Willemien J. M. Lommen[1]
Sytze Elzinga[1]
Francel W. A. Verstappen[2]
Harro J. Bouwmeester[2]

Artemisinin and Sesquiterpene Precursors in Dead and Green Leaves of *Artemisia annua* L. Crops

Abstract

This paper analyses the accumulation and concentrations of the antimalarial artemisinin in green and dead leaves of *Artemisia annua* crops in two field experiments. Concentration differences were analysed as being determined by (a) the total production of artemisinin plus its upstream precursors dihydroartemisinic acid, dihydroartemisinic aldehyde, artemisinic aldehyde and artemisinic alcohol and (b) the conversion of precursors towards artemisinin. Concentrations of the total of artemisinin plus its precursors were higher in green leaves than in dead leaves in the younger crop stages, but were comparable at the final harvests. In every crop stage, the conversion of precursors to artemisinin was more advanced in dead leaves than in green leaves. This resulted in the molar concentrations of artemisinin being higher in dead leaves than in green leaves at the final harvests. The molar quantity of dihydroartemisinic acid, the last enzymatically produced precursor, was higher than that of artemisinin in green leaves, but only 19–27% of that of artemisinin in dead leaves. Dead leaves were very important for the final artemisinin yield. They constituted on average 34% of the total leaf dry matter and 47% of the total artemisinin yield at the final harvests. The possibility to convert a larger part of dihydroartemisinic acid into artemisinin during post-harvest handling is discussed.

Key words
Artemisia annua · Asteraceae · artemisinin biosynthesis · dihydroartemisinic acid

Introduction

Artemisinin, a sesquiterpene lactone with an endoperoxide bridge, is an important antimalarial produced by *Artemisia annua* L. (annual wormwood, Asteraceae). *A. annua* is an annual herb originating from Asia [1] and thus far the only economical source of artemisinin. Artemisinin and some of its semi-synthetic derivatives, e.g., dihydroartemisinin, artesunate, arteether and artemether [2], are highly effective against *Plasmodium falciparum* and *P. vivax*, two of the four *Plasmodium* species causing malaria, with *P. falciparum* causing the severe malaria tropica. Artemisinin is produced almost exclusively in the leaves and inflorescences [3], in glandular trichomes [4], [5]. Leaves are the most important organs for commercial production, because the crop is generally harvested before full flowering.

The biosynthetic pathway by which artemisinin is formed (Fig. **1**) was recently elucidated for the Vietnamese genotype that was used in our study [6]. The first step in artemisinin formation is the cyclisation of farnesyl diphosphate to amorpha-4,11-diene [7]. This sesquiterpene is oxidised to artemisinic alcohol and artemisinic aldehyde [6], [7], [8]. Artemisinic aldehyde is now either reduced at the C-11/C-12 double bound to yield dihydroartemisinic aldehyde and subsequently oxidised to dihydroartemisinic acid [6] or it is further oxidised to yield artemisinic acid [9]. It is as yet unclear how artemisinic acid would then be further converted to dihydroartemisinic acid as Bertea et al. [6] did not find any enzymatic evidence that supports such a conversion. Dihydroartemisinic acid can be converted non-enzymatically into artemisinin [10], [11], [12].

Affiliation
[1] Crop and Weed Ecology Group, Wageningen University, Wageningen, The Netherlands
[2] Plant Research International, Wageningen University and Research Centre, Wageningen, The Netherlands

Correspondence
Dr. W. J. M. Lommen · Crop and Weed Ecology Group · Wageningen University · Haarweg 333 · 6709 RZ Wageningen · The Netherlands · Fax: +31-317-485-572 · E-mail: Willemien.Lommen@wur.nl

Received February 22, 2007 · **Revised** May 11, 2007 · **Accepted** June 11, 2007

Bibliography
Planta Med 2007; 73: 1133–1139 © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2007-981567 · Published online July 12, 2007
ISSN 0032-0943

