Yields of Antimalarial *Artemisia annua* L. Species

P.M. De Magalhães, B. Pereira and A. Sartoratto
Centro de Pesquisas Químicas, Biológicas e Agrícolas – CPQBA
Universidade Estadual de Campinas. pedro@cpqba.unicamp.br
C.P. 6171. CEP: 3.081-970 Campinas –SP Brazil

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Abstract

Since the development of the selection program for *Artemisia annua* L. hybrid lines of high artemisinin content and agricultural improvement within the program at CPQBA-UNICAMP, new selections have been annually evaluated for biomass yields, rates between leaves and stem, artemisinin content, and essential oil (composition and yields). Among the genotypes were evaluated during the period of November, 2000, to March, 2001, at CPQBA-UNICAMP, in Campinas-SP, Brazil, artemisinin ranged from 1.69 to 2.01 g/m^2. The essential oil yield and composition of a population cultivated in large-scale exhibited variations by phenologic stages: at booming, 0.40 %; in mid flowering, 0.30 %; and close to senescence stage 0.21 %. In the same phenological stages, some of the major compounds of essential oil also varied, respectively: 1, 8 cineole, 17.06 %, 21.88 %, and 28.6 %; camphor: 28.44 %, 14.89 % and 30.87 %. The results provide parameters to develop raw material with antimalarial applications as well as to characterize the essential oil obtained from large-scale cultivation.

INTRODUCTION

At least two new products have been acquired from the genetic improvement of the *A. annua* L. (Asteraceae), first achieved in Switzerland by MEDIPLANT and later established in Brazil by CPQBA-UNICAMP where the species has been studied in experimental cultivations (Magalhães, 1996; Delabays, 1997). One of these products has a strong social component which is the possibility to produce low cost medicine for the treatment of the malaria from standardized raw material with a high level of the antimalarial active principle, artemisinin, to be used by populations that live in those affected regions. In this direction, preliminary projects are being conducted in Congo, Africa, where simple and good quality medicines are obtained from standardized leaves of *A. annua* (Mueller et al., 2000). Spitteler (1999) reported the use of pills mainly made with dry leaves of *A. annua* genotypes rich in artemisinin in Tanzania for this same purpose. Another product is the essential oil extracted from leaves and inflorescence of this species. In India, *A. annua* oil is largely used in the composition of cosmetic and hygienic products, which could lead to the opportunity to be produced and commercialized also in the West, as long as one has the superior genotypes and technology of cultivation. The breeding program of *A. annua* conducted at CPQBA started from a great variability of populations where the artemisinin yields were approximately of 1 Kg of artemisinin per hectare. The plants displayed heterogeneous morphology, growing cycle, and composition. With such great variability, the development of the standard raw material was impossible.

With the introduction of superior genotypes from Vietnam and the selection techniques at Mediplant, Switzerland, development of specific hybrids for Brazilian conditions, which are today homogeneous and with high biomass, essential oil, and artemisinin yields was possible. The current goal is to obtain the phytomedicines where the uniformity is essential for a precise dose in not so elaborated medicine forms. However the process is a long and arduous one requiring many years and still requires significant additional work to be conducted. The leaf:stem ratio, the yields from essential oil composition during the growth and artemisinin percentage of the new hybrids are some of the parameters evaluated in this work.

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MATERIALS AND METHODS

In 2000-2001 a field trial with 3 new hybrids of *A. annua* and a culture of 0.5 ha was conducted at CPQBA-UNICAMP. The sowing was done in a greenhouse with 50 % shade in the beginning of September, 2000. After 2.5 months, due to low fertility of the used substratum, the plants were transferred to the experimental station. The field plots were established as randomized block design with 4 replications. Each plot consisted of 25 plants, at 0.6 m between plants and 1 m between the rows. Only the 9 interior plants of each plot were evaluated. The hybrid lines from CPQBA were: 3 Mx5; ½ B2; and H x 3 M. The parameters measured included: weight of dry leaves per plant; weight of small stems per plant, artemisinin percentage and artemisinin yield per square meter. The harvest was done in March, 2001, by manually cutting the plants at the ground level and discarding the main stems. The drying done through in a gas drier at 40 C^{o} until reaching constant weight. The artemisinin analyses were done by HPLC (Prass et al., 1991). The large-scale production was transplanted into the field in December, 2000 and harvested in April, 2001. In this area, yields and the composition of essential oil were analyzed. The oil was distilled in a 210 L still of by steam passing through the fresh material for one hour. Approximately 35 Kg of fresh material was distilled in each one of the three phenological stages of the culture: close to the early bloom (only leaves and stems), at full bloom (flowering branches), and post bloom when both, flowers and fruits/seeds, were present.

Qualitative analysis of the essential oil was done using a HP-5890 II GC coupled to a HP-5971 mass selective detector fitted with a 25 m × 0.20 mm × 0.33 μm HP-5 capillary column. The operational temperatures were: injector = 220 C°; detector = 280 C° and column oven = 60 C° $\rightarrow$ 3 C°·min^{-1} to 240 C° $\rightarrow$ 7 min at 240 C°. Chromatographic grade helium (1.0 mL·min^{-1}) was the carrier gas. Identification of the detected oil constituents was accomplished by matching their mass spectra with the available databases (NIST-98 and Wiley-198), and confirmed by comparing their retention indexes with literature data and by co-injection of the pure compounds, when available.

