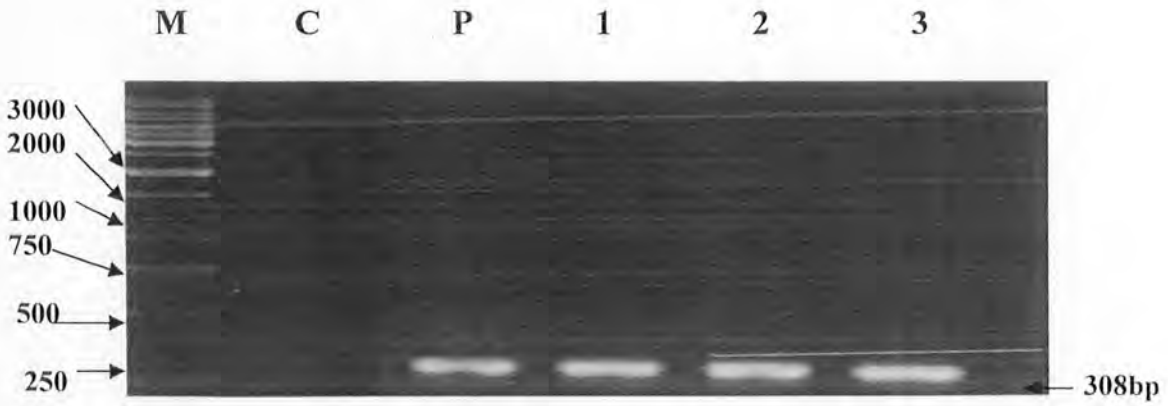


Fig.3.9: PCR analysis showing amplified product of *rol A* gene in transgenic *Artemisia* plant. Lanes 1-3 represent the bands obtained from the DNA of transgenic plants. Lane P represents the band from plasmid DNA. Lane C refers to the non transformed control plants. Lane M corresponds to 1 kbp Ladder (Sigma).

3.8: Acclimatization

Transformed rooted plantlets were shifted to small pots containing a mixture of soil and peat moss. Plants were then acclimatized in larger pots in growth chamber at 25 ±2°C, 16 h of photoperiod, illumination of 45 μE m^{-2} s^{-1} and 60 % relative humidity before transfer to the harsher conditions of the green house environment. Out of 15 seedlings shifted to pots, only 8-10 seedlings were able to survive the growth room conditions, so the percentage of seedlings acclimatized to the outer environment was 53-66%. Rest of the seedlings could not withstand the environment of the soil in the growth room (Fig: 3.10).

Fig.3.10: Transformed plant in green house

3.9: Growth of transformed roots on solid B5 medium

After transformation the roots were transferred to solid B5 medium for further root proliferation. The roots were harvested after ten days interval from each explants in triplicate and analyzed for biomass production.

There is large variation in biomass production of fresh roots from day 10 to day 20; the fresh weight from day 10 to days 20 fluctuates from 0.75 g to 3.31 g showing rapid multiplication of roots. In this time period average biomass increase was 2.4±0.46. Untransformed roots of *Artemisia dubia* attained fresh weight 1.9 g. From day 20 to day 30, increase in fresh weight was from 3.1 g to 4.16 g with average increase in 0.9±0.42 in transformed roots. It can be stated that during this time period the root cultures entered in stationary phase and production of biomass was less. In control production of biomass was much less as compared to transformed roots. After 30 days maximum fresh weight (4.16g) was attained as shown in figure 3.11, *Artemisia dubia* attained 3.5g fresh root weight as control (Fig. 3.11). These roots were dried in vacuum oven at 60°c for three days, than dry weight of these roots was taken. The dry weight of transformed roots was 3.4 g and of untransformed roots was 3.0g (Table. 3.3).