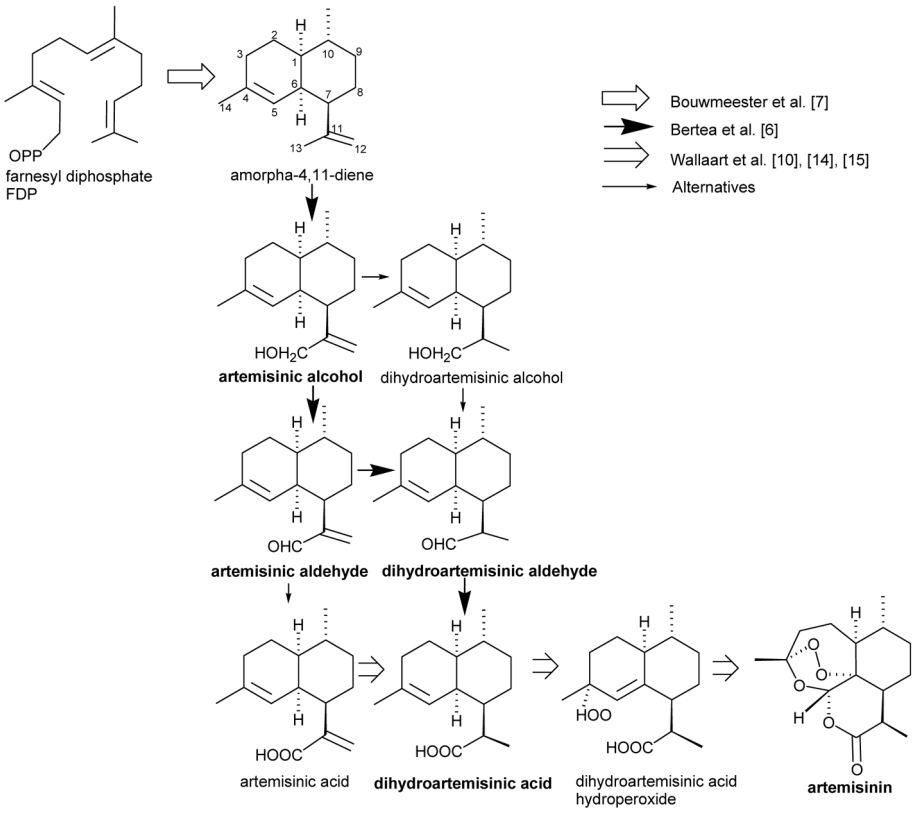

Fig. **1** Biosynthetic pathway for conversion of farnesyl diphosphate to dihydroartemisinic acid and proposed pathway for conversion of dihydroartemisinic acid to artemisinin, as suggested by different authors. **Bold:** intermediates assessed in this paper.

Recent studies on artemisinin accumulation in individual leaves within a crop have shown that during the life cycle of an individual leaf, artemisinin quantities per leaf were low at leaf appearance and increased steadily thereafter [13]. In leaves studied until after senescence, maximum quantities and concentrations were always achieved when the leaf had already turned brown. This suggests that the dead leaves within an *A. annua* crop might be an important source of artemisinin. In these leaves the conversion of precursors to artemisinin might be more advanced than in green leaves. However, not just the degree to which the conversion to artemisinin is completed will determine artemisinin yields and concentrations, but also the total production of precursors.

This paper studies how concentrations of artemisinin and its precursors change during crop development in dead and green leaves, compares the two leaf fractions and analyses the artemisinin production as a function of (a) the production of artemisinin plus precursors and (b) the degree to which the compounds in this pool are converted to artemisinin. This will show the importance of dead leaves for the total artemisinin production and will reveal if there are still possibilities of increasing artemisinin concentrations after harvest by stimulating further conversion of precursors into artemisinin.

Materials and Methods

Plant material and experimental design

A. annua crops (Asteraceae) were grown in two field experiments. Both experiments were block experiments with five replicate blocks and two (Experiment 1) or three (Experiment 2) harvest dates. Experiment 1 was carried out from May 21, 2002 – September 10, 2002 in Wageningen (N51°59′31″ E005°39′08″), using 49-day-old transplants from a Vietnamese genotype described earlier [6], [7], [13], [14]. Experiment 2 was carried out from June 19, 2002 – October 29, 2002 in Achterberg (N51°59′24″ E05°34′58″), using 53-day-old transplants from the hybrid cultivar Anamed A3 (Anamed; Winnenden, Germany), described earlier by Lommen et al. [13]. Transplants were raised under natural day length in glasshouses from April 2, 2002 – May 14, 2002 (Experiment 1) and April 26, 2002 – June 7, 2002 (Experiment 2) and were hardened outside for 7 (Experiment 1) and 12 (Experiment 2) days. Transplants were planted mechanically at a distance of 75 cm between rows and 20 cm within rows to 45 m^2 plots (4.5 × 10 m) in sandy soil with KCl pH 5.0 (Experiment 1) and 4.5 (Experiment 2). Net plots were 2.16 m^2 and were surrounded by at least 2 guard rows and 7 guard plants in a row. The crops were fertilised with 74 kg ha^{-1} N, 94 kg ha^{-1} P_2O_5, and 62 kg ha^{-1} K_2O (Experiment 1), and 95 kg ha^{-1} N (Experiment 2). Harvests took place on July 16 (56 DAT, days after transplanting) and September 10 (112 DAT) in Experiment 1, and July 22 (33 DAT), September 2 (75 DAT) and October 29 (132 DAT) in Experiment 2. Average temperatures during the field period of the respective experiments were 17.2 and 15.3 °C, average photoperiods 15.4 and 13.6 h and average global radiation 1627 and 1296 J m^{-2} day^{-1}. Net plants did not flower during the experiments.