RESULTS AND DISCUSSION

The artemisinin levels of the genotypes (Table 1) are relatively smaller than the average reached in previous years, which was of 1 % (Magalhães et al., 1997). This occurred because of a delay in planting, therefore, the plants had less time to develop as the harvest point is fixed when the day length is smaller than 12 and this species begins flowering. Ferreira, 1995, made studies on day length inducing flowering in *A. annua,* showing that a photoperiod of 12 hours induces flowering.

But even with these artemisinin contents, smaller than the tested hybrids´ potential, we observed no significant difference among the selections. The same observation was valid for other parameters, such as leaves and stem biomass, which do not differ among these genotypes. The biomass yields were also limited by fewer days of cultivation in the field, while the average of the other hybrids coming from the same introduced population has been of 150 g of dry leaves/plant (Magalhães et al., 1997). The artemisinin yield could have profited from the practice of leaving the harvested material exposed to the sun for a week Under such condition, Simonnet et al., 2001 demonstrates that the artemisinin content can increase by 20 %, probably due to the transformation of the artemisinin precursors' active molecules by photo-oxidative reactions. In the large-scale cultivation that was transferred to the field even later, almost in the inductive period of flowering, we decided to follow the essential oil composition and yields in the different stages of culture.

A. annua essential oil has potential use in the making of sanitary and cosmetic products, already commercialized in India. Possibly, the oil could have a protective action on the artemisinin molecule; hypothesis based on the fact that the tea obtained from the *A. annua* leaves has presented therapeutic effect in the malaria treatment with much smaller doses than the recommended when using artemisinin purely. One possibility to explain it, is that the oil could be protecting the active molecule, artemisinin, from degradation in the

human organism, leaving the molecule acting longer or even acting synergistically, increasing the artemisinin penetration into the *Plasmodium* membrane. We observed that the major volatile aromatic constituents were: camphor, 1,8 cineole, β-myrcene and β-germacrene. Little change in concentration was observed across the phenologic stages. The 1,8 cineole increases with plant maturity while β-myrcene decreases. The β-germacrene and trans-cariophylene had a large decline in phase 3. The essential oil content was greatest at the beginning of flowering; however, the productivity (weight of plants per area) is greater closer to senescence due to the heavier weight of the seeds.

The yield of essential oil per area was also greater closer to senescence as shown by Foglio, 1996. In the essential oil, 2 groups of compounds of the essential oil of *A. annua* (region of Bio-TLC) exhibit activity against *Staphylococcus aureus and Enterococcus faecium* and 3 groups of compounds that exhibit action against *Streptococcus faecium* and *Bacillus subtilis* (Table 3).

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Tables

Table 1. Yields of *Artemisia annua* hybrids evaluated in 2000-2001, Brazil.

Hybrids	Artemisinin (%)	Leaves (g dw/plant)	Small stems (g dw/plant)	Artemisinin (g/m^2)
3M x 5	0.84a	143.2a	144.2a	2.01a
½ B_2	0.77a	131.7a	127.8a	1.69a
Hybrid x 3M	0.87a	126.4a	130.6a	1.84a

Means followed of the same letter were not different at level of 5 % of significant.

Table 2. Relative volatile oil composition from leaves of *A. annua* at 3 phenologic stages: (1) beginning of flowering, (2) branches with inflorescence and (3) post-flowering, close to senescence.

Compounds	**R.I.**	**(1)%**	**(2)%**	**(3)%**
α-pinene	931	1.89	1.84	2.38
canphene	945	2.86	2.67	4.24
sabinene	971	4.14	4.58	3.97
β-myrcene	990	7.89	12.41	4.09
α-terpinene	1018	0.41	0.39	0.53
p-cymene	1022	1.23	N.E.	N.E.
1,8 cineole	1028	17.06	21.88	28.76
γ-terpinene	1060	1.03	1.56	0.73
α-terpinolene	1085	0.11	0.10	0.16
camphor	1139	28.44	14.89	30.87
α-terpineol	1187	1.46	1.31	1.15
carvone	1240	0.10	0.12	0.13
trans-caryophyllene	1414	3.28	2.97	1.68
β-farnesene	1457	3.18	2.92	2.40
germacrene B	1552	5.85	5.30	0.34
Essential Oil (total %)	----	0.40	0.30	0.21

N.E.= "the value was not evaluated" R.I. = Retention Index

Table 3. Anti microorganism activity from essential oil of *A. annua.*

Microorganisms	**Standard (Cloranfenicol)**	**Essential Oil of *A. annua*** (20 mg/mL)
Staphylococcus aureus	+	++
Streptococcus faecium	+	+++
Salmonella	+	-
Pseudomonas	+	-
Bacillus subtilis	+	+++
Enterococcus faecium	+	++
Staphylococcus epidermides	+	-
Rhodococcus	+	-
Micrococcus	+	-
Escherichia coli	+	-
Candida albicans	+	-

(-) "There was no activity", (+) The number of positive signs means that there was biologic activity at many different regions of the TLC plaque, indicating different chemistry groups to control the microorganism in question.