Table. 3.3: Root length, fresh weight (gms) and dry weight (gms) of *Artemisia dubia* transformed with *Agrobacterium tumefaciens* in comparison to control

	Time interval	**Root length(cm)**	**Fresh wt. (gms)**	**Dry wt.(gms)**
Transformed plant A	Day 10	3	1.4	0.5
	Day 20	5.5	2.3	1.0
	Day 30	7	3.3	2.5
Transformed plant B	Day 10	2.5	1.2	0.4
	Day 20	6	2.5	1.3
	Day 30	7.5	3.7	2.3
Control plant	Day 10	1	1.2	0.6
	Day 20	2.5	2.6	1.0
	Day 30	5	3.0	2.4

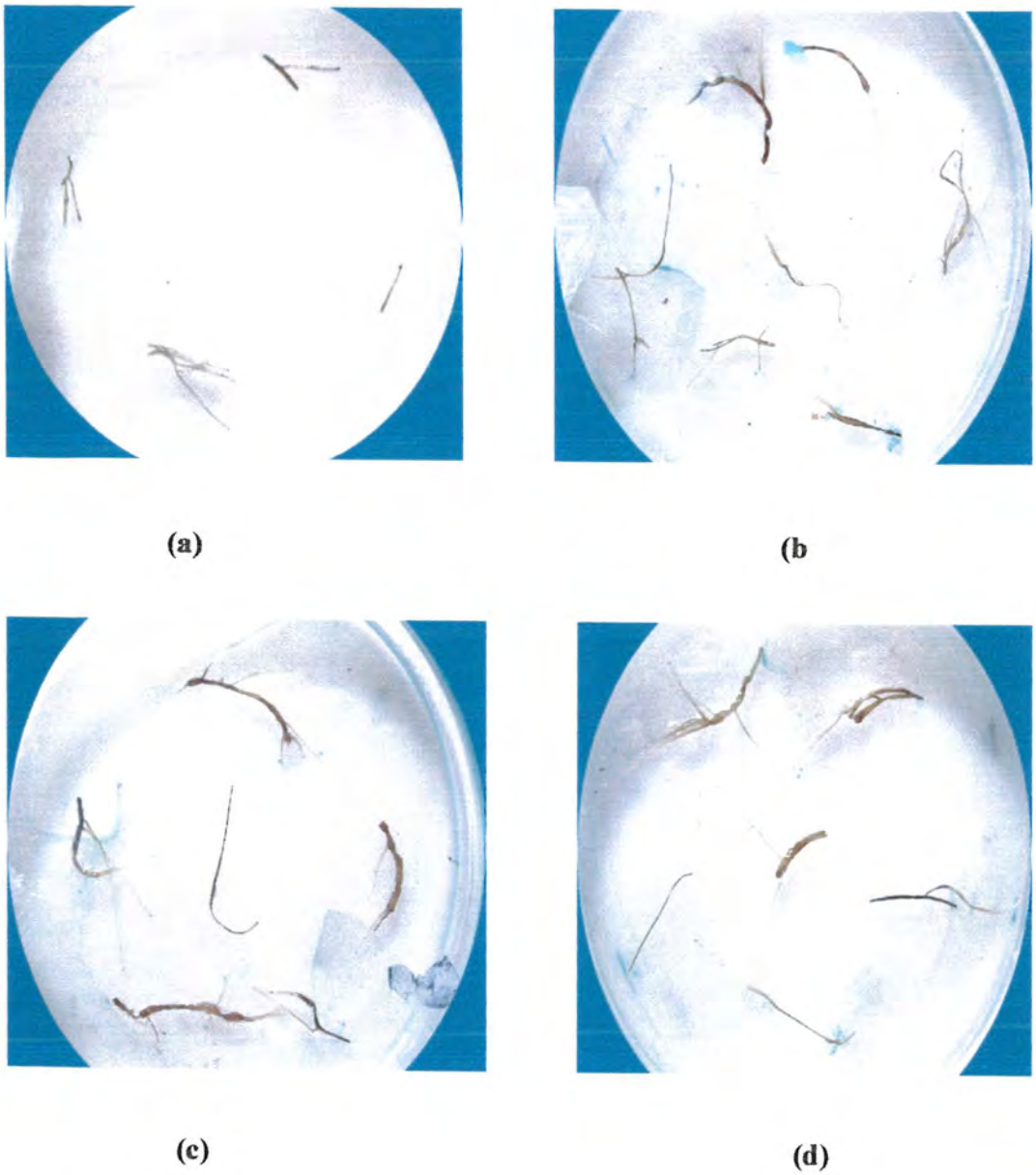
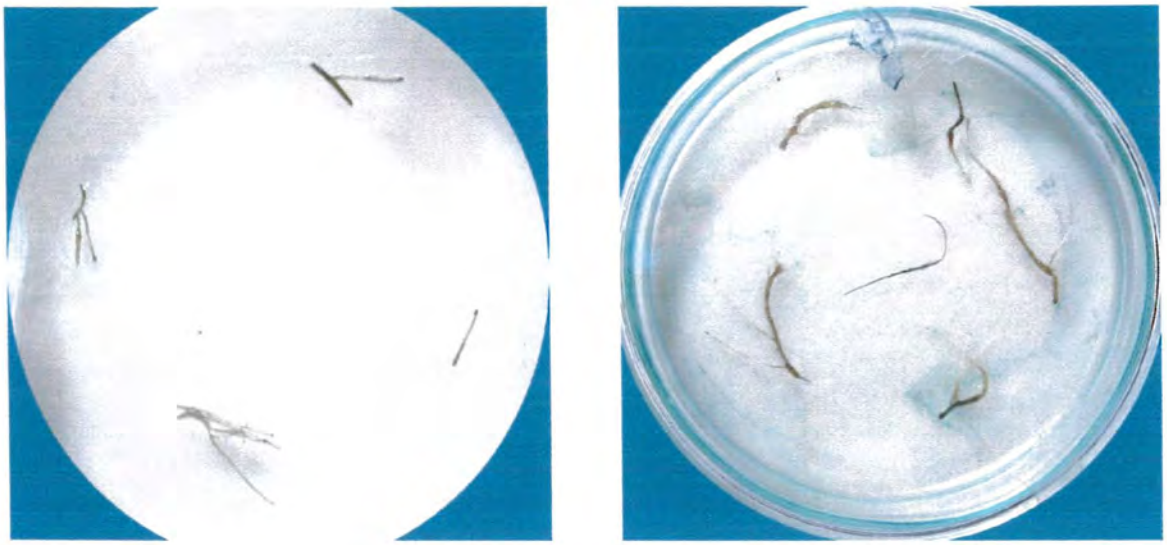


