mitosis may account for irregular chromosome distribution in somatic cells. This phenomenon of chromosomal inconstancy (mosaicism) has been reported in a number of vegetatively propagated monocotyledons[16]. Mosaicism is often associated with the occurrence of polyploidy, hybridity and chemical treatment. However, these variations have not resulted in any appreciable phenotypic variation. Some of the gametes behave normally where the complements are balanced, as is often the case in polyploids. Union of these gametes can produce considerable variation in chromosome numbers in the next generation.

Pollen germination and pollen tube growth are prerequisites for fertilization and seed development. An earlier attempt on *in vitro* germination[7] in *B. vulgaris* proved futile. However, the present investigation has shown that at least a very small percentage of pollen grains in this species is viable.

It is pertinent to note that the stigma in *B. vulgaris* is dry and no pollen was found on it under natural conditions[8]. From this, it can be inferred that there are some physical barriers adversely affecting pollination in this species. The inability of the stigma to get exposed or even if exposed, the stigmatic portion being hidden by the staminal filaments and the role of bristle-like hairs on palea preventing the pollen to fall on the stigma, could be reasons for the absence of natural pollination, as reported earlier[6]. The percentage of *in vivo* pollen germination though low, was very significant. In spite of germination, the pollen tube did not find its way into the style, to effect fertilization. This appears to be the result of self-incompatibility. Self-incompatibility can be confirmed only when pollen grains of a different clone are available for effective cross-pollination[17].

Factors responsible for failure of seed set in *B. vulgaris* seem to be manifold. High rate of pollen sterility, absence of natural pollination and inhibition of pollen tubes in the stigmatic papillae act as cumulative factors for lack of seed set in this species.

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Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua*

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Different strains of *Agrobacterium rhizogenes*, viz. A_4, 15834, K_{599}, LBA 9402, 9365 and 9340, were evaluated for induction of transformed hairy roots in *Artemisia annua* using shoot-tip meristem explants. Incorporation of acetosyringone in bacterial culture and co-cultivation medium increased the frequency of hairy root induction in *A. annua*. Different strains of *A. rhizogenes* varied in their virulence for induction of hairy roots. Growth kinetics of transgenic hairy roots induced by different strains indicated a similar pattern of growth, with maximum growth occurring between 14 and 16 days. Transformed hairy root cultures showed significant differences in artemisinin content. A hairy root line induced by strain 9365 was found to contain highest amount of artemisinin (0.23%). Artemisinin content of different hairy root lines was also found to be growth-related.

MALARIA ranks among the most prevalent and severe infectious diseases of the tropics and has been responsible for

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over 2.7 million deaths every year[1]. Artemisinin has proved to be one of the most promising drugs. It has also shown to possess considerable antimicrobial and antifungal activities[2]. Artemisinin, a sesquiterpene lactone endoperoxide, is produced by aerial parts of *Artemisia annua* L. (Asteraceae). It has been used in Chinese traditional medicine for centuries to treat chills and fever. Chemical synthesis of artemisinin has proved to be complex and uneconomical. Currently, the leaves and flowers of *A. annua* form the only source of this drug. Artemisinin is found in very low quantities (0.05–1.1%) in different cultivars of *Artemisia annua*[3]. 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[1].

The soil-borne plant pathogen *Agrobacterium rhizogenes* responsible for adventitious (hairy) root formation at the site of infection also causes certain biochemical changes in the plant metabolism[4]. Hairy root cultures have several properties that have promoted their use for plant biotechnological applications. Their fast growth and genetic and biosynthetic stability offer an additional advantage for their use as an alternative to plant cell suspension cultures, for production of secondary metabolites of interest[5,6]. A few studies have been carried out for the induction of hairy roots in *A. annua* and varying levels of artemisinin (0.001–0.45%) were reported in cultures[7–10]. Production of antimalarial compound from transgenic hairy roots of the related species, *A. absinthum*, has also been reported. However, the potent drug artemisinin could not be produced in these transformants[11]. A systematic study using different strains of *A. rhizogenes* for the evaluation of transformation frequency, growth and sesquiterpene production from hairy roots has not been carried out till date. In the present study, we have examined the possibility of generating high artemisinin-yielding hairy root cultures of *A. annua* using different strains of *A. rhizogenes*. We have also studied the influence of acetosyringone on frequency of transformation and hairy root induction.

