Plant Cell, Tissue and Organ Culture **32**: 247–257. 1993.

Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L.

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Received 28 April 1992; accepted in revised form 29 July 1992

Key words: antimalarial drugs, *Artemisa annua*, artemisinin, sesquiterpene lactones, shoot culture

Abstract

From aseptically grown *Artemisia annua* plantlets, shoot cultures were initiated. Using different concentrations of auxine, cytokinine and sucrose, a suitable culture medium was developed, with respect to the growth of the shoots and their artemisinin accumulation. Nitrate concentration and conductivity appeared to be suitable growth parameters. The artemisinin content was measured gas chromatographically. The shoot cultures were maintained in the developed standard medium, consisting of a half concentration of MS-salts with vitamins, 0.2 mg l^{-1} BAP, 0.05 mg l^{-1} NAA and 1% sucrose. The growth of the shoots and the artemisinin content remained stable for a longer period. They showed considerable photosynthetic activity and generally contained ca. 0.08% artemisinin on a dry weight basis. The highest artemisinin content found was 0.16% in the above mentioned standard medium, but also on the same medium with 0.5% sucrose. Attempts were made to further improve the artemisinin production by varying the medium composition through addition of gibberellic acid or casein hydrolysate; by omitting plant growth regulators; by precursor feeding, i.e. mevalonic acid; by influencing the biosynthesis routing through inhibition of the sterol synthesis by miconazole, naftifine or terbinafine; by changing gene expression with 5-azacytidine or colchicine; and by elicitation, using cellulase, chitosan, glutathione or nigeran. Enhanced artemisinin production was found with 10 mg l^{-1} gibberellic acid, 0.5 g l^{-1} casein hydrolysate, 10 mg l^{-1} or 20 mg l^{-1} naftifine. Relative increases of 154%, 169%, 140% and 120% were found, respectively. Other additions caused the growth to cease and the artemisinin contents to drop.

Abbreviations: BAP – benzylaminopurine, DW – dry weight, FW – fresh weight, GA_3 – gibberellic acid, MS – Murashige & Skoog basal medium, NAA – naphthaleneacetic acid

Introduction

Malaria is an infectious disease, mainly caused by *Plasmodium falciparum*, and affects over 200 million people annually. Due to the remorseless rise in the resistance of *P. falciparum* to commonly used antimalarial drugs, this disease is a serious threat to the tropical world. Especially in South East Asia and in West Africa this drug resistance forms a huge problem in public health care and is a major cause of death. Therefore, new drugs to which the resistant parasites are sensitive, are urgently needed (Anonymous 1992).

Since the isolation of the endoperoxide sesquiterpene lactone artemisinin (Fig. 1) from aerial parts of *Artemisia annua* L., a traditionally used medicinal plant from China, and the determination of its antimalarial activity (Qinghaosu Antimalarial Coordinating Research Group 1979; Li et al. 1982), much effort is being made to develop a new class of antimalarials, based on this compound (Klayman 1985; Woerdenbag et al. 1990; Woerdenbag & Pras 1991). Extensive clinical studies with artemisinin and a series of semisynthetic derivatives (artemether, arteether, artelinic acid and sodium artesunate) have been conducted in China, Burma, Vietnam, Thailand and Gambia during the last decade, proving these drugs to be rapidly effective in severe falciparum malaria (WHO 1986; Anonymous 1992; White et al. 1992).

However, on a global scale artemisinin is hardly available, and drug registration requirements are not yet met (Anonymous 1992). The total organic synthesis has been established, but is very complicated, and low yields, that are economically unattractive, are obtained (Schmid & Hofheinz 1983; Xu et al. 1986; Avery et al. 1987; Ravindranathan et al. 1990; Avery et al. 1992). Therefore, the plant remains the only valid source of artemisinin. Next to selection procedures of *A. annua* plants (Singh et al. 1988; ElSohly et al. 1990; Simon et al. 1990; Pras et al. 1991; Elhag et al. 1992), a biotechnological approach has been considered to be an alternative for the production of artemisinin. However, results from experiments with undifferentiated callus and cell suspension cultures of *A. annua* are rather disappointing with respect to the artemisinin production, as most traces of this compound have been found (He et al. 1983; Nair et al. 1986; Kudakasseril et al. 1987; Jha et al. 1988; Martinez & Staba 1988; Tawfiq et al. 1989; Fulzele et al. 1991; Woerdenbag et al. 1992). Apparently, a certain degree of differentiation of the cultures is a prerequisite (Martinez & Staba 1988; Fulzele et al. 1991). In this communication we wish to report on the establishment of well-growing *A. annua* shoot cultures, accumulating significant amounts of artemisinin.

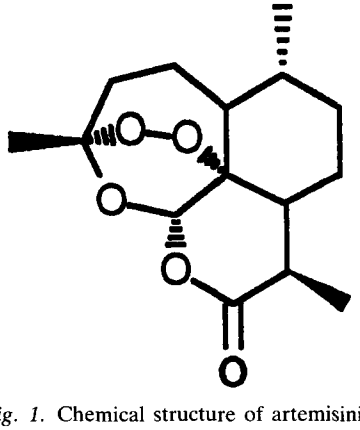

Fig. 1. Chemical structure of artemisinin.

