Agrobacterium rhizogenes-Mediated Transformation of *Artemisia annua*: Production of Transgenic Plants

S. Banerjee[1,3], M. Zehra[1], M. M. Gupta[2], and S. Kumar[1]

[1] Genetic Resources and Biotechnology Division, Central Institute of Medicinal and Aromatic Plants (CIMAP), P. O. CIMAP, Lucknow-226 015, India
[2] Analytical Chemistry, Central Institute of Medicinal and Aromatic Plants (CIMAP), P.O. CIMAP, Lucknow-226 015, India
[3] Address for correspondence

Received: November 19, 1996; Revision accepted: April 6, 1997

Abstract: **Transgenic hairy roots were induced in the leaves of *Artemisia annua* by treatment with the LBA 9402 strain of *Agrobacterium rhizogenes*. The axenic hairy root cultures were found to produce the sesquiterpenes artemisinic acid and arteannuin B. The hairy root cultures were observed to spontaneously regenerate into plantlets on solid hormone-free MS medium. The regenerated plants had phenotypic characteristics typical to the transformed plants. Among the plants of the age of one month in culture, the transgenic plant was bigger (2.643 g/plant) than the normal (0.856 g/plant). Both these kinds of *in vitro* plants carried sesquiterpenes-artemisinic acid and arteannuin B.**

The highly aromatic annual herb *Artemisia annua* L. (Asteraceae) produces artemisinin, an endoperoxide sesquiterpene lactone in its aerial parts (1–4). Since the establishment of the antimalarial property of this compound against both chloroquine- and mafloquine-resistant strains of *Plasmodium falciparum*, efforts are in progress in several laboratories to improve the economics of artemisinin production. The chemical synthesis of artemisinin being complex and costly, the approaches being tried include the production of artemisinin through tissue culture, increasing the yield within the plant system through genetical interference and biotransformation of its precursors (5–10). Although many investigators have successfully micropropagated *A. annua*, there are mixed reports regarding the *in vitro* production of artemisinin in the cultured organs and tissues (11–15). In tissue culture, the biosynthesis of artemisinin has been found to occur in differentiated plants possessing normal looking shoot and root systems (11–13, 16–17). The production of artemisinin could not be detected in the isolated roots by most of the workers (12–13, 18–19), and presence of trace amounts has been reported in a few studies (15–16). In addition, production of artemisinin like compounds of unknown identity has been noted in the transformed hairy roots by ELISA analysis (10). Among the three studies in which the presence of artemisinin was investigated in *A. rhizogenes* transformed hairy root cultures, artemisinin was not detected in two of them (9–10), while the third reported 0.4% artemisinin content (20). There is as yet no report on the regeneration of transgenic plantlets from the *A. rhizogenes* transformed hairy root cultures. However, β-glucoronidase positive transgenic shoots have been recovered following *A. tumefaciens*-mediated transformation (21). The present paper reports experiments on the production of transformed hairy roots of *A. annua*, regeneration of plants for the first time, from hairy roots and synthesis of artemisinic acid and arteannuin B in both the hairy roots and plants derived from them.

Primary hairy roots were visible within 25–28 days of culture initiation at 98% frequency. Forty explants were used in three separate experiments, out of which 39 produced 4–5 roots per explant. These roots were excised and sub-cultured initially in hormone-free MS3 liquid medium and grown on rotatory shaker at 25 ± 2 °C under constant light conditions (3,000 Lux). Subsequently the hairy roots were maintained in hormone-free MS1 and MS3 liquid medium, respectively. The established hairy root lines exhibited a high degree of lateral branching and required subculturing at 30 days intervals. The genetic transformation was proved by the opine assay. The fresh weight of the roots increased by approximately 86-fold (4.32 g F.W.) and 185-fold (9.27 g F.W.) in MS1 and MS3 media, respectively from an original inoculum of 50 mg over 30 days duration. Control roots obtained from the non-transformed *in vitro* cultured plants, grown in MS3 medium and the hairy roots grown in MS1 and MS3 media were analyzed for the sesquiterpenes at 1 and 3 months growth period. Artemisinin could not be detected at either of the stages through a TLC densitometric procedure in either of them. However, artemisinic acid and arteannuin B could be detected in the samples through HPLC (Table **1**). One month old hairy root cultures grown in MS1 medium had more artemisinic acid and arteannuin B than those grown in MS3 medium, as well as than that of the respective control roots.