Seeds of *A. annua* obtained from Elsa Cappetteti, Orto Botanico, Italy, were sown and plants grown to maturity in field. Shoot tips of two-month-old field-grown *A. annua* plants were surface sterilized with 0.1% (w/v) mercuric chloride for 2 min, rinsed thoroughly with sterile distilled water 7–8 times and cultured on MS medium[12].

A. rhizogenes strains, viz. A_4, 15834, K_{599}, LBA 9402, 9365 and 9340, were used for induction of hairy roots in the present study. The cultures were maintained on nutrient agar and were subsequently cultured in nutrient broth (Hi media) for use in transformation experiments.

Leaves, petiole sections and shoot tips from *in vitro* grown *A. annua* shoot cultures were excised and used for transformation. Actively growing bacterial cultures of different *A. rhizogenes* strains on nutrient broth (cell density was adjusted to 5×10^9 cells/ml) with and without acetosyringone (50 μM) were used for infecting the *A. annua* explants by incubating them for 20 min. After infection, the shoot tips were transferred to hormone-free MS basal medium and also MS basal medium supplemented with acetosyringone (50 μM) for co-cultivation. After 48 h, the shoot tips were transferred to MS basal medium containing cefotaxime (300 μg/ml) to eliminate the excess bacterial growth, and the resultant axenic cultures of hairy roots were later inoculated into different strengths (full, half, one-fourth) of MS liquid medium (50 ml in 250 ml flask). These hairy root cultures were placed on a gyratory shaker at 120 rpm at 25 ± 2°C under continuous light (cool white fluorescent light, 35 μmol m^{-2} s^{-2}) and subcultured at a four-week interval. The growth and development of hairy roots were studied during a 4-week period. Hairy roots were cultured initially on full, half and one-fourth strength of MS media to evaluate the optimal medium.

To determine the growth rate of the transformed cultures, root tips 2 cm long were excised from 8-day-old cultures and an inoculum size of 50 mg fresh weight was inoculated into 50 ml of one-fourth strength MS liquid medium supplemented with 3% sucrose in 250 ml flask in triplicates. Growth rate was determined by checking the fresh weight of hairy roots every fourth day up to 25 days. Untransformed control roots obtained from shoot tip explants on MS basal medium were also grown similarly.

Control and all the transformed root lines were tested for the presence of opines. Opine analysis was done by paper electrophoresis, according to the protocol reported earlier[13,14]. The contents of the flasks were harvested, blotted-dry and weighed. Transformed and control root tissues were dried, ground and extracted with hexane in a soxhlet apparatus at 60°C for 8 h. The extract was concentrated by evaporation. The extract was redissolved in analytical grade methanol prior to analysis.

Artemisinin produced was quantified by HPLC[15]. HPLC was performed on Shimadzu – LC-10AT VP Series using a Supelco column (250 × 4.6 mm, C18, ODS with particle size of 5 μm) with a mobile phase of 1% TFA in water : acetonitrile (30 : 70), at a flow rate of 1 ml/min. Artemisinin was monitored at 220 nm with an UV-VIS detector (Shimadzu UV-Visible SPD-LC 10A VP Series). The retention time for artemisinin was between 5.9 and 6.0 min.

All the strains of *A. rhizogenes*, except K_{599}, produced hairy roots at the site of infection of explants, when cultured on MS basal medium. Transformed nature of hairy root lines was confirmed by the presence of agropine and mannopine. The number of roots produced at the site of infection after a specific time span of *in vitro* culture (7–8 weeks) has been used as measure of virulence. Efficiency of transformation is known to differ with different bacterial strains[16,17]. Certain phenolic compounds, e.g. acetosyrin-

Table 1. Performance of different strains of *Agrobacterium rhizogenes* on induction of hairy roots

Bacterial strain	Days for induction of hairy roots		Frequency of transformation (%)	Number of hairy roots after six weeks of culture
	With acetosyringone	Without acetosyringone		
LBA 9402	6–8	14–15	100	25
9365	5–8	14–15	95	24
9340	5–9	16–18	95	18
15834	10–15	15–20	80	22
A_4	8–10	15–20	75	16

A minimum of 15 explants was used for the transformation experiment.

Figure 1. Morphological characteristics of hairy roots of *Artemisia annua* induced by different strains of *Agrobacterium rhizogenes* in *A. annua* after 6 weeks of culture. ***a***, 9365; ***b***, 15834; ***c***, A_4; ***d***, 9340; ***e***, non-transformed control roots; ***f***, LBA 9402.