Materials and methods

Shoot cultures of A. annua

Seeds of *Artemisia annua* L. (Asteraceae) of Vietnamese origin (obtained from ACF Beheer BV, Maarssen, The Netherlands) were surface sterilised by immersion in 70% ethanol, followed by stirring for 20 min in a 3% w/v sodium hypochlorite solution. The seeds were rinsed three times with sterile, deionised water and gently dried on tissue paper. Seeds were plated on Petri-dishes, containing medium with a quarter concentration of MS-salts (Murashige & Skoog 1962) without vitamins and plant growth regulators, 2% w/v sucrose, solidified with 0.8% w/v agar, pH 5.9. They were incubated at 25 °C at a 16 h light (ca. 2,500 lux) and 8 h dark cycle. Germination started within 2–3 days.

Of 1–2 week-old plantlets, the green parts were cut off and cultured further in 100 ml Erlenmeyer flasks, in ca. 30 ml medium composed of a half concentration of MS-salts with vitamins, 3% w/v sucrose, different concentrations of naphthaleneacetic acid (NAA) (0–0.5 mg l^{-1}) and benzylaminopurine (BAP) (0–2.0 mg l^{-1}). The cultures were incubated on a rotary shaker at ca. 40 rpm, at 25°C at 16 h light (ca. 2,500 lux) and 8 h dark cycle. Subsequently, shoot and root formation, callus formation, colour and growth of the cultures were judged visually. After 6 weeks their artemisinin content was measured.

The sucrose percentage of the medium with the NAA and BAP concentration in which good growth was accompanied with a significant ar-

temisinin production, was then varied from 0.5–8% w/v. After 2 growth cycles of 14 days each, the cultures were judged visually for their green colour and growth, analysed for their artemisinin content, and the ratio fresh weight/dry weight (FW/DW) was determined.

For maintainance of the cultures, a medium composed of a half concentration of MS-salts with vitamins, 0.2 mg l^{-1} BAP, 0.05 mg l^{-1} NAA and 1% sucrose, was considered to be optimal, and further designated as the 'standard medium'. The cultures were maintained by subculturing every two weeks, by transferring 2–3 g shoots (FW) into ca. 30 ml of fresh medium.

Determination of the growth

The growth of cultures, maintained on the standard medium with different sucrose concentrations, was determined by measuring the following parameters in the culture medium during and after a growth cycle of 14 days: the conductivity, the nitrate concentration using a test-combination for the enzymatic determination of nitrate in foodstuffs and other materials (Boehringer Mannheim, Almere, The Netherlands; cat. no. 905 658) and the sucrose concentration using the testcombination for the enzymatic determination of sucrose/D-glucose in foodstuffs and other materials (Boehringer Mannheim; cat. no. 139 041). During the growth cycle, small samples of culture medium (ca. 0.3 ml) were taken aseptically at regular intervals.

Measurement of the photosynthetic activity

The photosynthetic oxygen evolution was measured at 25°C with an oxygen electrode (Schott Geräte, Hofheim a. Ts., Germany, Digital O_2 Meter CG 867). Ca. 2 g fresh shoots of *A. annua*, grown in the standard medium with different sucrose concentrations (0–3%) for two weeks, were cut into small pieces and suspended in 5 mM sodium phosphate buffer, pH 7.8, in a 23 ml incubation vessel and illuminated with a slide projector at a light intensity of ca. 100,000 lux. The oxygen consumption rate of the stirred mixture was monitored until it was constant. As a source of carbon dioxide, 1 ml of a 0.1 M stock solution of sodium hydrogen carbonate was added, yielding a final concentration of ca. 5 mM. The decrease in oxygen consumption rate after this addition was used as a criterion for photosynthetic oxygen evolution. By switching off the light, the nett oxygen consumption could be calculated. The photosynthetic activity was defined as the difference between oxygen production (in light) and oxygen consumption (in the dark) and expressed in mg O_2 g^{-1} FW h^{-1}.

Analysis of artemisinin

Lyophilised shoots were powdered in a mortar. Ca. 100 mg, accurately weighed, was extracted for 30 min with 3.0 ml toluene in an ultrasonic bath. After centrifugation, 1.0 ml of the toluene extract was taken and evaporated to dryness. The residue was dissolved in 100 µl methanol, containing 10 µg docosane ($CH_3(CH_2)_{20}CH_3$; Fluka, Buchs, Switzerland) as the internal standard. Then, 2 µl was submitted to gas chromatographic analysis, under conditions as described earlier (Woerdenbag et al. 1991). A calibration curve was obtained using pure reference artemisinin (ACF Beheer BV).

Improvement of the artemisinin production

In order to improve the artemisinin production in shoot cultures, growing in the standard medium, the following additions or changes were made. All changes were introduced at the time of subculturing (day 0), unless otherwise stated.

Gibberellic acid (GA_3; Serva, Heidelberg, Germany) 10 mg l^{-1}, 20 mg l^{-1}; casein hydrolysate (United States Biochemical Corporation, Cleveland, USA) 0.5 g l^{-1}, 1.0 g l^{-1}; mevalonic acid lactone (Sigma, St. Louis, USA) 0.25 g l^{-1}, 0.5 g l^{-1}, 1 g l^{-1}, 2 g l^{-1}; 5 azacytidine (Janssen, Beerse, Belgium) 1 mg l^{-1}, 2 mg l^{-1} on day 2, 4 and 7; colchicine (Fluka) 0.2 g l^{-1}, 2 g l^{-1}; miconazole (Sigma) 10 mg l^{-1}, 20 mg l^{-1}, 50 mg l^{-1}, 100 mg l^{-1}; naftifine (Sandoz, Basle, Switzerland) 10 mg l^{-1}, 20 mg l^{-1}, 50 mg l^{-1}, 100 mg l^{-1}; terbinafine (Sandoz) 10 mg l^{-1}, 20 mg l^{-1}; cellulase (BDH Chemicals, Poole, UK) 20 mg l^{-1}, 50 mg l^{-1}, 100 mg l^{-1} on day 10; chitosan (Sigma) 0.5 g l^{-1}, 1 g l^{-1} on day 10; glutathione (Sigma) 0.3 g l^{-1}, 0.6 g l^{-1} on day 10; nigeran (Sigma) 0.2 g l^{-1}, 0.5 g l^{-1}, 1 g l^{-1} on day 10. An

additional variation was made by the total omittance of plant growth regulators from the medium.

