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Selection and Micropropagation of High Artemisinin Producing Clones of *Artemisia annua* L. Part II*. Follow up of the Performance of the Micropropagated Clones

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ABSTRACT. *Artemisia annua* plants were selected from seed-grown plants in the season 1990/1991 according to the morphological characters previously identified as markers for high artemisinin production. These were micropropagated by *in vitro* techniques as previously described. The performance of these micropropagated clones and those of the seasons 1989-1991 was followed up after transfer to the field for two seasons. They were compared to the mother plants in terms of morphological characters and their content of artemisinin and related sesquiterpenes, as determined by reversed-phase HPLC. It was found that the identified morphological characters are suitable for selection of high artemisinin-producing clones of *A. annua*. Several micropropagated clones with relatively high contents of artemisinin and/or artemisinic acid are reported. The optimum time for the transfer of the micropropagated plants to the field in Riyadh was identified to be late October to early November. One to two years of maintenance in culture during micropropagation was consistant with the preservation of the plant's characters and sesquiterpenes contents. The parasite, *Orobanche cernua* Loefl. was identified as a root parasite of *A. annua* that may cause heavy losses in the biomass yield.

The Chinese herbal plant *Artemisia annua* L. (Compositae) has received increased attention in recent years as the source of the reputed antimalarial agent artemisinin (qinghaosu) **1** (Klayman 1985). Artemisinin and its semisynthetic derivatives are

*Part I (Elhag *et al.* 1992).

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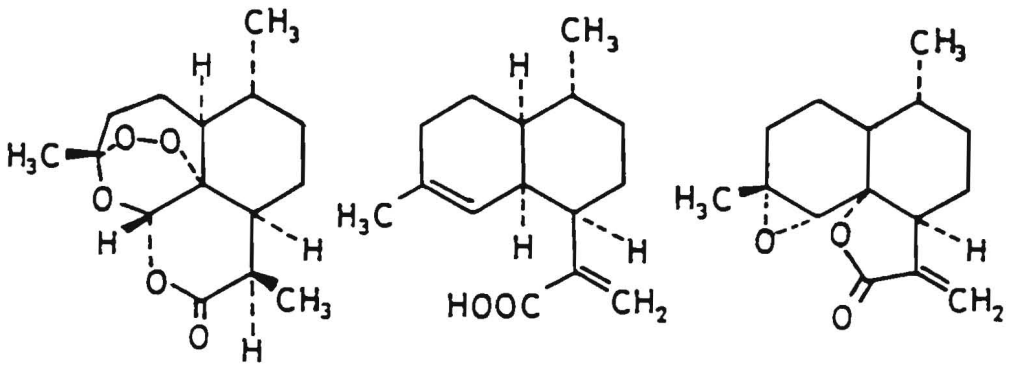
clinically important since they are effective in the treatment of acute cerebral malaria (Klayman 1985). These compounds are particularly effective against most chronic forms of malaria resistant to traditional antimalarial chemotherapeutic agents, including chloroquine and mefloquine. They have the added advantage of not presenting cross-resistance with most existing antimalarial agents (Klayman 1985, China Co-operative Research Group 1982).



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The yield of artemisinin from *A. Annua* has been shown to be variable and low to be suitable for commercial production (Martinez and Staba 1988, Charles *et al.* 1990, Fulzele *et al.* 1991). Moreover, although chemical synthesis of artemisinin has been reported (Brown 1994), it does not warrant its economical production. Therefore, efforts have been recently directed towards enhancing the yield of artemisinin within the plant itself or through the bioconversion of its natural precursors artemisinic acid **2** and arteannuin B **3** (Roth and Acton 1989, El-Feraly *et al.* 1986, Akhila *et al.* 1990, Jung *et al.* 1990, Haynes and Vonwiller 1994).

In a previous publication, certain morphological characteristics were identified as taxonomic markers associated with relatively high artemisinin production (Elhag *et al.* 1992). This was described as tall robust plants, with long internodes, open branching, dense leaves and thick stems.