Fig 3.11: Growth of Transformed roots on solid B5 medium

1- First day
2- After 10 days
3- After 20 days
4- After 30 days



(a) At first day **(b) After 30 days**

Fig 3.12: Growth of Control roots on solid B5 medium

3.10: Analysis of Artemisinin content in transformed roots grown on B5 medium

Artemisinin content was analyzed with the help of spectrophotometer in transformed and untransformed roots collected after ten days interval grown on solid B5 medium. As depicted in table 3.4, transformed *Artemisia dubia* showed more production of artemisinin as compared with non-transformed root cultures. The table describes that during the first interval, artemisinin content was less as compared with second interval (from day 20 to day 30). *Artemisia dubia* non-transformed roots produced minimum artemisinin while maximum artemisinin was detected in transformed roots after 30 days when infected with *A. tumefaciens.*

Table 3.4: Comparison of Artemisinin content in Transformed and Non-tansformed *Artemisia dubia* Plants

Plants	Time interval	Absorbance at 218 nm	Artemisinin content (µg/ml)
Transformed Plant A	Day 10	0.03	0.13
	Day 20	0.072	0.32
	Day 30	0.51	2.23
Transformed Plant B	Day 10	0.01	0.04
	Day 20	0.22	0.96
	Day 30	0.60	2.60
Control Plant	Day 10	00	00
	Day 20	0.001	0.004
	Day 30	0.13	0.56