One of the hairy root clones spontaneously regenerated into plantlets on hormone-free MS medium after 45 days of root induction under constant light condition (3,000 Lux). Both non-transformed plants (obtained from the nodal explants on hormone-containing medium) and transformed plants (10 in number) were maintained on hormone-free solid MS medium. Transformed plants showed distinct variation in their morphology, such as a massive ageotropic root system, reduced apical dominance, and wrinkled leaves. Transformed shoots gave a higher biomass (2.643 g/plant) as compared to the non-transformed shoots (0.856 g/plant). Although the one month old *in vitro* grown transformed plants produced more artemisinic acid and arteannuin B than the respective hairy root cultures, the amounts were less than those of the *in vitro* grown non-transformed control plants of the same age (Table **1**). Since the synthesis or accumulation of artemisinin has already been proved to be highly variable under tissue culture conditions (22) and selection for artemisinin producing clones is not feasible at this time (23), the manipulation of media and culture conditions as well as shifting of the transgenic plants to glass house and subsequently to the field offer possibilities for improving the yield of the sesquiterpenes from the clones in which the pathway is expressed.

Several reports have identified artemisinic acid as a biogenetic precursor of artemisinin (6–7, 24) and considered arteannuin B to be a late precursor of artemisinin (5). On the background of this information, it can be presumed that the presence of comparatively more artemisinic acid than arteannuin B in the hairy root culture of the present study and the reverse in the regenerants might indicate that the regenerants were more close towards the expression of artemisinin than the hairy roots.

Planta Medica 63 (1997) 467–469

Table 1 *In vitro* studies of growth and sesquiterpene production by *A. annua* hairy root cultures and its regenerants.

Experimental material	Age of cultured material	Fresh weight (g)[a]	Content (percent DW × 1000)	
			AA[b]	AB[b]
Normal roots	1 month	0.731 ± 0.15	ND	ND
(from nontransformed plant)	3 months	1.112 ± 0.44	0.10	0.10
Transformed roots developed in MS	1 month	4.320 ± 0.35	0.85	0.58
medium containing 1 % sucrose	3 months	3.773 ± 0.22	0.65	0.53
Transformed roots developed in MS	1 month	9.272 ± 0.54	T	T
medium containing 3 % sucrose	3 month2	8.460 ± 0.42	0.51	0.57
Normal nontransformed plant (*in vitro*)	1 month	0.856 ± 0.10	66 ± 0.16[a]	102 ± 3.7[a]
Transformed plant (*in vitro*)	1 month	2.643 ± 0.38	33 ± 2.9[a]	72 ± 3.2[a]

ND = Not detected.
T = In trace amounts.
AA = Artemisinic acid.
AB = Arteannuin B.
a = Values are the mean of 3 replicates.
b = Values are the mean of 2 replicates.

In contrast to the report of a high level of artemisinin production in a hairy root culture (20), our data indicate for the first time that both artemisinic acid and arteannuin B may be produced in hairy root cultures as well as in regenerants therefrom, although artemisinin could not be detected in either of them, which is consistent with other reports (9–10). The above observation is promising in that it indicates that the artemisinin pathway does express partially or fully in hairy roots. Screening of a large number of transformants and regeneration with or without mutagenesis may provide means of developing high artemisinin and/or precursor producing clones. Demonstration in this paper for the first time that plants can be regenerated from transgenic hairy roots has provided a tool for experimentation in this direction.

Materials and Methods

Nodal explants (2–2.5 cm) collected from the field at the flowering stage were washed with 10% teepol solution for 5 min and kept under running water for 2 h. Such explants, surface sterilized with 0.1% $HgCl_2$ solution for 3 min, rinsed thoroughly with sterile distilled water, were inoculated in MS medium containing 0.2 mg/l BAP and 0.05 mg/l NAA: For rhizogenesis the shoots were transferred to half strength hormone-free MS medium. Leaves from these *in vitro* regenerated shoots served as the explants for genetic transformation.

Agrobacterium rhizogenes strain LBA 9402 (a kind gift of Dr. David Tepfer, INRA, France) were maintained on YMB (25) plates. 24-h-old suspension culture grown in YMB liquid medium on rotary shaker at 100 rpm was used for transformation.