Casein hydrolysate, colchicine, and miconazole were autoclaved together with the medium; GA_3, mevalonic acid lactone, 5-azacytidine, naftifine, terbinafine, cellulase, and glutathione were aseptically added from concentrated stock solutions, by passing them through a 0.2 μm filter. Chitosan and nigeran were added from an autoclaved stock suspension.

Results and discussion

Establishment of A. annua shoot cultures

First experiments were directed to initiate *A. annua* shoot cultures and to develop a standard medium in which a good growth was exhibited, accompanied with significant artemisinin contents. Based on the few available literature data on the cultivation of more or less organized tissue cultures of *Artemisia* species (Kudakasseril et al. 1987; Martinez & Staba 1988; Benjamin et al. 1990; Simon et al. 1990; Fulzele et al. 1991), combinations of the auxine NAA and the cytokinine BAP were chosen, in a medium supplemented with 3% sucrose. This series of cultures was judged visually on their general appearance (shoot, root, or callus formation), as well as on their growth and colour. The results of these experiments are listed in Table 1.

Over a period of 6 weeks, it generally appeared that the cultures on a low BAP concentration (I–V) grew better than those on a high BAP concentration (VI–X). The latter cultures became yellow-green first and finally turned brown. In cultures IX and X, this process proceeded slightly slower. Cultures I–IV kept a dark-green colour. They initially changed into a callus-like structure, but after 4–5 weeks, 5–10 mm long shoots appeared. Only culture V turned brown. Culture XI, grown without growth regulators, abundantly developed roots. Shoot formation did not occur however, and no artemisinin could be detected.

Based on these results, cultures I–IV were grown further on their respective BAP and NAA concentrations with 3% sucrose, and for comparison also with the lower sucrose concentration of 2%. The effect on the artemisinin content is depicted in Fig. 2. As can be seen from this figure, the artemisinin content varies, depending on both the NAA and sucrose concentration. The shoot cultures showed a better growth and produced more artemisinin on 2% sucrose. Because of the better growth, as compared with 0.1

Table 1. Growth and artemisinin content of 6-week-old *A. annua* shoot cultures, in media with 3% sucrose and different BAP and NAA concentrations.

Culutre	BAP ($mg\,l^{-1}$)	NAA ($mg\,l^{-1}$)	Artemisinin (% DW)	Growth[a]
I	0.2	0	0.029	+
II	0.2	0.05	0.051	+
III	0.2	0.1	0.039	+
IV	0.2	0.2	0.061	+
V	0.2	0.5	nd[b]	–
VI	2.0	0	nd[b]	–
VII	2.0	0.05	nd[b]	–
VIII	2.0	0.1	nd[b]	–
IX	2.0	0.2	0.027	±
X	2.0	0.5	0.046	±
XI	0	0	nd[b]	root formation

[a]growth was judged visually; + = good; ± = moderate; – = bad; [b]nd = not detectable

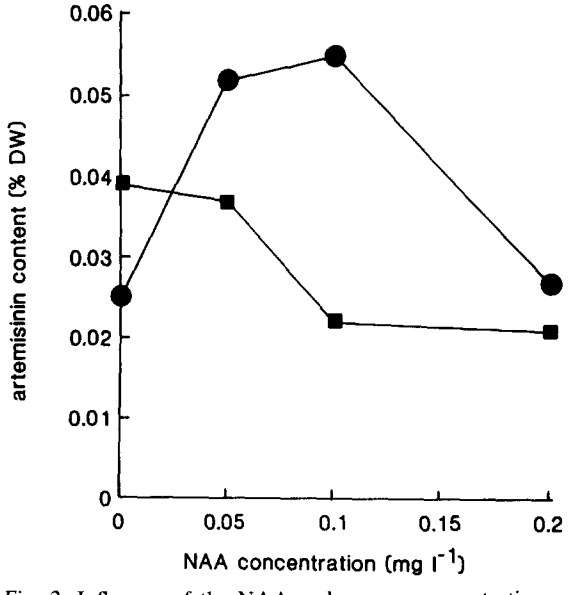

Fig. 2. Influence of the NAA and sucrose concentration on the artemisinin content in *A. annua* shoot cultures. Sucrose concentrations: 2% (●); 3% (■). The BAP concentration was 0.2 mg l^{-1}. Culture period: 2 weeks.

mg l^{-1} NAA, a concentration of 0.05 mg l^{-1} NAA was found to be optimal.

Subsequently, the influence of the sucrose concentration was studied, in a broad range from 0 to 8%, using a culture medium with 0.05 mg l^{-1} NAA and 0.2 mg l^{-1} BAP. After 2 passages the artemisinin content and the ratio FW/DW were determined. The results are reflected in Fig. 3. At sucrose contents < 2%, both the artemisinin content and the FW/DW ratio increased. As can be seen from Fig. 3, high FW/DW ratio co-occurred with a high artemisinin content. Cultures growing on 0.5 and 1% sucrose were darkgreen and generally consisted of shoots with a length of 1–2 cm, whereas cultures growing in a medium with sucrose concentrations ≥ 2% for a culture period of 2 weeks were light-green and tended to callus formation.