The purpose of the present investigation was to select new clones of *A. annua* conforming with the taxonomic characteristics identified as associated with high

artemisinin production and to follow the performance of the micropropagated clones thereof and the previously selected clones, in terms of their contents of artemisinin and related sesquiterpenes, in comparison to the mother plants.

Experimental

Plant Material. Selection of *A. annua* mother plants (MP) during 1989/1990 season was previously reported (Elhag *et al.* 1992). During 1990/1991 season, however, additional mother plants were selected so as to conform with the morphological characteristics identified to be associated with relatively high productivity of artemisinin **1** and the related sesquiterpenes artemisinic acid **2** and arteannuin B **3**. Selection was done before flowering when the plants were 4 months old.

Micropropagation. Shoot tips from each of the selected plants were collected, surface-disinfected and cultured on MS-A (Murashige and Skoog's shoot multiplication medium A) as previously reported (Elhag *et al.* 1991). Shoot multiplication has also been achieved on MS B5 basal medium, supplemented with 3.0 mg. L^{-1} benzyladenine (BA) and 0.3 mg. L^{-1} indoleacetic acid (IAA) (data not presented). Multiplied shoots were rooted on 1/2 MS medium without hormones and were maintained in culture for 1-2 years by regular transfer of shoot tips every 4-6 weeks to fresh 1/2 MS rooting medium.

The micropropagated clones (~ 20 plants per clone), were transferred from the rooting medium by gently washing away the adhering agar from the roots and planting into a soil mixture [peat-sand (2:1)] in small plastic pots. After one month in the greenhouse, the cloned plants (CP) were transplanted to the field and grown on ridges. The proper time of transfer to the field was late October to early November. During their growth in the field, the plants (CP as well as MP) received three applications of urea fertilizer (100 kg/hectar) and several waterings and weedings as necessary.

At the onset of flowering, each plant (MP or CP) was harvested and dried separately at R.T. in the shade. The dried leaves stripped off from each micropropogated clone were pooled and the average dry weight of leaves per plant was determined, while the leaves from each mother plant were kept separate. The dried leaves from each MP and CP were subjected to analysis.

Extraction. The method of extraction and column chromatographic separation of the main sesquiterpenes was performed as previously reported (Elhag *et al.* 1992).

HPLC determination of artemisinin 1, artemisinic acid 2 and arteannuin B 3. HPLC determination of artemisinin and the two other main sesquiterpenes was done as previously reported (Elhag *et al.* 1992). The HPLC apparatus and operating conditions were also the same; an internally standardized reversed-phase HPLC system, using a UV detector, was used to determine artemisinin **1**, artemisinic acid **2** and arteannuin B **3** in the various clones and in the respective mother plants.

Results and Discussion

The data in Tables 2 and 3 represent the clones which have been successfully established in culture and in the field and therefore were subjected to HPLC analysis for artemisinin and the related sesquiterpenes in comparison with the respective mother plants (Table 1). The vegetative growth period in the field extended from late October or early November until the onset of flowering in late April. Under our field conditions, the onset of flowering coincided with the rise in temperature which marks the beginning of summer in Saudi Arabia. When the time of transfer to the field was delayed to December or January (off season), the resulting plants were weak, small, of low biomass and of low sesquiterpene content (data not shown). Thus the time of transfer of the clones to the greenhouse and subsequently to the field seems to be critical under our conditions.

Maintenance of the micropropagated clones in culture for one (1990/1991 MP selection) or two years (1989/1990 MP selection) did not seem to affect the characteristics of the clones regarding the morphological characters or the sesquiterpene contents, provided that the clones were transferred to the field at the proper time (late October to early November). However, it was observed that prolonged maintenance in culture, resulted in changes in the growth habit of certain clones from robust plants of high biomass to small rosette plants of low biomass. Rosette plants lacked the main branch and consisted of several lateral branches originating from the top of the root system. The factor(s) responsible for the induction of rosetteness in *A. annua* is not known at present and would be the subject of a future investigation.