On a harvest date, total above-ground fresh weight of the net plot was recorded and its dry weight was determined by means of a dry matter sample. A separate sample of 4 plants from the net plot was taken and separated into green leaves (> 50% green area), dead leaves (≤ 50% green area; in general brown) and stems. Subsamples of the leaf fractions for phytochemical analy-

sis were taken at the latest on the second day after harvest after storing the fresh plant material at 4 °C, were dried in a fan-forced oven at 30 °C during 24 h and were kept dark and dry until analysis. About 300 mg of crushed dry leaf material were accurately weighed, ground with sea sand in a mortar and extracted three times with a total of 5 mL dichloromethane containing 20 µg mL^{-1} *cis*-nerolidol ($C_{15}H_{26}O$; Sigma; Zwijndrecht, The Netherlands) as internal standard for quantification of the GS-MS results. Phytochemicals analysed were artemisinic alcohol, dihydroartemisinic alcohol, artemisinic aldehyde, dihydroartemisinic aldehyde, artemisinic acid, dihydroartemisinic acid and artemisinin. Synthesis, isolation and analysis of the reference compounds have been described by Bertea et al. [6]. Samples of 2 µL were analysed by GC-MS on an HP 5980 series II gas chromatograph and HP5972A mass selective detector (70 eV) equipped with a HP-5MS column (95% dimethylpolysilane, 30 m × 0.25 mm i.d., film thickness 0.25 µm). The carrier gas was He (1 mL min^{-1}), the injection temperature 250 °C and detector temperature 290 °C. The oven was programmed for 2 min at 80 °C, then from 80 to 235 °C at 5 °C per min, then from 235 to 280 °C at 25 °C per min and kept at 280 °C for a final time of 5 min.

Statistical analysis

Analysis of variance (ANOVA) was conducted using Genstat 8.11 (Lawes Agricultural Trust; Rothamsted, UK) for comparisons between harvest dates. Means in Experiment 2 were compared using LSD tests at $\alpha < 0.05$. Paired t-tests were used to compare concentrations in green leaves to those in dead leaves.

Results

In green leaves, molar concentrations of artemisinin and the total quantity of artemisinin and its precursors in the leaf dry matter were higher in later harvests than in the earliest harvest in both experiments (Table **1**). The artemisinin precursors dihydroartemisinic acid, dihydroartemisinic aldehyde, artemisinic aldehyde and artemisinic alcohol were all detected in the green leaf fraction on all harvest dates, with dihydroartemisinic acid being the most abundant precursor (Table **1**). Molar concentrations of dihydroartemisinic acid did not differ significantly over harvest dates; those of dihydroartemisinic aldehyde were significantly higher in later harvests only in Experiment 2, of artemisinic aldehyde only in Experiment 1 and of artemisinic alcohol only in Experiment 2, although for the latter the concentration in the last harvest did not differ significantly from that in the first harvest (Table **1**). Just as for artemisinin, concentrations of precursors did not differ significantly between the last two harvests in Experiment 2. Molar concentrations of dihydroartemisinic alcohol and artemisinic acid in the green leaf fraction were not affected significantly by the harvest time (Table **1**).

In the dead leaves, the molar concentration of artemisinin increased with time in both experiments (Table **1**). In contrast to the concentrations in the green leaves where the increase levelled off in Experiment 2, the concentration in the dead leaves continued to increase over the whole season. The same was found for the total of artemisinin and its precursors. Some artemisinin precursors were not detected in the dead leaves of the youngest plants, 33 DAP in Experiment 2 (Table **1**), but at later harvest dates they were. Just as for artemisinin, their concentrations increased over time (Table **1**).

