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Short Communication

Establishment of Normal and Transformed Root Cultures of *Artemisia annua* L. for Artemisinin Production

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Summary

Transformed cultures of *Artemisia annua* L. (Asteraceae) were established by the co-culture method using leaf segments of *A. annua* and *Agrobacterium rhizogenes* NCIB 8196 or MAFF 03-01724. The hairy root clones thus obtained grew vigorously on hormone-free medium, showing the typical transformed morphology. The genetic transformation of the root was proved by the opine assay. Normal root and shoot cultures were also established. 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. The presence of artemisinin was confirmed by gas chromatography coupled to mass spectrometry (GC-MS) analysis. The hairy roots cultured in the dark produced no detectable level of artemisinin as shown by the adventitious shoots cultured under light conditions. The ELISA analysis of the green hairy roots cultured in liquid medium under a 16 h light/day photoperiod showed the existence of compound(s) structurally related to artemisinin, though normal and hairy roots cultured in the dark give no detectable levels of immuno-signal.

Key words: Agrobacterium rhizogenes; Artemisia annua; artemisinin; hairy root cultures; shoot cultures; transformation.

Introduction

Artemisinin, a sesquiterpene lactone, is the active antimalarial drug isolated from the aerial part of the Chinese herb Qinghao (*Artemisia annua* L.; Asteraceae). Artemisinin and its derivatives (artemether, arteether and artesunate) are effective against both chloroquine-resistant and -sensitive strains of *Plasmodium* [Li et al., 1982; Klayman, 1985; Woerdenbag et al., 1990]. The reported yield of artemisinin from leaves of *A. annua* has varied from a low yield of 0.01% dry wt. (in the European, Indian and American types) to a high yield of 0.5–0.7% dry wt. (in the Chinese type) [WHO report, 1981]. As an alternative source for the production of artemisinin, many laboratories have been engaged in developing biotechnological approaches based on cell and shoot cultures of *A. annua* [He et al., 1983; Jha et al., 1988; Martinez et al., 1988; Fulzele et al., 1991; Woerdenbag et al., 1993]. However, artemisinin was not found to be accumulated in callus and cell cultures; it is presumed that the biosynthesis of this compound is correlated with cell differentiation and/or intracellular organization. The biosynthesis of artemisinin seems to be restricted to the green part of the plant; indeed, this compound was not found in the root system [Martinez et al., 1988; Fulzele et al. 1991].

As part of an ongoing study on *A. annua*, we describe here the establishment of hairy root cultures of *A. annua*, which has not previously been reported. In order to expand the application of hairy root cultures, we established green hairy root cultures of *A. annua* and evaluated the capability of these root cultures to accumulate artemisinin and related compounds.

Materials and Methods

Establishment of normal and transformed root cultures

Normal root cultures were established by transferring root tips (1–2 cm in length) of sterile *A. annua* plantlets onto half-strength Murashige and Skoog (½ MS) [Murashige and Skoog, 1962] solid or liquid medium supplemented with 0.1 mg/L of indole acetic acid (IAA).

Hairy root cultures were established by the co-culture method using leaf discs of *A. annua* and *A. rhizogenes* MAFF 03-01724 or NCIB 8196. The transformed roots were cultured in ½ MS liquid medium and maintained at 25 °C in the dark on a rotatory shaker (100 rpm).

For the induction of greening, the hairy roots were cultured in the same medium at 25 °C in a 16 h light/day photoperiod (70 $\mu mol\, m^{-2} s^{-1}$, cool white fluorescent lamp) for 4 weeks before analysis. Effects of media on growth rate and artemisinin production of hairy roots were studied by inoculating hairy root segments from hormone-free ½ MS medium into hormone-free Woody Plant (WP) [Lloyd and McCown, 1980], Gamborg (B5) [Gamborg et al., 1968], MS or ½ MS medium. The hairy roots were cultured twice for 4 weeks in the corresponding medium before harvesting for analysis.

Establishment of shoot cultures

Internode segments (ca. 0.5 cm) of sterile *A. annua* plantlets obtained by seed germination were cultured on either ½ MS medium or WP solid medium supplemented with various combinations of benzyladenine (BA) and naphthalene acetic acid (NAA). The cultures were incubated at 25 °C under a 16 h light/day photoperiod (54 $\mu mol\, m^{-2} s^{-1}$).

Detection of opines

The detection of mannopine and mikimopine, for root clones transformed with *A. rhizogenes* NCIB 8196 and MAFF 03-01724, respectively, was performed as described previously [Jaziri et al., 1994; Yoshimatsu and Shimomura, 1992].

ELISA analysis

Dried material was weighed accurately and extracted with methanol. An aliquot of the methanolic extract was diluted with an appropriate volume of phosphate buffered saline and subjected to ELISA as described previously [Jaziri et al., 1993].

Artemisinin analysis

Gas chromatography combined with mass spectroscopy (GC-MS) was applied under conditions as described by Woerdenbag et al. (1991).