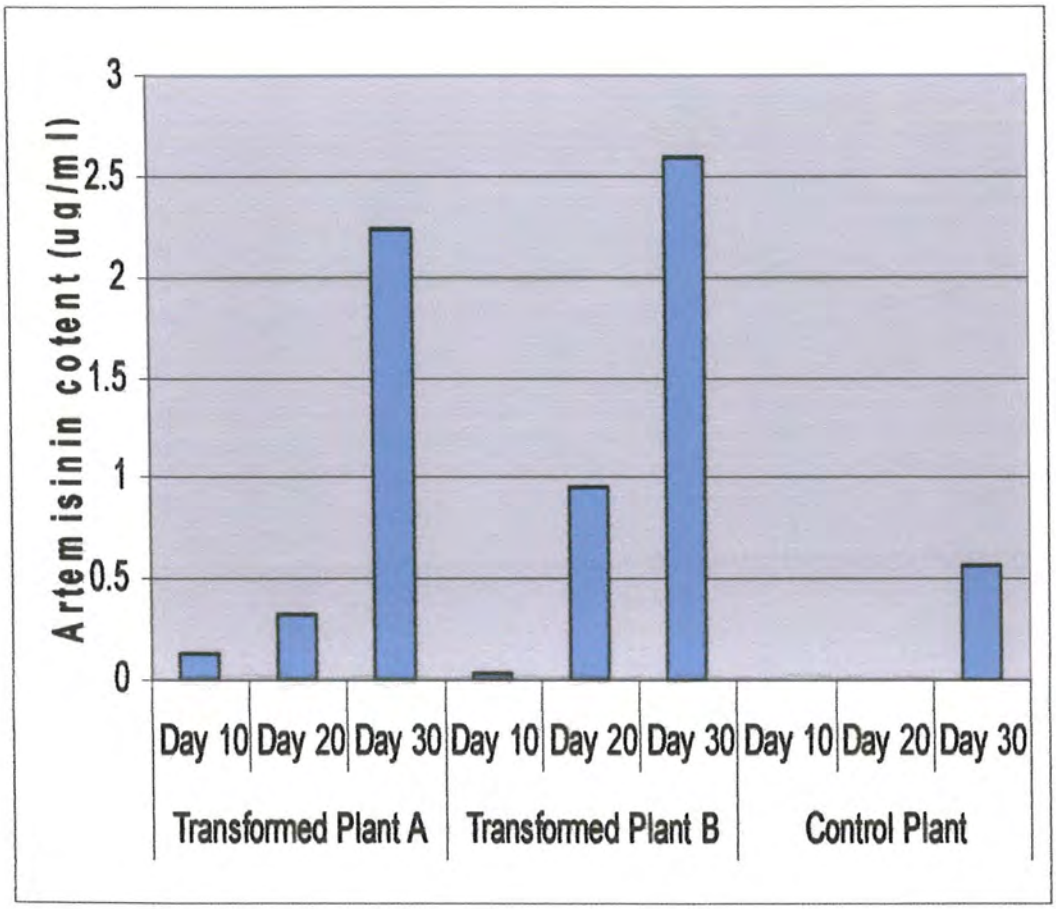


Figure. 3.13: Artemisinin content in transformed and control *Artemisia dubia* plants

DISCUSSION

DISCUSSION

Throughout the history plants have been of great importance to medicine. Plants with medicinal properties have been utilized successfully in the treatment of ailments of varying degrees of severity. The compounds that are responsible for medicinal property of the drugs are usually secondary metabolites (Vormfelde, and Paser, 2000). The use of the medicinal plants for curing disease has been documented in history of all civilizations (Street, 1977). One such plant is *Artemisia dubia* L. belonging to genes *Artemisia*. *Artemisia dubia* L. is an annual, biennial or perennial herb or small to large shrub and was famous in the past for its antihelminthic, insecticidal, stomachic, bitter and aromatic properties (Anderson, 1977; Smith and Secoy, 1988). Over the past decade, novel features of plant cells transformed with the *rol A*, *B* and *C* genes have been revealed, such as increased production of secondary metabolites. The *rol A* gene has emerged as a stimulator of growth and secondary metabolism (Altvorst *et al.*, 1992; Schmulling *et al.*, 2006). The *rol B* protein on the other hand, has been shown to have a tyrosine phosphatase activity and therefore a possible role in the auxin signal transduction pathway (Filippini *et al.*, 2005). Estruch *et al.* (1999) have demonstrated that *rol C* can be involved in the released of active cytokinins from their inactive glucosides due to its cytokinin glucosidase activity and therefore involved in activation of secondary metabolism. Collectively, these genes play a major role in the pathway that leads to high levels of secondary metabolites. Production of transgenic plants seems to be the most appropriate choice to improve production of secondary metabolites of any plant. For this purpose *Agrobacterium* mediated transformation method is being used for several medicinal plants including *Artemisia* species (Nin *et al.*, 1996; Nin *et al.*, 1997; Vergauwe *et al.*, 1998). Therefore, the aim of this project was to compare artemisinin production in the roots of *Artemisia dubia* transformed with *role ABC* genes by using *Agrobacterium tumefaciens* with the non transformed plants.

In this report, successful comparison of artemisinin content from roots of transformed and non transformed plants of *Artemisia dubia* has been achieved. In this

study transformation of *Artemisia dubia* has been carried out using *Agrobacterium tumefaciens* harbouring *rol ABC* genes. This report also covers the effect of different factors, such as type of explants, sterilization period and co-cultivation period, on transformation efficiency.