Determination of the growth

For the development of a standard medium, on which *A. annua* shoots exhibited an optimal growth, measurement of the growth was necessary. Moreover, when several factors, possibly improving the artemisinin production, are examined, the growth has to be determined more accurately. Unlike suspension-grown cells, from which samples can easily be taken during the growth cycle for the determination of biomass production in order to check growth, shoot cultures do not form such a dispersed system. Therefore, other parameters than packed cell volume or weight have to be used. The weight increase of the tissue during the growth or the mitotic index may be determined, but these parameters are inconvenient for routine determination. By determining certain parameters in the culture medium, it should also be possible to obtain an estimation of the growth of the shoots. As nutrients are absorbed from the medium during the growth, resulting in a decline in conductivity and a decrease of nitrate and sucrose concentrations, measuring this may render good growth parameters. Estimation of the growth of *A. annua* plantlets cultured in a bioreactor, using the nutrient consumption as a measure, has earlier been reported to be suitable (Park et al. 1989).

The three parameters mentioned were measured regularly in an *A. annua* shoot culture on standard medium (1% sucrose) during one growth cycle of 14 days. Figure 4 shows the time course of nutrient absorption and decline of the conductivity. The decrease of these three growth parameters corresponded well with each other. As soon as on day 7, halfway the growth cycle, all nutrients had vanished from the medium. This did not implicate that the growth stopped at this stage. Apparently, it took time before ab-

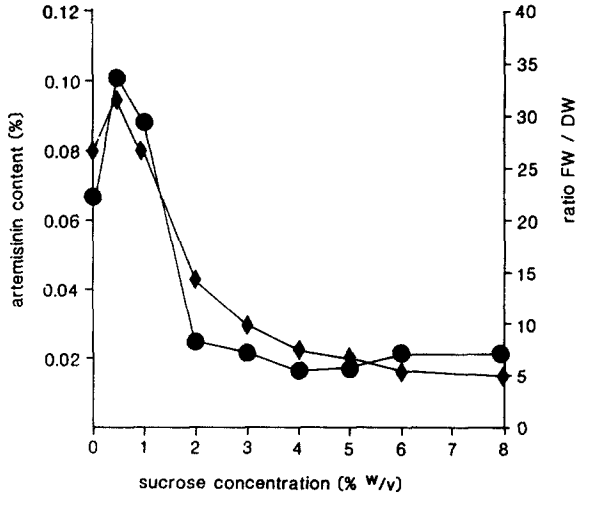

Fig. 3. Influence of the sucrose percentage in the culture medium containing 0.2 mg l^{-1} BAP and 0.05 mg l^{-1} NAA on the artemisinin content (●) and the ratio FW/DW (◆) of *A. annua* shoot cultures. Culture period: 2 weeks.

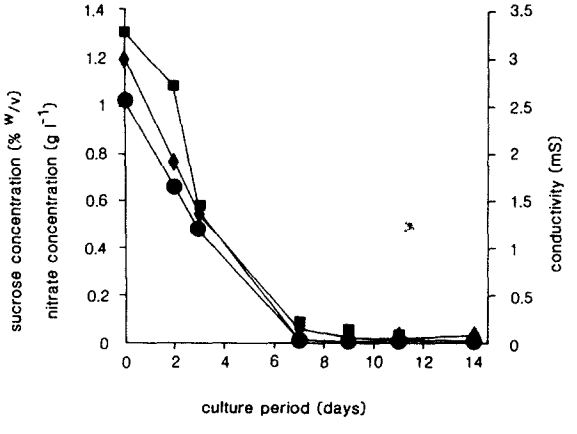

Fig. 4. Time course of the sucrose (●) and nitrate (■) concentration, and conductivity (◆) of the medium of an *A. annua* shoot culture in standard medium (1% sucrose) during one growth cycle of 14 days.

sorbed nutrients were used for the growth. This was confirmed by the observation that, visually, the growth clearly proceeded between day 7 and 14. The formation of secondary metabolites is often expressed towards the end of a growth cycle, at the time primary nutrients become limited. Based on these results, a cycle of 14 days was judged to be appropriate for *A. annua* shoot cultures, with respect to the artemisinin production.

The growth parameters conductivity, nitrate and sucrose concentration were then checked routinely at the end of a cycle of 14 days in the spent medium. This method was applied to the *A. annua* shoot cultures, grown with various sucrose concentrations (0–8%). Figure 5 shows the conductivity, the sucrose and the nitrate concentration of the spent medium of these shoot cultures on day 14. A good correlation was found between the decrease of sucrose and nitrate, and the decline of the conductivity. The amount of nutrients that had vanished from the medium on day 14 was highest with an initial sucrose concentration of 1%, indicating the best growth (compare Fig. 4). Also, from the media with 0.5, 2 and 3% sucrose large amounts of nutrients were taken up by the shoots. At sucrose concentrations of 4% and higher, the shoots hardly absorbed any nitrate or sucrose, and the conductivity became hardly lowered. However,