In the early and mid-term harvests, molar concentrations of artemisinin, the total of artemisinin plus precursors and all individual precursors and other relevant sesquiterpenes were higher in green leaves than in dead leaves (Table **1**), except for the concentrations of dihydroartemisinic aldehyde at 56 DAT in Experiment 1 and of artemisinic alcohol and artemisinic acid at 75 DAT in Experiment 2 for which the differences were not significant.

At the final harvests, however, artemisinin concentrations in both experiments were higher in dead leaves than in green leaves (Table **1**), whereas the total concentration of artemisinin and precursors did not differ over leaf fractions (Table **1**). The concentrations of the most abundant precursor dihydroartemisinic acid were lower in dead leaves than in green leaves also at the final harvests (Table **1**). Concentrations of the other precursors and of the other sesquiterpenes analysed did not differ between leaf fractions or did not show a consistent difference over experiments.

Profiles showing the relative molar fractions of artemisinin and its precursors indicate that the partitioning of the metabolites towards artemisinin was always more advanced in dead leaves than in green leaves (Figs. **2A** and **B**). The fraction dihydroartemisinic acid consistently was much higher in green leaves than in dead leaves (Figs. **2A** and **B**). At the final harvests, dihydroartemisinic acid constituted on average 48 and 15% of the total of precursors plus artemisinin in green and dead leaves respectively, whereas artemisinin constituted on average 39 and 67% of the total in green and dead leaves. The other precursors were less important.

Dead leaves constituted only a minor part of the total leaf dry mass in *A. annua* crops early in the field period, but by the final harvests the total dead leaf mass had reached almost 100 g/m^2, i.e., 1 t/ha (Figs. **3A** and **B**). Dead leaves constituted at that moment 31.4% and 37.0% of the total leaf mass in the respective experiments. Comparably, the contribution of the dead leaves to the total artemisinin production was small in early and mid-term harvests, but by the final harvests, dead leaves accounted for 41.3 and 53.4% of the artemisinin yield in the two experiments (Figs. **3C** and **D**).

Discussion

This paper analyses the accumulation of artemisinin in *A. annua* leaves as the result of (a) the total production of artemisinin and its precursors per unit of leaf dry weight and (b) the conversion of these precursors towards artemisinin. Artemisinic alcohol, artemisinic aldehyde, dihydroartemisinic aldehyde and dihydroartemisinic acid were assessed as the most relevant precursors, in line with the biosynthetic pathway postulated by Bertea et al. [6]. The last precursor in this pathway, dihydroartemisinic acid, can be converted non-enzymatically to artemisinin [10], [11], [12], most likely through dihydroartemisinic acid hydroperoxide [15], which can be converted via Hock cleavage to an enol that undergoes spontaneous autoxidation to form a hydroperoxyal-

Table **1** Differences between green and dead leaves in molar concentrations (nmol g^{-1} dry weight) of artemisinin and precursors (total and individual) and other relevant sesquiterpenes during crop growth in two experiments. DAT = days after transplanting to the field

DAT	*Total of artemisinin and its precursors*	*Artemisinin*	*Artemisinin precursors*				*Other sesquiterpenes*	
			Dihydro-artemisinic acid	*Dihydro-artemisinic aldehyde*	*Artemisinic aldehyde*	*Artemisinic alcohol*	*Dihydro-artemisinic alcohol*	*Artemisinic acid*
Experiment 1 *(Vietnamese selection)*								
56 DAT								
Green leaves	4991	924	3117	116	629	204	310	226
Dead leaves	875	326	254	108	134	53	50	21[b]
Paired t-test[a]	**	**	*	ns	***	**	**	*
112 DAT								
Green leaves	9071	3363	4303	385	817	203	266	325
Dead leaves	8263	5108	1369	624	780	381	197 [b]	327
Paired t-test[a]	ns	**	***	*	ns	**	ns	ns
Significance of the time effect								
P[a] Green leaves	*	**	ns	ns	*	ns	ns	ns
P[a] Dead leaves	***	***	ns	***	***	***	ns	**
Experiment 2 *(cv. Anamed A3)*								
33 DAT								
Green leaves	6444	508	5078	194	505	159	344	396
Dead leaves	459	362	78[b]	0	0	19[b]	19[b]	0
Paired t-test[a]	**	*	**	***	***	*	***	*
75 DAT								
Green leaves	15788	6724	6954	581	862	666	433	641
Dead leaves	4852	2971	937	154	463	327	0	297
Paired t-test[a]	*	*	*	***	**	ns	**	ns
132 DAT								
Green leaves	13867	5665	6590	444	745	423	341	346
Dead leaves	14710	10684	2055	543	827	601	315	591
Paired t-test[a]	ns	*	*	ns	ns	ns	ns	*
Significance of the time effect								
P[a] Green leaves	*	**	ns	**	ns	*	ns	ns
$LSD_{0.05}$	2975.7	2657.7		152.4		333.4		
P[a] Dead leaves	***	***	***	***	***	***	***	***
$LSD_{0.05}$	1076.6	442.8	476.8	86.1	153.4	131.3	56.6	131.2