Sections of leaves of 25-days-old *in vitro* grown plants were pricked 4–5 times with a sterile needle loaded with bacterial suspension and placed on the hormone-free MS medium. After 2 days of co-cultivation the leaves were transferred to antibiotic-containing MS solid medium (1 g/l of cephalexin). The transformed roots (50 mg) were cultured in 50 ml of liquid hormone-free MS medium containing 1 % (MS1) and 3 % (MS3) sucrose, resepctively, in 250-ml Erlenmayer flasks. The nontransformed roots obtained from *in vitro* cultured plants on half strength MS medium were cultured in MS3 medium under the same conditions. Shoots that were spontaneously regenerated from hairy roots on solid MS3 medium were subcultured on the same medium.

Artemisinin content was analyzed on a Desaga CD-50 densitometer using a dual wave length TLC-densitometric procedure (26). Artemisinic acid and arteannuin B were analysed (27), using a Shimadzu LC-10 HPLC model equipped with ODS column and a UV detector (220 nm), mobile phase: water : acetonitrile (30 : 70) + 0.1 trifluoroacetic acid at the flow rate of 1 ml/min. The variation in estimated values of the sesquiterpenes between the replication of a sample by the methods used was less than 5 %.

The detection of opine in the hairy root clones and the regenerated plantlets was performed as described by Petit et al. (28).

Acknowledgements

Financial assistance from Department of Biotechnology, New Delhi, is gratefully acknowledged.

References

[1] Ferreira, J. F. S., Simon, J. E., Janick, J. (1995) Planta Med. 61, 167–170.
[2] Pras, N., Visser, J. F., Batterman, S., Woerdenbag, H. J., Malingre, T. (1991) Phytochem. Anal. 2, 80–83.
[3] Singh, A., Vishwakarma, R. A., Husain, A. (1988) Planta Med. 54, 475–476.
[4] Morales, M. R., Charles, D. J., Simon, J. E. (1993) Acta Hort. 330, 203–206.
[5] Akhila, A., Thakur, R. S., Popli, S. P. (1987) Phytochemistry 26, 1927–1930.
[6] Elmarakby, S. A., El-Feraly, F. S., ElSohly, H. N., Croom, E. M., Hufford, C. D. (1988) Phytochemistry 27, 3089–3091.
[7] Akhila, A., Rani, K., Thakur, R. S. (1990) Phytochemistry 19, 2129–2132.
[8] Brown, G. D. (1994) Phytochemistry 36, 637–641.
[9] Mukherjee, S., Ghosh, B., Jha, T. B., Jha, S. (1995) Indian J. Expt. Biol. 33, 868–871.
[10] Jaziri, M., Shimomura, K., Yoshimatsu, K., Fauconnier, M. L., Marlier, M., Homes, J. (1995) J. Plant Physiol. 145, 175–177.

[11] He, X-C., Zengh, M-Y., Li, G. F., Liang, Z. (1983) Acta Bot. Sin. 25, 87–90.
[12] Martinez, B. C., Staba, J. (1988) Adv. Cell Cult. 6, 69–87.
[13] Fulzele, D. P., Sipahimalani, A. T., Heble, M. R. (1991) Phytother. Res. 1, 149–153.
[14] Whipkey, A., Simon, J. E., Charles, D. J., Janick, J. (1992) Herbs Spices Med. Plants 1, 15–25.
[15] Jha, S., Jha, T. B., Mahato, S. B. (1988) Curr. Sci. 57, 344–346.
[16] Nair, M. S. R., Acton, N., Klayman, D. I., Kendsick, K., Basile, D. V., Mante, S. (1986) J. Nat. Prod. 49, 504–507.
[17] Woerdenbag, H. J., Luers, J. F. J., Uden, W. V., Pras, N., Malingre, T. M., Alferman, A. W. (1993) Plant Cell Tiss. Org. Cult. 32, 247–257.
[18] Tawfiq, N. K., Anderson, L. A., Roberts, M. F., Phillipson, J. D., Bray, D. H., Warhurst, D. C. (1989) Plant Cell Rep. 8, 425–428.
[19] Kim, N. C., Kim, J. G., Lim, H. J., Hahn, T. R., Kim, S. U. (1992) J. Korean Agr. Chem. Soc. 35, 99–105.
[20] Weathers, P. J., Cheetham, R. D., Follansbee, E., Teoh, K. (1994) Biotech. Lett. 16, 1281–1286.
[21] Vergauwe, A., Cammaert, R., Vendernberghe, D., Genetello, C., Inze, D., Montagu, M. V., Eeckhout, E. V. D. (1996) Plant Cell Rep. 15, 929–933.
[22] Ferreira, J. F. S., Janick, J. (1995) Acta Horticul. 390, 41–47.
[23] Elhag, H. M., El-Domiaty, M. M., El-Feraly, F. S., Mossa, J. S., El-Olemy, M. M. (1992) Phytother. Res. 6, 20–24.
[24] El-Feraly, F. S., Al-Mesahl. I. A., Al-Yahya, M. L., Hifnawy, M. S. (1986) Phytochemistry 25, 2577.
[25] Hooykaas, P. J. J., Klapwick, P., Nuti, M. P., Schilperoort, R. A., Rorsch, A. (1977) J. Gen. Microbiol. 98, 477–484.
[26] Gupta, M. M., Jain, D. C., Verma, R. K., Gupta, A. P. (1996) J. Med. Arom. Pl. Sci. 18, 5–6.
[27] Gupta, M. M., Jain, D. C., Mathur, A. K., Singh, A. K., Verma, R. K., Kumar, S. (1996) Planta Med. 62, 280–281.
[28] Petit, A., David, C., Dahl, G. A., Ellis, J. C., Greyon, P., Casse-Delbert, F., Tempe, J. (1983) Mol. Gen. Genet. 190, 1204–1214.