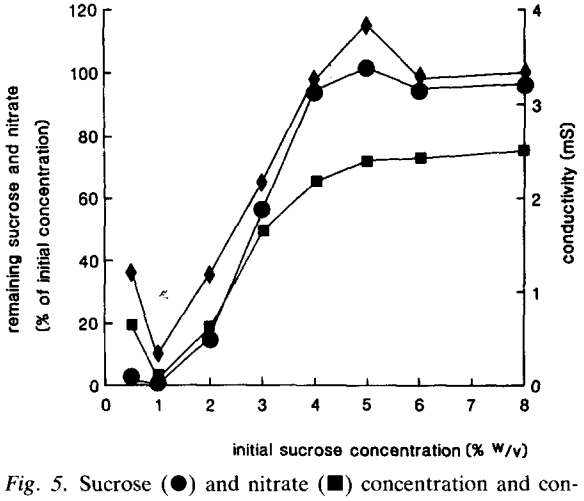

Fig. 5. Sucrose (●) and nitrate (■) concentration and conductivity (◆) on day 14 of the growth cycle of *A. annua* shoot cultures, grown on 0–8% sucrose. The remaining amounts of sucrose and nitrate are calculated as the percentage their initial concentration (day 0, 100%).

from the experiments directed to further improvement of the artemisinin production (see below), sucrose appeared to be an unreliable parameter here to check growth, because it was also consumed to some extent by poorly growing shoot cultures. This was accompanied with very slow growth, observed visually. Apparently, high sucrose levels were toxic for the shoots. At lower sucrose concentrations, chlorophyll synthesis may be stimulated, resulting in more photosynthetic activity and better growth of the shoots.

Measurement of the photosynthesis

Expression of photosynthetic activity may be considered essential for the artemisinin production, as the biosynthesis of sesquiterpenoids proceeds via mevalonic acid, that is linked with the photosynthesis by acetyl-coenzyme A (Akhila et al. 1987). Therefore, shoots growing on 0–3% sucrose, combined with 0.05 mg l^{-1} and 0.2 mg l^{-1} BAP, were checked for their photosynthetic capacity.

In Table 2 the photosynthetic activities of the shoot cultures are listed and compared with leaves of an intact plant. Considerable photosynthetic activity was measured at the sucrose concentrations used and, as could be expected, in all cases artemisinin was accumulated endogenously. As with 1% sucrose relatively high artemisinin contents were found combined with a good growth, this medium composition was chosen for further work, and designated as the standard medium. It was a prerequisite for good growth that the shoots were not fully submerged in the growth medium.

Further improvement of the artemisinin production

The highest artemisinin content found in our shoot cultures was 0.16% (DW) in the standard medium, supplemented with 1% as well as with 0.5% sucrose. The cultures with 0.5% sucrose, however, exhibited a slow growth, with slow biomass production. The average artemisinin content of shoots growing on the standard medium was 0.088 ± 0.041% (DW) (n = 11), and was accompanied with a good growth. No

Table 2. Photosynthetic activity of *A. annua* shoots and of leaves from the intact plant.

Sucrose (%)	Photosynthetic activity ($mg\ O_2\ g^{-1}\ FW\ h^{-1}$)	ratio FW/DW	Artemisinin content (% DW)
0	0.305	26.7	0.067
0.5	0.153	31.7	0.105
1.0	0.150	26.7	0.088
2.0	0.117	14.0	0.025
3.0	0.131	10.0	0.022
leaves	0.162	4.5	0.30

artemisinin was ever detected in the spent medium. In Fig. 6 the morphology of these shoot cultures is shown. Root formation did not occur under these conditions.

The artemisinin contents that have been found so far in *A. annua* shoot cultures were lower than in the intact plant. In shoot tips, Fulzele et al. (1991) found 0.012% (DW) and Simon et al. (1990) reported concentrations ranging from 0.03 to 0.05% (DW). Other studies on more differentiated tissue cultures revealed at most a few thousands of percents (He et al. 1983; Nair et al. 1986; Kudakasseril et al. 1987; Martinez & Staba 1988). It can be concluded that the artemisinin contents in *A. annua* shoot cultures, as presented in the current study, are superior to earlier published results. In intact plants usually concentrations around 0.1% (DW) have been found, although 0.5–0.8% has been reported for Chinese and Vietnamese plants (Klayman 1985; Martinez & Staba 1988; Dung & Loi 1991).

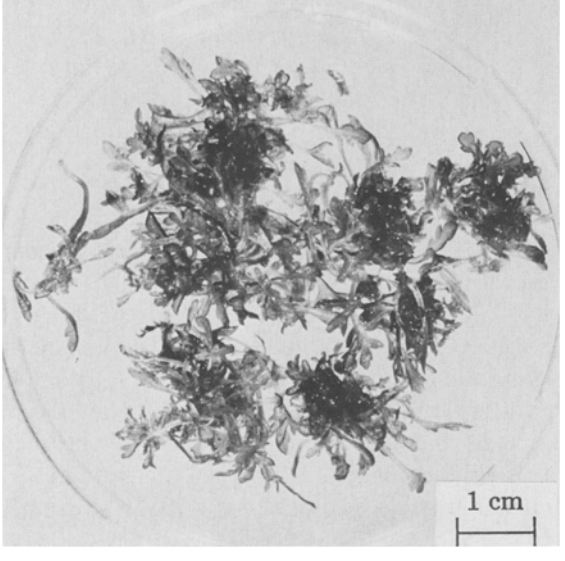

Fig. 6. Petri dish with *A. annua* shoot cultures.

Attempts were made to improve the artemisinin production by omittance or addition of medium components (plant growth regulators, casein hydrolysate), by precursor feeding (mevalonic acid), by influencing the artemisinin biosynthesis routing (addition of sterol synthesis inhibitors, mutagenic compounds) or by elicitation. In Table 3 the results from these experiments are listed.