During *Agrobacterium tumefaciens* mediated transformation; proper sterilization procedure is required for *in vitro* germination of seeds. Mercuric chloride was used as a sterilizing agent. Sterilizing agents play important role in the germination of seeds, long duration had an inhibitory effect on seed germination and the seeds became dead while in short time treatment the percentage germination of seeds increased with minimum germination period. Two minutes treatment was found to be the most suitable duration for the sterilization of seeds (Table 3.1).

Explants from in vitro grown seedlings of 20-days old seedlings were used for the transformation procedure. Explants were precultured on MS medium containing 50 ul acetosyringone. Co-cultivation period affect the transformation efficiency. MS medium (Murashige and Sakoog, 1962) containing 50 µl acetosyringone and 50mg/l kanamycin was used as cocultivation medium and after cocultivation; explants were placed on cocultivation medium for 2-days. Schmid *et al.* (2005) showed that this treatment increases the transformation efficiency up to 20%. It seems that after cocultivation of explants with *Agrobacterium,* a certain period of time prior to the shifting of explants to the selection medium positively influence the transformation efficiency, probably to allow the T-DNA transfer, integration, transcription and sufficient enzyme production leading to the expression of kanamycin resistant phenotypes.

In order to select the suitable types of explants for regeneration, different type of explants were regenerated on media in order to check their regeneration efficiencies. Leaf and stem explants were used on MS medium containing 0.1mg/l BAP, 20mg/l kanamycin and 500mg/l cefotaxime. Maximum regeneration efficiency was observed by stem explants on this medium. So this media was used as selection medium after the cocultivation of explants.

After the infection with *Agrobacterium*, explants were placed on cocultivation medium for 2-days. Then these explants were shifted to selection medium. In selection medium 20mg/l kanamycin was used for the selection of transformed explants as used by Baroncelli *et al.* (1992), Jorsobe *et al.* (2003) and Vatsya *et al.* (2002). In case of explants infected with *Agrobacterium tumefaciens* strain LBA 4404 with plasmid RT99 harbouring *rol ABC* genes, among the two types of explants used, stem explants showed maximum transformation efficiency on this SM (MS medium containing 0.1mg/l BAP, 20mg/l kanamycin and 500mg/l cefotaxime). Leaf explants were not found to be much efficient in generating transgenic shoots and most of these died on the selection medium. Thus it appears that leaf explants are not much worth in carrying out transformation experiments. This increased transformation efficiency of stem explants as compared to leaf explants has also been reported by Skoog and Miller (1997), Robertson and Earle (2000), Murata (1998), Gamborg *et al.* (2006) and Bidney *et al.* (2007).

For the selection of transformed explants 20mg/L kanamycin and 500µg/l cefotaxime were used in the selection media. Various reports (Davioud *et al.*, 1988, Maurel *et al.*, 1991, Morgan *et al.*, 2004) have shown that 20mg/l kanamycin is sufficient for the selection of transformed plants. The control of *Agrobacterium* growth at 500µg/l of cefotaxime has been shown by Vatsya *et al.* (2002), Jorsobe *et al.* (2003), Bettini *et al.* (2003), and Sambrook *et al.* (2006).

For determination of best explants for shoot regeneration, we have tried different type of explants. Considering the different kinds of explants, the use of stem explants seems best for the formation of shoots as well as production of *Artemisia dubia* plants. Shoot regeneration response observed on MS medium containing BAP 0.1mg/L, 20mg/l Kanamycin and 500µg/l cefotaxime with stem explants was 80-100%. Shoot regeneration response of leaf explants was not good as the leaves mostly formed calli on this medium. It is in accordance with Schmid *et al,* (2005) who worked on *A. dubia* and reported that within three weeks all stem explants of *A. dubia* developed shoots. However the shoot regeneration continued further and one explants led to the formation of clusters of several

shoots, so that the number of regenerated shoots was higher than the original number of leaf explants (Schmid *et al.*, 2005). Nair *et al.* (2001) reported the regeneration of *Artemisia* plants starting from leaf explants by using a hormone combination of the auxin NAA (0.05) mg/L and the cytokinin BAP (0.1 to 0.2 mg/L). In about 60 days distinct shoots were obtained in this procedure, but the rooting of the shoots took 3 months, suggesting that an optimum concentration of hormone in the regeneration medium highly effects the time of regeneration of explants. The BAP (0.1mg/L) was found to be best for regeneration of shoots.