Results and Discussion

Establishment of adventitious shoot cultures and their artemisinin production

Internode segments of sterile plantlets, obtained by *in vitro* seed germination, were incubated in either ½ MS or WP media supplemented with various concentrations of BA and NAA. After 4 weeks of culture, the most interesting results were obtained with WP medium supplemented with 1 mg/L BA and 0.01 mg/L NAA: an average of 4 new regenerated shoots/explant (n = 10), no callus formation and the highest artemisinin equivalent content (0.02 % dry wt. = 2.62 µg artemisinin equivalent/flask). The presence of artemisinin in the extracts was confirmed by GC-MS analysis.

Establishment of transformed and normal root culture and the evaluation of artemisinin production

The axenic hairy root clones obtained grew vigorously in hormone-free medium, showing the characteristic high degree of lateral branching. The genetic transformation of the cultures was proved by the opine assay. All root clones established were confirmed to be transformed; neither mannopine nor mikimopine could be detected in the non-transformed root cultures. Several hairy root clones of Asteraceae species have already been established, but this is the first report of genetic transformation of *A. annua* by *A. rhizogenes.*

It has been reported that green hairy roots from some plant species grown in the light produced certain levels of useful secondary metabolites characteristic of aerial parts of the plant rather than to the root system grown in the dark [Sauerwein et al., 1991; Flores et al., 1993; Yoshimatsu et al., 1990]. We therefore examined the physiological behavior of the *A. annua* normal and transformed root clones cultured on solid ½ MS medium in the light.

Adventitious normal roots were induced from internode segments cultured on ½ MS medium containing 0.1 mg/L IAA and turned green when cultured under a 16 h light/day photoperiod. The ELISA analysis showed that normal green roots accumulated artemisinin (0.001 % dry wt.); the presence of artemisinin in the extract was confirmed by GC-MS analysis. In contrast, no artemisinin-like compound was detected by ELISA in the light-brownish roots cultured in the dark.

Hairy root clones that were induced by *A. rhizogenes* NCIB 8196 and cultured in hormone-free ½ MS solid medium under a 16 h light/day photoperiod also turned green, but to a lesser extent than the normal root cultures. The chlorophyll content of the green hairy roots was 162 mg/kg fresh wt., which corresponds to 25 % of the chlorophyll level found in shoot cultures. This greening capacity was particularly observed in the root clone named NCIB 5. We therefore selected this root clone for further experiments.

The effect of various liquid media on growth and accumulation of artemisinin-related compounds by hairy root clone NCIB 5 was investigated. Table 1 indicates the growth and

Table 1: Growth and artemisinin equivalent content of *Artemisia annua* hairy roots cultured in various liquid media.

medium	Fresh weight (g)	Dry weight (g)	Artemisinin equivalent content ($\times 10^{-3}$ % dry wt.)
WP	7.50±0.30	0.71±0.09	0.06±0.020 (0.42)[a]
B5	13.70±0.20	1.51±0.23	0.01±0.001 (0.15)
MS	11.91±0.70	0.60±0.07	0.40±0.005 (2.40)
1/2 MS	4.95±0.20	0.33±0.10	0.10±0.020 (0.33)

[a] numbers in parentheses indicate the artemisinin equivalent content expresssed in µg/flask.

artemisinin equivalent content of the hairy roots grown in various media. Superior growth of hairy roots was obtained from cultures in MS and B5 media (11.9 g and 13.7 g, respectively), and poor growth from cultures in ½ MS and WP media (4.95 g and 7.50 g, respectively). On the other hand, the highest artemisinin equivalent contents were observed in ½ MS and MS media, 0.1×10^{-3} and 0.4×10^{-3} % dry wt., respectively. In MS medium, we observed the highest artemisinin equivalent content/flask (2.40 µg/flask), almost 8 times higher than in ½ MS medium (0.33 µg/flask). In WP and B5 media, the artemisinin equivalent content was approximatively 10 times lower (0.06 and 0.01×10^{-3} % dry wt., respectively) than in the other two media. This difference might be attributed to the high nitrogen (nitrate and ammonium) concentration in the MS medium as compared with the other media tested.

Unfortunately, the GC-MS analysis did not allow identification of artemisinin in the hairy root extracts. We were not able to identify the compound(s) in the hairy root extracts that cross-react with anti-artemisinin antibodies, as the amount was too small to carry out the purification and spectroscopic analysis. On the other hand, the preparative thin layer chromatography (TLC) of the methanolic extract from hairy roots cultured in MS medium allowed localization of the immunopositive fraction (*Rf* value between 0.3 and 0.6; artemisinin *Rf* = 0.85; TLC mobile phase: $CHCl_3$-MeOH (97 : 3)). A large-scale culture of hairy root clone NCIB 5 is actually being undertaken in order to isolate the unknown compound structurally related to artemisinin.

On the basis of these results, it can be suggested that the normal and/or transformed root cultures can be used as an experimental system for studies related to the induction of secondary metabolite biosynthesis, compounds that are not found in the root system of the plant. Further investigations on this topic are under way in our laboratory.

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