GA_3 is a plant growth regulator, capable of inducing blooming (Evans 1989). As the artemisinin contents in intact plants are maximal just before or at the time of flowering (Liersch et al. 1986; Sing et al. 1988; Woerdenbag et al. 1991), we added this regulator to the shoot cultures. With 10 $mg\ l^{-1}$ an enhancement of the artemisinin production was found, whereas the growth remained unaffected. At higher concentrations, growth and production were negatively influenced. The shoots became very light-green and transluscent by the addition of GA_3, and a high FW/DW ratio of 35 was found. When the cultures were maintained in the presence of GA_3 for more than one growth cycle, the artemisinin production ceased again, whereas the growth remained unaffected. Earlier, the addition of GA_3 has been reported not to affect the artemisinin content of *A. annua* shoot cultures (Martinez & Staba 1988).

It has been suggested in the literature (Fulzele et al. 1991; Elhag et al. 1992) that differentiation into shoots, or preferably shoots with roots, is necessary for significant artemisinin biosynthesis in *A. annua* shoot cultures. Furthermore, artemisinin contents increased when shoots developed into plants with a properly developed root system (Martinez & Staba 1988). The omitt-

Table 3. Influence of changes of the medium on the growth and artemisinin production of *A. annua* shoot cultures. The artemisinin contents were calculated on their corresponding control value (100%). The average artemisinin content found in control shoot cultures (standard medium) was 0.088 ± 0.041% (DW) (n = 11). The growth was quantitated by relating the decrease in conductivity and nitrate consumption, as measured at the end of the growth cycle, of the controls (100%) to the test cultures. The initial conductivity of the controls was 2.9 mS; the nitrate concentration 1.3 g l^{-1}. Culture period: 2 weeks.

Addition/change	Artemisinin content (% of control)	Growth (% of control)	
		Nitrate	Conductivity
Control	100	100	100
Gibberellic acid 10 mg l^{-1}	154	99	98
Gibberellic acid 20 mg l^{-1}	60	80	75
Casein hydrolysate 0.5 g l^{-1}	169	101	100
Casein hydrolysate 1.0 g l^{-1}	98	100	101
Mevalonic acid 0.25 g l^{-1}	54	88	83
Mevalonic acid 0.5 g l^{-1}	44	81	70
Mevalonic acid 1 g l^{-1}	75	80	75
Mevalonic acid 2 g l^{-1}	62	74	63
5-Azacytidine 1 mg l^{-1} (3x)	61	91	83
5-Azacytidine 2 mg l^{-1} (3x)	69	92	75
Colchicine 0.2 g l^{-1}	85	–[a]	18
Colchicine 2 g l^{-1}	76	–[a]	63
Miconazole 10 mg l^{-1}	65	93	92
Miconazole 20 mg l^{-1}	57	72	65
Miconazole 50 mg l^{-1}	41	73	52
Miconazole 100 mg l^{-1}	32	74	32
Naftifine 10 mg l^{-1}	140	97	92
Naftifine 20 mg l^{-1}	120	83	74
Naftifine 50 mg l^{-1}	35	71	41
Naftifine 100 mg l^{-1}	40	72	25
Terbinafine 10 mg l^{-1}	48	74	58
Terbinafine 20 mg l^{-1}	40	72	36
Cellulase 20 mg l^{-1}	63	102	101
Cellulase 50 mg l^{-1}	60	99	100
Cellulase 100 mg l^{-1}	45	102	101
Chitosan 0.5 g l^{-1}	54	99	99
Chitosan 1 g l^{-1}	82	101	101
Glutathione 0.3 g l^{-1}	89	92	89
Glutathione 0.6 g l^{-1}	49	88	82
Nigeran 0.2 g l^{-1}	77	100	102
Nigeran 0.5 g l^{-1}	85	101	101
Nigeran 1 g l^{-1}	76	101	102
Omission of growth regulators	53	73	65

[a]The nitrate concentration could not be measured in the presence of colchicine, as the absorption from the latter compound interferes in the assay used.

ance of growth regulators from the medium resulted in slight root formation, but the growth of the shoots was poor, as was their artemisinin content. In contrast, in our artemisinin-producing shoot cultures, growing on the standard medium, roots were completely absent.

Casein hydrolysate is a product, prepared from cheese proteins. It serves as an additional source of amino acids and oligopeptides. With 0.5 g l^{-1} an enhancement of the artemisinin content was found, but the growth of the shoot cultures and the biomass production became rapidly impaired upon longer exposure to casein hydrolysate.

Mevalonic acid is an intermediate in the biosynthesis of isoprene units. However, by feeding of mevalonic acid in the form of its lactone, the growth and the artemisinin content were negatively influenced. This is in good agreement with earlier findings (Kudakasseril 1987; Martinez & Staba 1988).

It is known that, starting from the C_{15}-inter-

mediate farnesyl diphosphate, the biosynthesis routing may follow two different pathways. One leads to sesquiterpenes and sesquiterpene lactones, the other to sterols. Theoretically, by blocking the enzymes involved in the sterol synthesis, the C_{15} pool principally becomes only available for the synthesis of sesquiterpenoids, including artemisinin, possibly resulting in an enhanced production of this compound. Antifungal drugs, such as miconazole, naftifine and terbinafine, are well-known inhibitors of the sterol biosynthesis in micro-organisms (Budvari et al. 1989). Miconazole inhibits the enzyme sterol desmethylase; naftifine and terbinafine inhibit the enzyme squalene epoxidase.