Once the transformed shoots were obtained, they were rooted on rooting medium. It occurred after using 0.025mg/L NAA in the rooting medium, this is in contrary to Vergauwe *et al.* (1998), who while working on the *Agrobacterium tumefaciens*-mediated transformation of *A. annua* reported high efficiencies of root induction of shoot grown on non-selective medium. Their study suggested that cocultivation of the explants with *Agrobacterium tumefaciens* did not affect rooting of regenerated shoots. Whereas we have faced great difficulty as both the transformed and untransformed shoots were not giving roots on the proposed medium of Vergauwe *et al.* (1998) that is 0.1mg/L NAA. We got rooting within 1-2 weeks in transformed and untransformed shooted explants by culturing them on rooting medium containing 0.025mg/L which is in accordance with the results of Nair *et al.* 2001 report that culture of young leaf segments on either modified MS or B5 medium with IBA or NAA (0.05-2.0mg/l) resulted in the development of roots in 6-14 days, mostly from proximal part of the midrib with best results of 0.1mg/L of NAA. According to our results the number of roots developed on transformed shoots was restricted whereas Nin *et al.*, (2004), while working on transformed *Artemisia annua in vitro* grown plants exhibited a more developed rooting system and roots appeared on the stem too. These differences could be due to the difference in the specie or more pronouncedly it is because they had used *Agrobacterium rhizogenes* for infection and as we know that it is a natural plant pathogen responsible for adventitious root formation at the site of infection (Hooykaas, 2004). While we have used *Agrobacterium tumefaciens*, which is a soil bacterium and causes crown gall disease (Brown, 2001).

Other factor studied that could affect the transformation efficiency was cocultivation period. Time of incubation of explants with *Agrobacterium* inoculums is an important factor affecting the transformation efficiency. An incubation time of five minutes was found to be best giving 70% transformation efficiency. Increase in incubation time led to decrease in transformation efficiency because of over growth of *Agrobacterium* that hinders the growth of the transformant and hence has a negative effect on transformation efficiency. Transformation efficiency is also influenced by cocultivation time. Explants were cocultivated for two or three days. The highest formation of transgenic plants was generally obtained after two days of cocultivation. All the leaf and stem explants showed a positive gene expression it is in agreement with the results of Vergauwe *et al.* 1998 as he did cocultivation of *Aretemisia annua* for 48 to 60 hours according to his findings longer cocultivation periods do not help the transformation efficiency it is also because of over growth of *Agrobacterium* as mentioned earlier.

For successful *Agrobacterium* transformation, elimination of bacteria from culture soon after transformed is necessary. This is achieved by the addition of antibiotics into the culture medium. Antibiotics, which are commonly used to eliminate *Agrobacterium tumefaciens* from plant tissues, have been shown to influence the morphogenesis either positively or negatively (Jun-Li *et al.*, 2005). Different doses of cefotaxime were used to optimize the standard dose which can control the overgrowth of Agrobacterium and also do not inhibit the regeneration. Cefotaxime at a concentration of 500mg/L controlled overgrowth of bacterium while doses higher than 500mg/L resulted in necrosis and hence no regeneration was observed. Even though this concentration of cefotaxime retarded the callus formation, but regenerated plants were able to grow successfully. This is an agreement with Vergauwe *et al*, (1996b), who used cefotaxime as decontaminating antibiotic and reported that it results in retardation of callus formation and inhibition of the shoot inducing capacity in *Artemisia annua*.

We have reported the some morphological differences that existed among transgenic and non-transgenic plants. Not all but morphological variations exist in some of the transgenic plants. Increased plant height, broad leaves, branched and hard stem are some of the features observed among transgenic plants. Such features were not found in control plants. Some of these changes were caused by *rol* genes such as increased plant height, broad leaves have been reported by Mercke *et al.* (2000) and Wallaart *et al.* (2001). These morphological changes could be because of the hormonal imbalance caused by the *rol* genes. The *rol* genes have been reported to cause functional imbalance in the auxin/cytoknin ratio in favor of cytokinins (Schmulling *et al.*, 2006).

Regenerated plantlets were potted in the greenhouse, and their survival rate was 98%, which is in accordance with the findings of Fazal *et al.* (2007) they acclimatized the *Artemisia absinthium* and 95% of the plantlets were successfully transplanted to soil and continued to grow in the field.