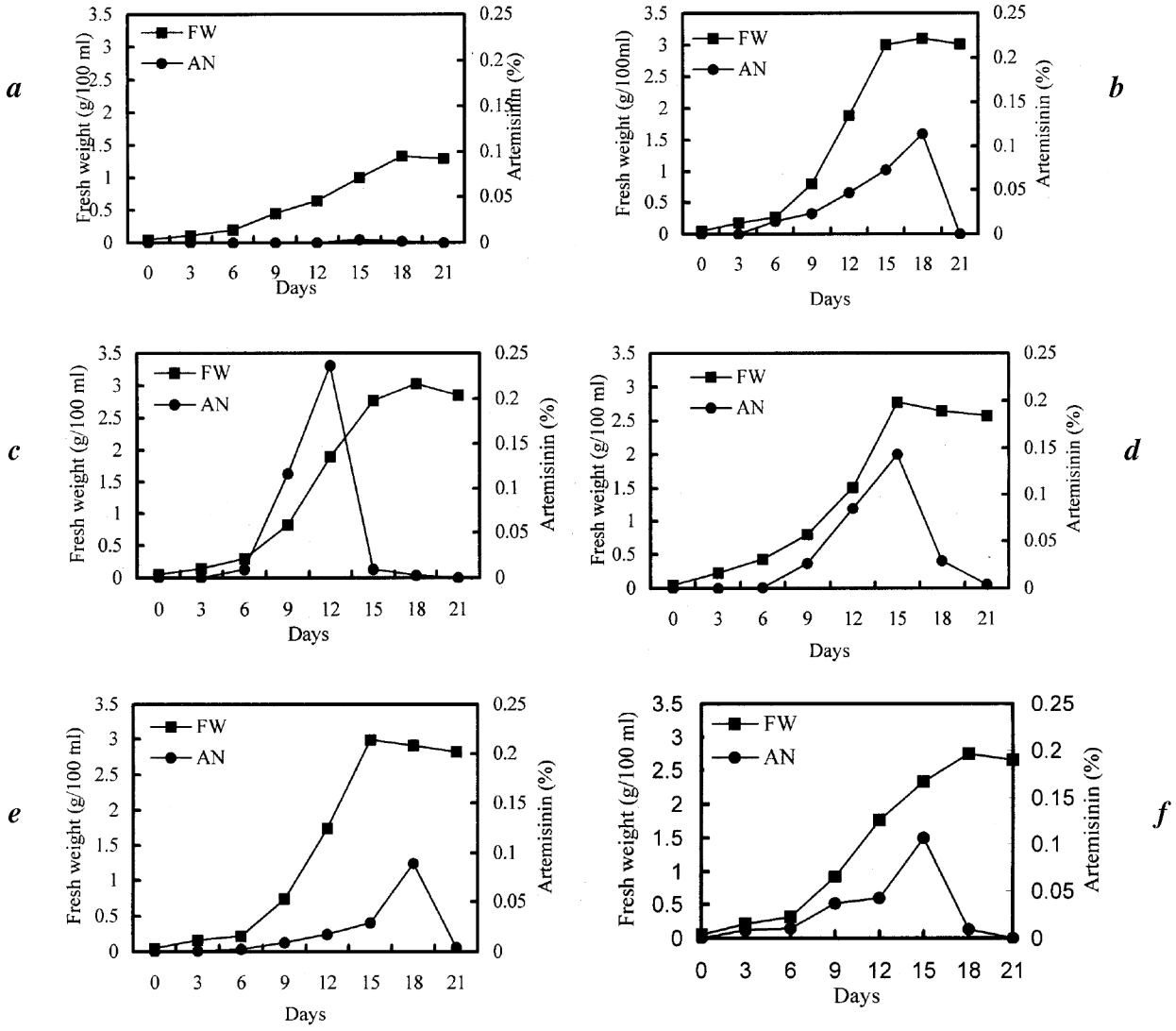

Figure 2. Relationship between growth and artemisinin production from control and transformed hairy root lines of *Artemisia annua* using different strains of *Agrobacterium rhizogenes*. ***a***, control; ***b***, 15834; ***c***, 9365; ***d***, LBA 9402; ***e***, 9340; ***f***, A_4; FW, fresh weight; AN, artemisinin.

gone, α-hydroxy acetosyringone, etc. are known to induce transcription of *vir* region[18]. Acetosyringone was found to radically affect the relative virulence of different bacterial strains. For all the strains, it reduced the time for induction of hairy roots. Bacterial cultures induced with acetosyringone and explants co-cultivated on MS basal medium with acetosyringone reduced the time of hairy root induction by a week, compared to explants infected with *A. rhizogenes* without *vir* gene induction.