Polyphenolic Constituents from the Leaves of two *Cynara* Species Growing in Greece

Ioanna Chinou[1,2] and Catherine Harvala[1]

[1] Department of Pharmacy, Division of Pharmacognosy, University of Athens, Panepistimiopolis Zografou, GR-Athens 157 71, Greece
[2] Address for correspondence

Received: October 21, 1996; Revision accepted: January 4, 1997

Abstract: **The chemical analyses of two *Cynara* species: *C. humilis* and *C. cornigera* revealed various combinations of flavones, flavonols, flavanones, coumarins, phenolic acids, and an aurone. All the identified constituents are reported for the first time from the two species, while the presence of the aurone is reported for the second time in the genus.**

Cynara is a genus of 7 annual taxa (1, 2) belonging to the family of Asteraceae, found mostly in all central and southern countries of Europe. For centuries, the genus is a popular medicinal plant, used in therapy for its strong choleretic activity causing substancial increase in the amount of bile excreted, as well as an increase in the concentration of biliary acids of the bile (3). the two studied taxa comprise *C. humilis* L. and *C. cornigera* Lindl, of which the former is common in Mediterranean countries, while the latter is an endemic species of Greece and the Aegian islands (4).

In the present work, we report the polyphenolic compounds composition from the leaves of the two above mentioned taxa from Greece. The chemical constituents of *C. humilis* and *C. cornigera* have never been the object of any kind of research study. The investigation of *Cynara* species was carried out on the leaves of the plants. From the methanolic extracts of the two species we observed interesting TLC profiles, differing from one another. This prompted us to undertake a more detailed study of their polyphenolic constituents. The structural groups of the isolated compounds were identified as phenolic acids, coumarins, flavonoids, and an aurone, the structures and occurrences of which are given in Table **1**. The phenolic acids: caffeic (**1**), chlorogenic (**2**), and isochlorogenic (the three possible isomers IUPAC: 3,3-, 3,5-, 4,5-di-*O*-caffeoylquinic acid) (**3**) were present in all tested taxa while the simple coumarins, aesculin (**4**) and scopoletin (**5**) were present only in

Table 1 Occurrence of the polyphenolic constituents in two species of *Cynara*.

Polyphenolic Constituents	*C. humilis*	*C. cornigera*
Luteolin	+	+
Luteolin 7-*O*-β-D-glucoside	+	+
Luteolin 7-*O*-β-D-rutinoside	–	+
Luteolin 7-*O*-β-gentiobioside	+	+
Apigenin	+	+
Apigenin 7-*O*-β-D-glucoside	+	+
Apigenin 7-*O*-β-D-rutinoside	+	+
Quercetin	+	+
Quercetin 3-*O*-β-rutinoside	–	+
Quercetin 7-methyl ether	+	–
Hesperitin	–	+
Hesperitin 7-*O*-β-neohesperidoside	+	+
Maritimein	+	–
Scopoletin	–	+
Aesculin	–	+
Caffeic acid	+	+
Chlorogenic acid	+	+
Isochlorogenic acid	+	+