Agrbacterium mediated transformation is an effective and widely used approach to introduce foreign DNA into dicotyledons plants. For different plant species different gene transfer protocol are applicable. Various researchers have reported transformation of *Artemisia* species with *Agrobacterium* but mostly they transformed *Artemisia annua.* (Biswajit *et al.*, 2000; Vergauwe *et al.*, 1998; Vergauwe *et al.*, 2002, Vergauwe *et al.*, 2005). Little work has been done on *Artemisia dubia* (Jun-Li *et al.*, 2005, Jin *et al.*, 1987, Kaul *et al.*, 1976., Kovacs *et al.*, 1994,; Zafar *et al.*, 1990; Nin *et al.*, 1976; 1996; 1997; Rizwana *et al.*, 2002, Altvorst et al., 1992, Charles *et al.*, 1990, Davioud *et al.*, 1988, Giri and Narasu, 2000) Before the transformation protocol established by Vergauwe *et al.* (1996b) no successful procedure had been developed to regenerate Artemisia plants in short time. Nin *et al.* (1997) transformed the *Artemisia dubia* with *Agrobacterium rhizogenes* and obtained transformed regenerated plants and in 1998, Vergauwe studied some more important factors that can affect the transformation efficiency. The results from these studies were combined to produce an enhanced protocol for the transformation of *Artemisia dubia* L. The protocol includes incubation of the explants for five minutes in

Agrobacterium solution, containing and 50mg/L kanamycin having a pH of 5.8 and an optical density of 1 at 560nm, followed by a three days cocultivation period on MS medium supplied with 50 µl acetosyringone and 50mg/l kanamycin, and then transfer of the explants to selective regeneration medium containing 0.1 mg/L BAP, 50mg/l cefotaxime and 20 mg/L kanamycin. Rooting was obtained on half MS medium containing 0.025mg/L NAA.

After transformation the transformed and non-transformed roots were shifted to solid B5 medium for further proliferation, In the present investigation the transformed roots grow faster as compared to non transformed roots. Increase in fresh weight of roots in initial days is very fast and after word it slows down and enters into stationary phase. Present investigation also reveals that from day ten to day twenty, increase of biomass was higher while in next ten days the biomass production was less. In this time period growth of roots either transformed or untransformed was in log phase after that it entered into stationary phase. Transformation *of A.dubia* also affects production of artemisinin in as transformed plants produced more artemisinin in the roots as compared with non-transformed plants. The results also describe that artemisinin production was less in the first time of investigation (after ten days of culturing the roots on solid B5 medium) while it enhanced in the second (after twenty days) and third time (after thirty days) of investigation. Artemisinin could not be significantly detected in control roots but in transformed roots production of artemisinin was maximum at the day thirty of roots proliferation in *Artemisia dubia*. Norma and Ana Maria (1996) also reported that hairy root culture can significantly increase 2.5-3 fold the secondary metabolites in comparison with control and also reported that transformed and non-transformed roots exhibit a strong tendency towards proliferation in liquid B5 medium. Jaziri *et al.* (1995) found 0.001% artemisinin in hairy roots of *Artemisia annua* transformed with *A. rhizogenes* strain NCIB 8196. Whereas Weather *et al.* (1994; 1997) reported 0.42% DW artemisinin in hairy roots culture of *Artemisia annua*. Artemisinin detection method also interfere concentration of artemisinin in the sample. Detection methods, in the present study was by spectrophotometric examination while others detect artemisinin by HPLC or GC, that

are more sophisticated method of analysis. During spectrophotometric analysis other metabolites either derivatives of artemisinin as artemether, different forms of artemisinin and other metabolites may change the absorbance value. But rough estimation may be conducted by spectrophotometric analysis at specific wavelength.

Production of artemisinin was lower at initial stages of root proliferation in transformed roots but as the time passes it increases sufficiently. So synthesis of artemisinin is not dependent upon days of proliferation but it increases as the roots grow further. Wilson *et al.* (1987) stated that production of secondary metabolites in vitro can occur at almost any stage of culture growth. For example, nicotine concentration in hairy roots of *Nicotiana rustica* grown in shake flasks remains constant throughout the growth phase, and production is, therefore, growth-related. The production of tabersonine and ajmalicine by *Catharanthus roseus* hairy root cultures grown in the dark is also at maximum during the exponential phase (Bhadra and Shanks, 1997). Tabersonine derivatives, lochnericine and horhammericine, are also growth-associated and show linear relationships between the total yields (not specific yields) and biomass production (Bhadra *et al.*, 1998). On the other hand, the ajmalicine derivative, serpentine, was not growth-associated and exhibited the highest specific yield during the stationary phase (Bhadra and Shanks, 1997).