[a] ns: not significant, $P \geq 0.05$; *: $0.05 > P \geq 0{,}01$; **: $0.01 > P \geq 0.001$; ***: $P < 0.001$.

[b] Components were not detected in all dead leaf samples. At 75 DAT in Experiment 1, artemisinic acid was detected in 2 out of 5 plots and at 112 DAT in Experiment 1, dihydroartemisinic alcohol was only detected in 3 out of 5 samples. At 33 DAT in Experiment 2, dihydroartemisinic acid was detected in 4 out 5 plots, artemisinic alcohol in 2 out of 5 plots and dihydroartemisinic alcohol in 1 out of 5 plots.

dehyde and then artemisinin by ring closure and loss of water [12]. We have not assessed intermediates between dihydroartemisinic acid and artemisinin as only the conversion to the hydroperoxide is regarded to be a rate-limiting step and intermediates are thus not likely to accumulate [10]. However, the total production of artemisinin and its precursors in especially the younger leaves might have been slightly underestimated by this decision, because Lommen et al. [13] suggested that the hydroperoxide might accumulate in young leaves. Also from the graphs by Wallaart [15] on wild-type and tetraploid *A. annua* plants, it can be deduced that the hydroperoxide accumulated in their wild-type plants in the early part of the season and during the whole season in their tetraploid genotype.

At the final harvests, dead leaves had higher artemisinin concentrations than green leaves, even though the total concentrations of artemisinin plus its precursors were similar (Table **1**). This was due to the more advanced conversion of the intermediates to artemisinin in dead leaves (Fig. **2**). This more advanced conversion is likely the result of the natural ageing of the leaves, because artemisinin accumulation gradually proceeds during the life cycle of individual leaves, while especially the quantity of the last artemisinin precursor produced enzymatically, dihydroartemisinic acid, concomitantly decreases [13].

By contrast, earlier in the season artemisinin concentrations were lower in dead leaves than in green leaves (Table **1**). This was because the more advanced partitioning to artemisinin in the dead leaves (Fig. **2**) was counteracted by a much lower total quantity of artemisinin and its precursors produced on a leaf dry weight basis (Table **1**). This lower total quantity of artemisinin plus precursors in the dead leaf fraction likely was caused by the fact that these dead leaves had been initiated at earlier crop stages than the green leaves harvested at the same date. During

