Enhancement of artemisinin concentration and yield in response to optimization of nitrogen and potassium supply to *Artemisia annua*

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**Background and Aims** The resurgence of malaria, particularly in the developing world, is considerable and exacerbated by the development of single-gene multi-drug resistances to chemicals such as chloroquine. Drug therapies, as recommended by the World Health Organization, now include the use of antimalarial compounds derived from *Artemisia annua* – in particular, the use of artemisinin-based ingredients. Despite our limited knowledge of its mode of action or biosynthesis there is a need to secure a supply and enhance yields of artemisinin. The present study aims to determine how plant biomass can be enhanced while maximizing artemisinin concentration by understanding the plant’s nutritional requirements for nitrogen and potassium.

**Methods** Experiments were carried out, the first with differing concentrations of nitrogen, at 6, 31, 56, 106, 206 or 306 mg L⁻¹ being applied, while the other differing in potassium concentration (51, 153 or 301 mg L⁻¹). Nutrients were supplied in irrigation water to plants in pots and after a growth period biomass production and leaf artemisinin concentration were measured. These data were used to determine optimal nutrient requirements for artemisinin yield.

**Key Results** Nitrogen nutrition enhanced plant nitrogen concentration and biomass production successively up to 106 mg N L⁻¹ for biomass and 206 mg N L⁻¹ for leaf nitrogen; further increases in nitrogen had no influence. Artemisinin concentration in dried leaf material, measured by HPLC mass spectroscopy, was maximal at a nitrogen application of 106 mg L⁻¹, but declined at higher concentrations. Increasing potassium application from 51 to 153 mg L⁻¹ increased total plant biomass, but not at higher applications. Potassium application enhanced leaf potassium concentration, but there was no effect on leaf artemisinin concentration or leaf artemisinin yield.

**Conclusions** Artemisinin concentration declined beyond an optimal point with increasing plant nitrogen concentration. Maximization of artemisinin yield (amount per plant) requires optimization of plant biomass via control of nitrogen nutrition.

**Key words:** *Artemisia*, fertigation, malaria, nitrogen, nutrition, potassium.

INTRODUCTION

Current figures suggest that more than 2 billion people are at risk from malaria with somewhere between 300 and 500 million people affected, of whom 2 million die annually (1 million children) (WHO, 2001; Greenwood et al., 2008). Efforts to reduce the impact of the disease are now hampered by single-gene drug-resistant *Plasmodium* (Kindermans et al., 2007). Therapies now include the use of antimalarial compounds derived from *Artemisia annua* (*qinghao* or sweet wormwood), a member of the Asteraceae (Ferreira, 2007; Greenwood et al., 2008). The medicinal properties of this plant have been part of traditional Chinese medicine for at least 1000 years. The World Health Organization (WHO) now recommends the use of artemisinin-based combination therapies (ACT) in regions where the tropical malarial parasite has developed multi-drug resistance to the more common antimalarial drugs (WHO, 2000; Kindermans et al., 2007).

Experience clearly shows that single drug treatment approaches are always likely to induce potential resistance and artemisinin appears to be no exception (see Greenwood et al., 2008).

Originally a native of Asia, *Artemisia* now grows wild throughout Europe, North and South America and Australia. The active principal compound (0.1–0.6 % dry weight, Putalun et al., 2007) in *A. annua*, along with an array of other terpenoids, is ‘artemisinin’ or *qinghaosu* – ((3R-(3xa, 5aβ, 6β, 8β, 9α, 12β, 12αR))-octahydro-3,6,9-trimethyl-, 3,12-epoxy-12H-pyranol[4,3-j]-1,2-benzooxepin-10(3H)-one) – a sesquiterpene lactone (Klayman, 1985). This compound was only isolated, identified and clinically evaluated in the 1970s; its biosynthesis has not yet been completely elucidated, but is suggested to involve the cytosolic mevalonate pathway, and the plastid-located deoxyxylulose (also called the methylerythritol 4-phosphate, or MEP) pathway (Covello et al., 2007; Towler and Weathers, 2007). Artemisinin’s mode of action is also a subject of much debate and research, but it
is suggested to include the involvement of the peroxodic oxygen atom and the generation of reactive oxygen species (ROS; see mechanisms review of Krishna et al., 2004; Denisov et al., 2005). There is, however, clear evidence of the inhibitor effects of artemisinin and its derivatives on the development and viability of the protozoan parasite. The uses of plant-based artemisinin compounds are not, however, an insignificant cost, particularly in the developing world where the need is greatest. These costs are in part linked to limitations in the quality of plant material, the climate and general agronomic management. Despite the low productivity of *A. annua* (1.5–2 tonnes ha
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) and artemisinin concentrations (<1 %), production of between 6 and 14 kg of artemisinin per hectare is possible (Kindermans et al., 2007).