Both control and transformed plants had biomass production with marginal difference while the difference in artemisinin was varying. Control roots also showed growth in biomass but they failed to produces artemisinin. It is stated that specific plastids present in the apical and sub apical cells of capitate glandular trichomes of *Artemisia annua* have been proposed as the site of artemisinin synthesis (Duke and Paul, 1993).

Transformed roots grown on solid B5 medium produced artemisinin in different concentrations. Nutrients in the culture medium can be easily manipulated and have a great impact on secondary metabolite production. Different authors reported that alteration in culture media may vary production of secondary metabolites in transformed roots. For example, ammonium ion altered the secretion pattern of products from

transformed roots of *Lithospermum erythrorhizon*. In ammonium-free medium, *L. erythrorhizon* normally accumulates shikonin on the surface of roots and in border cells. When ammonium ion is present, however, hydroxyechinifurn B (Fukui et al., 1998) and rhizonone (Fukui et al., 1999) were secreted into the medium instead. Mannan *et al.*, (2007) also reported that transformed hairy roots grown on MS and half MS liquid medium produced more artemisinin as nutrients in the culture medium have great impact on secondary metabolites.

Other investigations also states that transformed roots are fast growing, and produce the secondary metabolites characteristic of normal roots at levels similar to or even exceeding those of normal roots. The transformed roots of *Artemisia dubia* can make a significance contribution to our understanding of secondary metabolism. The ability of transformed roots to produce similar or higher levels of the characteristic metabolites from the original plant is stable during subculture periods (Pitta-Alvarez and Giulietti, 2001).

Conclusion

This research project reports the successful transformation of *Artemisia dubia* with *Agrobacterium tumefaciens* strain LBA4404 harbouring *rol* ABC genes. The transformed plants obtained were shifted to green house and analyzed for their morphological characters. Artemisinin content was also evaluated in transformed and non-transformed plants. The transformed plants produced maximum artemisinin content in comparison to control.

Future Perspective

Future study involves the Southern Blotting of the *Artemisia dubia* transformed with *Agrobacterium tumefaciens*. The transformation of *Artemisia dubia* with *Agrobacterium rhizogens* will be carried out and the artemisinin content produced in these transformed roots will be compared by HPLC with the roots produced by *Agrobacterium tumefaciens* in *Artemisia dubia* plants.

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APPENDIX-I

YMB medium

K_2HPO_4	0.5mg/l
$MgSO_4$	2g/l
NaCl	0.1g/l
Manntol	10g/l
Yeast extract	10g/l
pH	7.0

APPENDIX-II

MYA Medium

Yeast extract	5g/l
Cas-amino acid	0.5mg/l
Mannitol	8g/l
$NH_4(SO_4)_2$	2g/l
NaCl	5g/l
pH	6.6

APPENDIX-III

Composition of Murashige and Skoog (1962) medium

S. No	Constituents	Formula	Conc. in stock solutions g/l	Volume of stock/l of medium (ml)
Macronutrients				
1	Potassium nitrate	KNO_3	38	50
2	Ammonium nitrate	NH_4NO_3	33	
3	Calcium chloride	$CaCl_2.2H_2O$	8.8	
4	Magnesium sulphate	$MgSO_4.7H_2O$	7.4	
5	Potassium Phosphate	KH_2PO_4	3.4	
Micronutrients				
6	Manganese sulphate	$MnSO_4.H_2O$	4.4	5
7	Zinc sulphate	$ZnSO_4.H_2O$	1.72	
8	Boric acid	H_3BO_3	1.24	
9	Potassium iodide	KI	1.67	
10	Sodium molybdate	$Na_2MoO_4.2H_2O$	0.05	
11	Copper Sulphate	$CuSO_4.2H_2O$	0.01	
12	Cobalt chloride	$CoCl_2.6H_2O$	0.005	
Iron Source				
13	Sodium EDTA	$Na_2EDTA.2H_2O$	7.46	5
14	Ferrous sulphate	$FeSO_4.7H_2O$	5.56	
Organic Supplements (Vitamins)				
15	Myo-inositol		20	5
16	Glycine		0.4	
17	Nicotinic acid		0.1	
18	Pyridoxine-HCl		0.1	