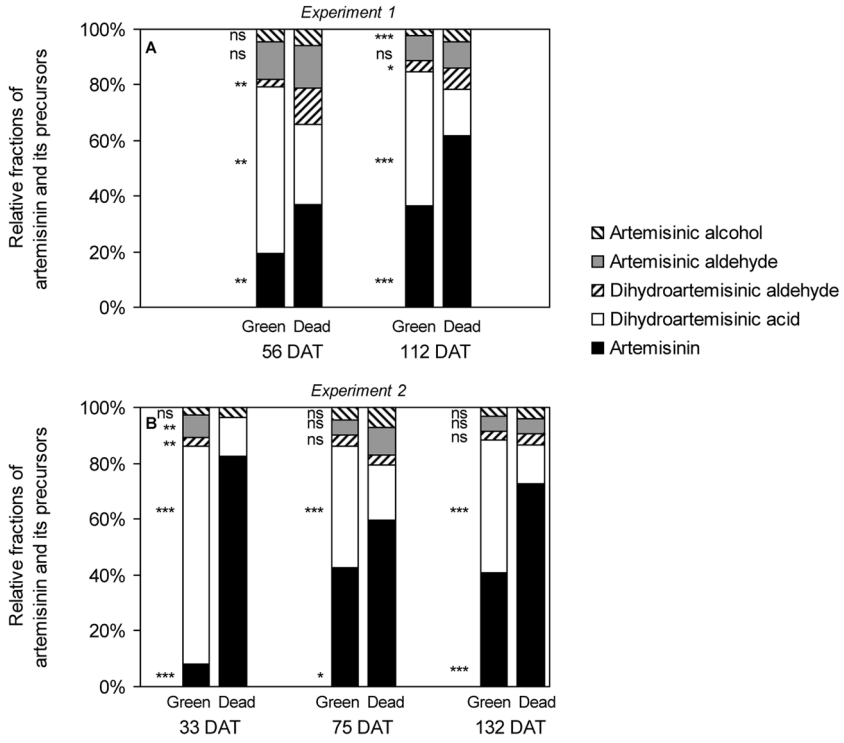

Fig. **2** Relative fractions of artemisinin and its precursors in green and dead leaves during development of *A. annua* crops in Experiment 1 (A, Vietnamese genotype) and Experiment 2 (B, cv. Anamed A3). Symbols denote significant differences in frequencies between dead and green leaves according to the paired t-test: ***: $P < 0001$, **: $0.001 \leq P < 0.01$, *: $0.01 \leq P < 0.05$, ns: not significant, $P \geq 0.05$. DAT = Days after transplanting to the field.

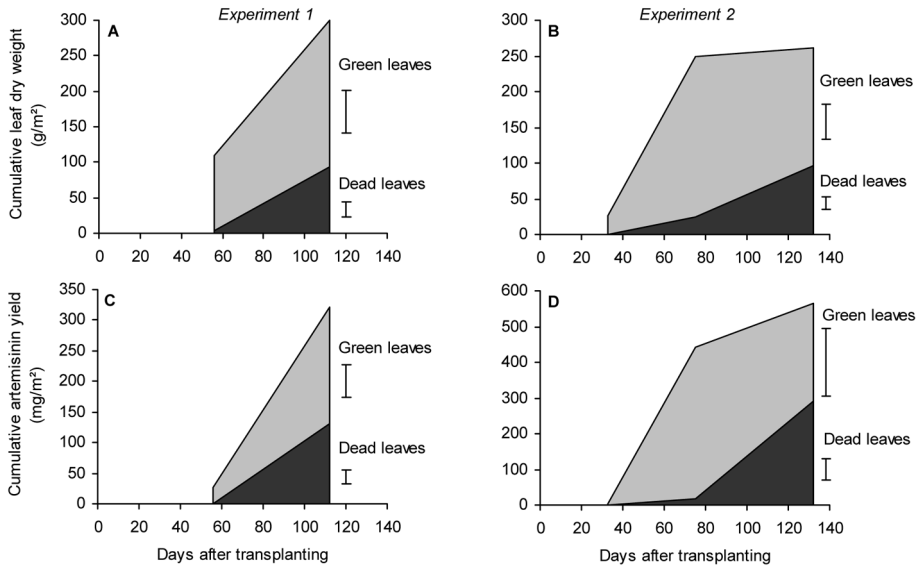

Fig. **3** Contribution of green and dead leaves to the leaf dry weight (A, B) and artemisinin weight (C, D) during development of *A. annua* crops in Experiment 1 (A, C, Vietnamese genotype) and Experiment 2 (B, C, cv. Anamed A3). Bar: $LSD_{0.05}$.

crop development, the total concentration of artemisinin and its precursors in new classes of leaves seems to increase up to a certain moment. This is supported by the increase found up to a certain moment in the green leaf fraction and the consistent increase up to the last harvest in the dead leaf fraction (Table **1**), and by the lower quantities accumulating in early appearing leaves at comparable or higher dry weights [13]. For artemisinin alone, concentrations usually first increase during crop growth, and then may decrease [3], [16], [17], [18], with highest concentrations being achieved during vegetative stages [19], [20], just before or around flowering [16] or during flowering [3], [18]. Also within a crop, artemisinin concentrations are often higher in the upper (later initiated) leaves compared to lower (earlier initiated) leaves when plants are still vegetative [20], [21], although not all authors find similar results [22]. We expect that a part of these differences is related to differences in trichome densities (cf. [13]).