Table 1. Selected *Artemisia annua* (Mother) Plants (Seasons 89/90 and 90/91)

Mother Plants (MP)	Morphological characteristics		Biomass (Dry wt. of leaves) g/plant)	Main sesquiterpenes g % (w/w)		
	Growth habit	Plant Height (cm)		Art. A	Art. B	Artemisinin
MP8	Dwarf (rosette), green stem and dark green leaves, strong odor	85	80.0	1.15	0.15	0.028
MP16	upright, robust, open branching, reddish thick stem, late flowering	100	210.0	0.92	0.14	0.047
MP27	upright, robust, open branching, thick stem and light green leaves	100	305.0	0.81	0.13	0.041
MP28	upright, robust, open branching, thick stem and dark green leaves	125	156.5	0.31	0.11	0.052
MP29	upright, robust, open branching, thick stem and dark green leaves	140	190.5	0.54	0.13	0.083
MP31	upright, robust, open branching, thick stem and dark green leaves	97	157.5	0.59	0.11	0.048
MP35	upright, robust, open branching, thick stem and dark green leaves	140	182.5	0.32	0.09	0.039
MP36	upright, robust, open branching, thick stem and dark green leaves	135	182.0	0.38	0.05	0.033
MP38	upright, robust, open branching, reddish thick stem and small dark green leaves	131	103.4	0.38	0.10	0.052

Table 2. Selected Micropropagated Clones of *A. annua* (Seasons 91/92)

Clone (cp)	Morphological characteristics		Biomass (Dry wt. of leaves) g/plant)	Main sesquiterpenes g % (w/w)		
	Growth habit	Plant Height (cm)		Art. A	Art. B	Artemisinin
CP8	Rosette, small, closed branching, dark green leaves,	86	41.18	0.54	0.15	0.077
CP16	upright, open branching, light green leaves (uniform plant size)	128	72.00	0.46	0.18	0.023
CP27	upright, robust, open branching, light green leaves	144	135.53	0.58	0.12	0.061
CP28	upright, robust, open branching, dark green leaves, tall plant	179	271.43	0.54	0.24	0.048
CP29	upright, tall, robust, closed branching, dark green stem and leaves	172	177.50	0.28	0.17	0.060
CP31	upright, robust, open branching, dark green leaves	149	127.77	0.50	0.14	0.050
CP35	rosette, robust, dark green leaves, tall branches	147	196.66	0.30	0.09	0.037
CP36	upright, robust, open branching, dark green leaves	150	154.55	0.42	0.07	0.032
CP38	upright, robust, open branching, dark green leaves	178	337.50	1.14	0.24	0.050

Table 3. Selected *micropropagated* clones of *A. annua* (Seasons 92/93*)

Clone (cp)	Morphological characteristics		Biomass** (Dry wt. of leaves) g/plant)	Main sesquiterpenes g % (w/w)		
	Growth habit	Plant Height (cm)		Art. A	Art. B	Artemisinin
CP8	small, mixed rosette and upright, closed branching, dark green leaves	85.0	11.0	0.75	0.16	0.051
CP27	medium, upright, open branching, light green leaves	116.3	115.0	0.60	0.16	0.102
CP28	small, upright, closed branching, angular stem, dark leaves	91.5	17.0	0.32	0.22	0.052
CP29	medium, upright, open branching, dark green leaves	141.0	91.0	0.54	0.16	0.060
CP35	medium, upright, open branching, angular stem, dark green leaves	112.0	65.0	1.20	0.17	0.039
CP36	medium, upright, open branching, angular stem, dark green leaves	124.6	52.0	1.06	0.15	0.044
CP38	medium, upright, open branching, angular stem, dark green leaves	124.0	101.0	0.64	0.13	0.032

*Clones CP16 and CP31 were lost in this season and are therefore not included.