The best root formation response as transformation frequency was achieved with the *A. rhizogenes* strains was in the following order: LBA 9402, 9340, 9365, 15834 and A_4. The transformation ability of different *A. rhizogenes* strains was found to be different (Table 1). Roots also showed difference in their morphology (Figure 1 *a–f*). Growth of transformed culture was checked for hairy roots in different strengths of MS medium supplemented with 3% sucrose. The fastest growth was obtained on one-fourth strength MS medium. All the roots turned green when transferred to light, indicating their photosynthetic capability. Green, hairy roots have also been observed in a few other plant species[19]. These green hairy roots are known to synthesize similar metabolites that are normally synthesized in the green parts of the plants.

The growth of hairy roots was similar on full, half and one-fourth strength MS media. On economical grounds, one-fourth strength MS medium was used for the experiments. The time course of growth and artemisinin production were investigated on one-fourth strength MS medium under continuous illumination. There was an increase in biomass from 50 mg at day one to 3.6 g at 15–18 days. The variation shown by different clones was marginal. The growth pattern of most clones was similar, except that cer-

tain clones showed a slightly prolonged exponential phase. However, there was a distinct difference in the artemisinin content of different clones. In *Hyoscyamus albus*, Vanhala *et al.*[20] found significant differences in hyoscyamine content of hairy root clones induced by different strains of *A. rhizogenes*. As depicted in Figure 2 (*a–f*) strains LBA 9402 and 9340 entered a stationary stage after fourteen days from inoculation, whereas strains 15834, A_4 and 9365 reached the stationary phase after eighteen days. The untransformed control roots reached the stationary stage after eighteen days. However, the biomass increase for untransformed control roots was very less when compared to transformed cultures (Figure 2 *a*). Artemisinin content appeared to be closely related to growth. Artemisinin production increased relatively slowly during the first six days for all the hairy root clones. Then all the lines behaved differently. For 9365 clone, there was a rapid rise after 6 days and artemisinin reached a value of 0.23% after 12 days. This is 20 times the amount detected in control roots. At 15 days, there was a sudden decrease in the artemisinin content, although the cultures reach the stationary phase after fifteen days. The medium was also analysed for the presence of artemisinin, but it was not detected in the medium, suggesting its retention within the tissue.

Generally, primary metabolites are more pronounced in log phase, whereas secondary products are produced in significant quantities only after the culture reaches the stationary phase. Smith *et al.*[21] reported lack of significant levels of artemisinin in their hairy root cultures, may be due to insufficient light. Jaziri *et al.*[8] found 0.001% of artemisinin in hairy roots of *A. annua* transformed with *A. rhizogenes* strain MAFF 03-01724 or NCIB 8196. They reported better growth of hairy roots on full strength MS medium and artemisinin content of these cultures was eight times higher than that in the half MS medium. They attributed the higher levels of artemisinin to high nitrogen concentration. Weathers *et al.*[9,10] reported 0.42% DW artemisinin in hairy root cultures and the effect of phosphate, nitrogen salts, sucrose and culture inoculum age on artemisinin production. However, owing to possible degradation of artemisinin by peroxidases a clear picture of optimum conditions for maximum production of artemisinin could not be presented. Woerdenbag *et al.*[22] reported that artemisinin has an endoperoxide bridge that is susceptible to peroxidases. Sudden product disappearance from cultures can be ascribed to peroxidases that are developmentally regulated and produced in great quantities by plant cell cultures[23]. Peroxidases may be responsible for destroying the endoperoxide bridge in artemisinin, thereby affecting the artemisinin levels of cultures[10]. 0.06% of artemisinin has been found in shooty teratomas of *A. annua*, whereas no artemisinin was found in undifferentiated callus[24]. This indicates that differentiation seems to be necessary for production of artemisinin.

Production of secondary metabolites from transformed cultures is influenced by bacterial strains, transformed clones and growth kinetics of *in vitro* cultures. The significant finding of this work was the production of artemisinin by the 9365-induced clone, which is higher than the level found in others.

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