The most striking result of our study is that under our conditions dead leaves contributed just as much to the final artemisinin yield as green leaves. Although dead leaves constituted only 31.4% and 37.0% of the total leaf mass at the final harvest in the two experiments, they constituted 41.3 and 53.4% of the artemisinin yield respectively (Fig. **3**), because of their higher artemisinin concentration (Table **1**). This stresses the importance of developing harvesting techniques that exclude loss of dead leaf material.

The high fraction of dihydroartemisinic acid, that occurred in molar concentrations of 130 – 115 % of that of artemisinin in green leaves and of 27 – 19 % of that of artemisinin in dead leaves in the two experiments (Fig. **2**), shows that there is theoretically room for a substantial increase in artemisinin production by further conversion of dihydroartemisinic acid into artemisinin. This could probably be achieved by optimising harvesting and drying methods after harvest, that now give varying results [23], [24], [25], [26]. Because *A. annua* is usually harvested in a premature stage, the crop has to be dried before further processing. The gradual advancement of conversion of precursors to artemisinin in individual leaves during ageing (cf. [13]) and in dead as compared with green leaves at the same crop stage (Fig. **2**) suggests that the conversion takes place during the natural ageing of the leaves within a crop and is interrupted by harvesting and drying. Further gradual conversion post-harvest may explain why increases in artemisinin concentrations were observed during several weeks of field drying under moderate temperatures [24], [25], although part of these increases in artemisinin concentrations could be due to a decrease in dry weight because of continued respiration.

Further conversion of dihydroartemisinic acid could perhaps also be stimulated by specific management decisions. Dihydroartemisinic acid has been suggested to act as an anti-oxidant in *A. annua* by quenching singlet oxygen ($^1O_2$), and the first – and rate-limiting – step in the conversion of dihydroartemisinic acid to artemisinin was postulated to be photo-oxidation to dihydroartemisinic acid hydroperoxide [15]. This conversion step requires light and singlet oxygen. Pre- or post-harvest conditions providing light and singlet oxygen therefore may enhance the conversion. In theory, circumstances leading to photodestruction in plants can lead to the generation and action of singlet oxygen [27]. Examples are stress in general [14], and more specifically absence of CO_2, chilling conditions, chlorophyll formation under high light conditions, or the presence of photosynthetic electron transport inhibitors (as summarized by Knox and Doyle, [27]). A considerable push in the conversion of dihydroartemisinic acid to artemisinin was observed after night frost [14], but could perhaps also be achieved by treatment of the crop with compounds inhibiting the photosynthetic electron flow. However, all these possibilities will only lead to more artemisinin when there are no spatial barriers between dihydroartemisinic acid and the singlet oxygen. Earlier, we postulated that artemisinin at least partly was formed after disruption of the trichomes [13]. This would suggest that, rather than with or in addition to endogenous (singlet) oxygen, artemisinin could be produced from dihydroartemisinic acid on the leaf surface.

In order to maximise artemisinin yield, also losses should be avoided. Thus, artemisinin should be protected from possible leaching, degradation or further conversion post-harvest (cf. [13]). There is no information on conditions under which artemisinin is further converted or degraded post-harvest *in planta*, but post-harvest reductions in yield or concentrations of artemisinin can occur, as summarised by Laughlin [26]. Further research on harvest and drying methods should demonstrate whether it is possible to improve the yield of artemisinin by enhancing the conversion of dihydroartemisinic acid to and/or to prevent losses of artemisinin after formation.

Acknowledgements

The authors would like to thank the teams of G. Versteeg and H. Masselink (Unifarm Wageningen) for their enthusiasm and help in raising the transplants and growing the field crops.