**The low biomass yield observed in this season is attributed to the *Orobanche* root parasite.

Although there were variations from season to season in terms of plant height and biomass, in each season each clone remained uniform. In 1991/1992 season, certain micropropagated clones (CP28, CP35 and CP38) outweighed their mother plants (Tables 1 and 2). This was often associated with increases in the content of artemisinic acid (CP28 and CP38), but not other sesquiterpenes (Table 2); in fact the contents of arteannuin B were lower in the three clones as compared to the mother plants. Clone CP38 (Table 2) showed the highest content of artemisinic acid (1.14%) in the 1991/1992 season and gave the highest biomass yield. In 1992/1993 season (Table 3), higher contents of artemisinic acid were observed for CP35 (1.20%) and CP36 (1.06%); it should also be noted that clone CP27 produced the highest content of artemisinin (0.102%) in that season. This reflects an improvement in the production of such sesquiterpenes over previous seasons. Whether such notable increases in the levels of artemisinic acid and artemisinin was brought about by the *in vitro* culture conditions or was due to a more effecient biogenetic conversion (Akhila *et al.* 1990, Sangwan *et al.* 1993) of the related sesquiterpenses within the plant, is not known at this point.

In conclusion, micropropagation of *A. annua* of high artemisinin and artemisinic acid contents, selected according to the previously set parameters (Elhag *et al.* 1992) would be useful for higher production of these useful sesquiterpenes. One to two years of maintenance in culture during micropropagation was consistant with preservation of the plant's characters and sesquiterpene contents, provided that the clones were transferred to the field at the proper time (late October to early November). Clones CP27 and CP38 may be useful for large scale production of artemisinin. Based on the possibility of the chemical conversion of artemisinic acid into artemisinin (Roth and Aton 1989, El-Feraly *et al.* 1986, Jung *et al.* 1990, Haynes and Vonwiller 1994), clones CP27, CP35, CP36 and CP38 could be useful for large scale production of artemisinic acid and hence artemisinin.

Finally, we wish to report on the appearance of a root parasite *Orobanche cernua* Loefl. (kindly identified by Dr. S. Ul-Abdin, Taxonomist, MAPPRC, College of Pharmacy, KSU, Riyadh, S.A.) with *A. Annua* plantations. In the absence of *Artemisia* plants in the field, the seeds of the *Orobanche* parasite failed to germinate. This parasite which grows on the root system of *A. annua,* was difficult to control, except by frequent weeding around the root system and resulted in severe reduction in the biomass yield particularly in 1992/1993 season (Table 3).

References

Akhila, A., Rani, K. and **Thakur, R.S.** (1990) Biosynthesis of artemisinic acid in *Artemisia annua*. *Phytochemistry*, **29:** 2129-2132.

Brown, G.D. (1994) Cadinanes from *Artemisia annua* that may be intermediates in the biosynthesis of artemisinin. *Phytochemistry*, **36:** 637-641.

Charles, D.J., Simon, J.E., Wood, K.V. and **Heinstein, P.** (1990) Germplasm variation in artemisinin content of *Artemisia annua* using an alternative method of artemisinin analysis from crude plant extracts. *J. Nat. Prod.*, **53:** 157-160.

China Co-operative Research Group (1982) Qinghaosu and its derivatives as antimalarials. *J. Trad. Chin. Med.*, **2:** 45-50.

El-Feraly, F.S., Al-Meshal, I.A., Al-Yahya, M.A. and **Hifnawy, M.S.** (1986) On the possible role of qinghao acid in the biosynthesis of artemisinin. *Phytochemistry*, **25:** 2777-2778.

Elhag, H., El-Feraly, F., Mossa, J.S. and **Hafez, M.** (1991) *In vitro* propagation of *Artemisia annua* L. *J. King Saud Univ., Agric. Sci.*, **3:** 251-259.

Elhag, H.M., El-Domiaty, M.M., El-Feraly, F.S., Mossa, J.S. and **El-Olemy, M.M.** (1992) Selection and micropropagation of high artemisinin producing clones of *Artemisia annua* L. *Phytother. Res.*, **6:** 20-24.

Fulzele, D.P., Sipahimalani, A.T. and **Heble, M.R.** (1991) Tissue Cultures of *Artemisia annua:* organogenesis and artemisinin production. *Phytother. Res.*, **5:** 149-153.

Haynes, R.K. and **Vonwiller, S.C.** (1994) Extraction of artemisinin and artemisinic acid: preparation of artemether and new analogues. *Transac. Royal Soc. Trop. Med. and Hyg.*, **88** (Supplement 1): 23-26.