Addition of miconazole negatively influenced the artemisinin production, as well as the growth of the cultures. This is inconsistent with a report from Kudakasseril et al. (1987), who found enhanced artemisinin production in shoot cultures of *A. annua*, even with 100 mg l^{-1}. We found naftifine to give a concentrationdependent increase in artemisinin contents, whereas the growth was hardly affected. Terbinafine, on the other hand, appeared to be toxic in the concentrations used.

The nucleoside analogue 5-azacytidine has been reported to function as a strong stimulator of the secondary metabolism in some cases (Arfmann et al. 1985). By incorporation of 5-azacytidine into DNA, unmethylated cytidine fragments are formed, possibly leading to the expression of genes, coding for enzymes in the biosynthesis of artemisinin. The addition of 5-azacytidine during the exponential phase of the growth, however, did not enhance the artemisinin production in *A. annua* shoot cultures.

Colchicine, an inducer of polyploidy in cell cultures (Chavadej & Becker 1984), strongly inhibited the growth of the shoot cultures, and the artemisinin production became impaired.

Elicitation has been reported to induce enhancement of the secondary metabolism in cell suspensions in certain cases (DiCosmo & Misawa 1985). Cellulase elicited the production of capsidiol in *Nicotiana tabacum* cultures (Watson et al. 1985; Threlfall et al. 1988). Addition of cellulase to our shoot cultures only resulted in a reduction of the artemisinin contents. Chitosan, successfully used to enhance the accumulation of acridone epoxides in cultures of *Ruta graveolens* (Eilert et al. 1984), also caused a reduction of the artemisinin contents. Glutathione, an ubiquitous tripeptide, has also been reported to possess eliciting properties (Wingate et al. 1988), but only a negative effect was found with respect to the artemisinin production in *A. annua* shoot cultures. Also nigeran, a polyglucan isolated from *Aspergillus japonicus*, negatively influenced the artemisinin content.

Conclusions

Shoot cultures of *A. annua* exhibit good growth as well as a significant artemisinin production in a medium composed of a half concentration of MS-salt with vitamins, 0.2 mg l^{-1} BAP, 0.05 mg l^{-1} NAA and 1% sucrose. The growth of the cultures may be well-characterized by measuring the decrease of nitrate concentration and the conductivity in the medium. During prolonged maintainance of the *A. annua* shoot cultures, they showed stability with respect to their growth and artemisinin accumulation. This offers perspectives to select high-producing shoots for upscaling procedures, cloning and regeneration experiments. The addition of GA_3, casein hydrolysate or naftifine to the medium improved the artemisinin production. Other additions, viz. of mevalonic acid, 5-azacytidine, colchicine, miconazole, terbinafine, cellulase, chitosan, glutathione and nigeran, were too toxic for the cultures, and did not induce an enhancement of the artemisinin production. Subtle optimization procedures, with respect to the basal medium or other culture conditions, such as light and temperature, are currently in progress.

Acknowledgements

Sandoz AG (Basle, Switzerland) is greatfully acknowledged for making naftifine and terbinafine available to us. This research was financially supported by the Netherlands Technology Foundation (STW) and was co-ordinated by the Foundation for Biological Research (BION). We

also thank the Federal Minister of Research and Technology (Bonn, Germany) as well as the Fonds of Chemical Industry (Frankfurt, Germany) for financial support.

References

Akhila A, Thakur RS & Popli SP (1987) Biosynthesis of artemisinin in *Artemisia annua*. Phytochemistry 26: 1927–1930

Anonymous (1992) Rediscovering wormwood: qinghaosu for malaria (editorial). Lancet 339: 649–651

Arfmann HA, Kohl W & Wray V (1985) Effect of 5-azacytidine on the formation of secondary metabolites in *Catharanthus roseus* cell suspension cultures. Z. Naturforsch. 40: 21–25

Avery MA, Chong WKM & Jennings-White C (1992) Stereoselective total synthesis of (+)-artemisinin, the antimalarial constituent of *Artemisia annua* L. J. Am. Chem. Soc. 114: 974–979

Avery MA, Jennings-White C & Chong WKM (1987) The total synthesis of (+)-artemisinin and (+)-9-desmethylartemisinin. Tetrahedron Lett. 28: 4629–4632

Benjamin BD, Sipahimalani AT & Heble MR (1990) Tissue cultures of *Artemisia pallens*: organogenesis, terpenoid production. Plant Cell Tiss. Org. Cult. 21: 159–164

Budvari S, O'Neil MJ, Smith A & Heckelman PE (Eds) The Merck Index, 11th edition. Merck & Co., Inc., Rahway

Chavadej S, Becker H (1984) Influence of colchicine treatment on chromosome number and growth rate of tissue cultures of *Valeriana wallichii* DC. Plant Cell Tiss. Org. Cult. 3: 265–272

DiCosmo F & Misawa M (1985) Eliciting secondary metabolism in plant cell cultures. Trends Biotechnol. 12: 318–322

Dung NG & Loi DT (1991) Selection of traditional medicines for study. J. Ethnopharmacol. 32: 57–70

Eilert U, Ehmke A & Wolters B (1984) Elicitor-induced accumulation of acridone alkaloid epoxides in *Ruta graveolens* suspension cultures. Planta Med. 50: 508–512

Elhag HM, El-Domiaty MM, El-Feraly FS, Mossa JS & El-Olemy MM (1992) Selection and micropropagation of high artemisinin producing clones of *Artemisia annua* L. Phytother. Res. 6: 20–24