References

[1] Ferreira JFS, Simon JE, Janick J. *Artemisia annua*: botany, horticulture, pharmacology. Hortic Rev 1997; 19: 319 – 71.
[2] Dhingra V, Rao KV, Narasu ML. Current status of artemisinin and its derivatives as antimalarial drugs. Life Sci 2000; 66: 279 – 300.
[3] Ferreira JFS, Simon JE, Janick J. Developmental studies of *Artemisia annua*: Flowering and artemisinin production under greenhouse and field conditions. Planta Med 1995; 61: 167 – 70.
[4] Duke SO, Paul RN. Development and fine structure of the glandular trichomes of *Artemisia annua* L. Int J Plant Sci 1993; 154: 107 – 18.
[5] Duke MV, Paul RN, Elsohly HN, Sturtz G, Duke SO. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L. Int J Plant Sci 1994; 155: 365 – 72.
[6] Bertea CM, Freije JR, van der Woude H, Verstappen FWA, Perk L, Marquez V et al. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua* L. Planta Med 2005; 71: 40 – 7.
[7] Bouwmeester HJ, Wallaart TE, Janssen MHA, van Loo B, Jansen BJM, Posthumus MA et al. Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. Phytochemistry 1999; 52: 843 – 54.
[8] Teoh KH, Polichuk DR, Reed DW, Nowak G, Covello PS. *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. FEBS Lett 2006; 580: 1411 – 6.
[9] Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 2006; 440: 940 – 3.
[10] Wallaart TE, Pras N, Quax WJ. Isolation and identification of dihydroartemisinic acid hydroperoxide from *Artemisia annua*: a novel biosynthetic precursor of artemisinin. J Nat Prod 1999; 62: 1160 – 2.
[11] Sy L-K, Brown GD. The mechanism of the spontaneous autoxidation of dihydroartemisinic acid. Tetrahedron 2002; 58: 897 – 908.
[12] Haynes RK. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. Curr Top Med Chem 2006; 6: 509 – 37.
[13] Lommen WJM, Schenk E, Bouwmeester HJ, Verstappen FWA. Trichome dynamics and artemisinin accumulation during development and senescence of *Artemisia annua* leaves. Planta Med 2006; 72: 336 – 45.
[14] Wallaart TE, Pras N, Beekman AC, Quax WJ. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: Proof for the existence of chemotypes. Planta Med 2000; 66: 57 – 62.
[15] Wallaart TE, Pras N, Quax WJ. Seasonal variations of artemisinin and its biosynthetic precursors in tetraploid *Artemisia annua* plants compared with the diploid wild-type. Planta Med 1999; 65: 723 – 8.
[16] Liersch R, Soicke H, Stehr C, Tüllner H-U. Formation of artemisinin in *Artemisia annua* during one vegetation period. Planta Med 1986; 52: 387 – 90.
[17] Laughlin JC. Effect of agronomic practices on plant yield and anti-malarial constituents of *Artemisia annua* L. Acta Hortic 1993; 331: 53 – 61.
[18] Morales MM, Charles DJ, Simon JE. Seasonal accumulation of artemisinin in *Artemisia annua* L. Acta Hortic 1993; 344: 416 – 20.
[19] Woerdenbag HJ, Pras N, Nguyen Gia Chan, Bui Thi Bang, Bos R, van Uden W et al. Artemisinin, related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetation period in Vietnam. Planta Med 1994; 60: 272 – 5.
[20] Gupta SK, Singh P, Bajpai P, Ram G, Singh D, Gupta MM et al. Morphogenetic variation for artemisinin and volatile oil in *Artemisia annua*. Ind Crop Prod 2002; 16: 217 – 24.

[21] Laughlin JC. The influence of distribution of antimalarial constituents in *Artemisia annua* L. on time and method of harvest. Acta Hortic 1995; 390: 67 – 73.
[22] Ferreira JFS, Janick J. Distribution of artemisinin in *Artemisia annua*; In: Janick J, editor. Progress in new crops. Arlington, VA.: ASHS Press; 1996: 579 – 84.
[23] Charles DJ, Simon JE, Shock CC, Feibert EBG, Smith RM. Effect of water stress and post-harvest handling on artemisinin content in the leaves of *Artemisia annua* L. In: Janick J, Simon JE, editors. New Crops. New York: Wiley; 1993: 640 – 3.
[24] Simonnet X, Gaudin M, Hausammann H, Vergeres C. Le fanage au champ d'*Artemisia annua* L.: élever la teneur en artémisinine et abaisser les coûts de production (Field drying of *Artemisia annua* L.: increasing artemisinin content and lowering production costs). Revue Suisse de Viticulture, Arboriculture et Horticulture 2001; 33: 263 – 8.
[25] Laughlin JC. Post-harvest drying treatment effects on antimalarial constituents of *Artemisia annua* L. Acta Hortic 2002; 576: 315 – 20.
[26] Laughlin JC, Heazlewood GN, Beattie BM. Cultivation of Artemisia annua L. In: Wright CW, editor. Artemisia. London and New York: Taylor & Francis; 2002: 159 – 95.
[27] Knox JP, Dodge AD. Singlet oxygen and plants. Phytochemistry 1985; 24: 889 – 96.