Jung, M., ElSohly, H.N. and **McChesney, J.D.** (1990) Artemisinic acid: a versatile chiral synthon and bioprecursor to natural products. *Planta Med.*, **56:** 624.

Klayman, D.L. (1985) Qinghaosu (Artemisinin): An antimalarial drug from China. *Science*, **228:** 1049-1055.

Martinez, B.C. and **Staba, J.E.** (1988) The production of artemisinin in *Artemisia annua* L. tissue cultures. *Adv. in Cell Cult.*, **6:** 69-87.

Roth, R.J. and **Acton, N.** (1989) A simple conversion of artemisinic acid into artemisinin. *J. Nat. Prod.*, **52:** 1183-1185.

Sangwan, R.S., Agarwal, K., Luthra, R., Thakur, R.S. and **Neelam, S.S.** (1993) Biotransformation of arteannuic acid into arteannuin-B and artemisinin in *Artemisia annua*. *Phytochemistry*, **34:** 1301-1302.

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انتخاب واكثار سلالات من نبات *Artemisia annua* L. ذات انتاج عال لمادة أرتميسينين ومشتقاته

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يعتبر نبات *Artemisia annua* L. من أهم النباتات الطبية والاقتصادية نظراً لاحتوائه على مادة الأرتميسينين والتي تستخدم ومشتقاتها بنجاح في علاج الملاريا ، وفي هذا البحث تم انتخاب عدد من نباتات *A. annua* من مجموعة النباتات المنزرعة في الحقل في موسمي ١٩٨٩/ ١٩٩٠ و ١٩٩٠/ ١٩٩١م . وكان الانتخاب على أساس صفات الشكل الظاهري التي سبق تحديدها مثل حجم وكثافة النبات وشكل ولون الساق والأوراق لارتباط ذلك بزيادة الكثافة الورقية وانتاج مادة الارتميسينين . كذلك تم اكثار وحفظ السلالات المنتخبة بطريقة زراعة الأنسجة التي تعتمد على نقل القمم الخضرية ، وفي موسمي ١٩٩١/ ١٩٩٢ و ١٩٩٢/ ١٩٩٣م نقلت السلالات إلى الحقل وتم تدوين ملاحظات نموها ، ووجد أنها مشابهة للنبات الأم من حيث الشكل الظاهري وأن حفظها لمدة عام أو عامين على بيئة غذائية مستحضرة لا يؤثر على هذه الصفات فيما عدا التباين الموسمي للكثافة الورقية ، وتبين أن بعض السلالات تفوقت على النبات الأم الذي أخذت منه من حيث كثافة الأوراق وانتاج مادة الأرتميسينين وحمض الأرتميسنيك ، ومن بين السلالات التي تم اكثارها ونقلها

إلى الحقل تعتبر سلالات ٢٧ و ٣٨ من أفضل السلالات انتاجاً للأرتميسينين وحمض الأرتميسنيك على أساس الانتاجية العالية للأوراق أو المحتوى العالي لتلك المواد ، والجدير بالذكر أن حمض الأرتميسينك والذي يوجد في النبات بنسبة تفوق بكثير نسبة الأرتميسينين ويمكن تحويله إلى الأرتميسينين بطرق كيميائية مناسبة ، وعلى هذا فتعتبر سلالات ٢٧ ، ٣٥ ، ٣٦ ، ٣٨ هامه في هذا المجال .

وقد وجد أن وقت نقل السلالات إلى الحقل له أهمية قصوى بالنسبة لنمو النبات وانتاجيته من الأوراق ، وحدد هذا الوقت بنهاية أكتوبر وأوائل نوفمبر حسب مناخ منطقة الرياض ، كذلك يلفت البحث الانتباه لوجود نبات طفيلي من نوع الهالوك *Orobanche cernua* Loefl. والذي ينمو فقط على جذور نبات الـ *Artemisia* دون سواها ويقلل من انتاجها الورقي بدرجة كبيرة .