ElSohly HN, Croom Jr EM, El-Feraly FS & El-Sherei MM (1990) A large-scale extraction technique of artemisinin from *Artemisia annua*. J. Nat. Prod. 53: 1560–1564

Evans FJ (1989) Trease and Evans' Pharmacognosy (pp 113–114). Ballière Tindall, London

Fulzele DP, Sipahimalani AT & Heble MR (1991) Tissue cultures of *Artemisia annua*: organogenesis and artemisinin production. Phytother. Res. 5: 149–153

He XC, Zeng MY, Li GF & Liang Z (1983) Callus induction and regeneration of plantlets from *Artemisia annua* and changes in qinhaosu contents. Acta Bot. Sin. 25: 87–90

Jha S, Jha TB & Mahato SB (1988) Tissue culture of *Artemisia annua* L. – a potential source of an antimalarial drug. Current Sci. 57: 344–346

Klayman DL (1985) Qinghaosu (artemisinin): an antimalarial drug from China. Science 228: 1049–1055

Kudakasseril GJ, Lam L & Staba EJ (1987) Effect of sterol inhibitors on the incorporation of ^{14}C-isopentenyl pyrophosphate into artemisinin by a cell-free system from *Artemisia annua* tissue cultures and plants. Planta Med. 53: 501–502

Li Q, Guo X, Jin R, Wang Z, Jian H & Li Z (1982) Clinical studies on treatment of cerebral malaria with qinghaosu and its derivatives. J. Trad. Chin. Med. 2: 125–130

Liersch R, Soicke H, Stehr C & Tüllner HU (1986) Formation of artemisinin during one vegetation period. Planta Med. 52: 387–390

Martinez BC & Staba EJ (1988) The production of artemisinin in *Artemisia annua* L. tissue cultures. Adv. Cell Cult. 6: 69–87

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 51: 473–497

Nair MSR, Acton N, Klayman DL, Kendrick K, Basile DV & Mante S (1986) Production of artemisinin in tissue culture of *Artemisia annua*. J. Nat. Prod. 49: 504–507

Park JM, Hu WS & Staba EJ (1989) Cultivation of *Artemisia annua* L. plantlets in a bioreactor containing a single carbon and a single nitrogen source. Biotechnol. Bioeng. 34: 1209–1213

Pras N, Visser JF, Batterman S, Woerdenbag HJ, Malingré ThM & Lugt ChB (1991) Laboratory selection of *Artemisia annua* L. for high artemisinin yielding types. Phytochem. Anal. 2: 80–83

Qinghaosu Antimalarial Coordinating Research Group (1979) Antimalarial studies on qinghaosu. Chin. Med. J. 92: 811–816

Ravindranathan T, Anil Kumar M, Menon R & Hiremath SV (1990) Stereo-selective synthesis of artemisinin. Tetrahedron Lett. 31: 755–758

Schmid G & Hofheinz W (1983) Total synthesis of qinghaosu. J. Am. Chem. Soc. 105: 624–625

Simon JE, Charles D, Cebert E, Grant L, Janick J & Whipkey A (1990) *Artemisia annua* L.: a promising aromatic and medicinal. In: Janick J & Simon JE (Eds) Advances in New Crops (pp 522–526). Timber Press, West Lafayette

Sing A, Vishwakarma RA & Husain A (1988) Evaluation of *Artemisia annua* strains for higher artemisinin production. Planta Med. 54: 475–476

Threlfall DR & Whitehead IM (1988) Co-ordinated inhibition of squalene synthetase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in cultures of *Nicotiana tabacum*. Phytochemistry 27: 2567–2580

Tawfiq NK, Anderson LA, Roberts MF, Phillipson JD, Bray DH & Warhurst DC (1989) Antiplasmodial activity of *Artemisia annua* plant cell cultures. Plant Cell Rep. 8: 425–428

Watson DG, Rycroft DS, Freer IM & Brooks CJW (1985) Sesquiterpenoid phytoalexins from suspended callus cultures of *Nicotiana tabacum*. Phytochemistry 24: 2195–2200

White NJ, Waller D, Crawley J, Nosten F, Chapman D, Brewster D & Greenwood BM (1992) Comparison of artemether and chloroquine for severe malaria in Gambian children. Lancet 339: 317–321

Wingate VPM, Lawton MA & Lamb CJ (1988) Glutathione causes a massive and selective induction of plant defense genes. Plant Physiol. 87: 206–210

Woerdenbag HJ, Lugt ChB & Pras N (1990) *Artemisia annua* L.: a source of novel antimalarial drugs. Pharm. Weekbl. Sci. Ed. 12: 169–181

Woerdenbag HJ & Pras N (1991) *Artemisia annua* L. – Der einjährige Beifuß. Eine traditionelle Arzneipflanze als Quelle für neue Antimalariamittel. Z. Phytother. 12: 133–139

Woerdenbag HJ, Pras N, Bos R, Visser JF, Hendriks H & Malingré ThM (1991) Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. Phytochem. Anal. 2: 215–219

Woerdenbag HJ, Pras N, Van Uden W, De Boer A, Batterman S, Visser JF & Malingré ThM (1992) High peroxidase activity in cell cultures of *Artemisia annua* with minute artemisinin contents. Nat. Prod. Lett. 1: 121–128

World Health Organization (1986) The development of artemisinin and its derivatives: report of a meeting of the scientific working group on the chemotherapy of malaria. WHO, Geneva

Xu XX, Zhu J, Hung DZ & Zhou WS (1986) Total synthesis of arteannuin and deoxyarteannuin. Tetrahedron 42: 819–828