There are a number of reasons why we should endeavour to enhance the concentration and yield of this beneficial compound, along with a number of routes by which enhancement might be achieved. However, the aim here is to examine the agronomic requirements for growing *A. annua* in temperate regions of northern Europe. This work forms part of a larger programme devised to enhance artemisinin concentration and supply by conventional breeding and field management.

Changes in the production of secondary metabolites are frequently linked to a number of aspects of the plant’s interaction with its environment. It is therefore no surprise that agronomic factors which influence plant growth and development may also influence the accumulation of some secondary metabolites. The involvement of nitrogen nutrition in influencing plant growth and development and biomass production is universally acknowledged (see review of Fernandes and Rossiello, 1995). Based on this knowledge, it seems entirely appropriate to examine the effects of nitrogen as an interactive factor in driving photosynthesis (via Rubisco content), growth and acting as resource of secondary metabolite production (Wang and Weathers, 2007). It has also be hypothesized that environmental stress, such as nutrient deficiency, via the production of ROS, which are themselves scavenged by dihydroartemisinic acid, produces the stable end product artemisinin (Ferreira, 2007).

Some attention has already been given to the importance of mineral nutrition (nitrogen and potassium) in perturbing artemisinin production (Ferreira, 2007; Ferreira et al., 2007). Given the importance of nitrogen in influencing plant biomass production, and of potassium on growth through its role in the regulation of carbohydrate and protein synthesis, it is critically important that we understand clearly how best to maximize biomass production against leaf artemisinin concentration to achieve optimal yield per plant of the active component. The aims of this experiment are to describe how artemisinin yield can be optimized through nitrogen and potassium nutrition when grown in the UK.

**MATERIALS AND METHODS**

**Plant material and experimental set-up**

Seeds of *Artemisia annua* L. were produced by Humber VHB (Chichester, UK) in a glasshouse in winter 2006/07 and pelleted with a specialist vegetable pellet and sown and grown individually in 2.5-cm × 2.5-cm and 3-cm-deep modules by Frontier Agriculture Ltd (lot B/2007). Six hundred individual seedlings of *A. annua* were transferred to East Malling Research, where the plants were subsequently grown. In late June, 400 seedlings were selected and potted into 7.5-L pots using Klassman medium Irish graded peat with no added N, P or K, to which 1.9 g of CaCO
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per litre of peat was added to raise the pH to between 5.8 and 6. Chemical analysis of the compost revealed that concentrations of N, P and K were respectively 30, <0.6 and 5.3 mg L
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of compost. Plants were placed in a naturally lit unheated glasshouse for 3 weeks to establish. They were then sorted according to size and placed outside onto a freely draining gravel bed (11 × 4.8 m) in 15 rows, on 19 July; the pots were staggered along the row, with 20 pots per row. Rain infiltration was excluded from the pots, which were covered with plastic. Plants at the end of each row acted as experimental guards; each experimental block was guarded by a complete row north and south, removing edge effects from the experiment.

**Experimental treatments**

**Nitrogen experiment.** Nitrogen was supplied to the plants, via a liquid feed system, at six different levels: 6 (N1), 31 (N2), 56 (N3), 106 (N4), 206 (N5) and 306 mg L
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(N6). Ammonium nitrate was used as the source of nitrogen. All other macro- and micronutrients were kept constant and were supplied, as liquid feed, at the following rates: P at 40 mg L
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, K at 156 mg L
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, Ca at 80 mg L
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, Na at 33 mg L
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, Zn at 0.1 mg L
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, B at 0.3 mg L
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, Cu at 0.1 mg L
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, S at 112 mg L
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, Fe at 2.8 mg L
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 and Cl at 3.5 mg L
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. The design was a randomized complete block: eight blocks × six treatments; each plot contained three plants, with a total 144 (8 × 6 × 3) plants. The accuracy of the feed systems used to deliver irrigation and nutrition was checked. Nutrient solutions were prepared within the tank and in manner to ensure no precipitation, evaporation (sealed around the injectors) or algal degradation (blackened tanks). Total irrigation volume, theoretically applied, was calculated based on measured inputs from the feed tank system to the pots, on a daily basis, and summed over the experiment. This calculated estimate agreed closely (within <10 %) with the actual quantity of nutrient solution used, based on measuring the tank contents. Total plant nutrient budgets for N and K calculated after chemical tissue analysis, at the end of the experiment, also agreed closely with calculated total applied N and K.

**Potassium experiment.** Potassium was supplied to the plants, via a fertigation system, at three different levels: 53 (K1), 155 (K2) and 301 mg L
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(K3). Potassium sulphate was used as the source of potassium. Sulphur was applied at a concentration of between 69 and 170 mg L
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, with K1 plants having the lowest concentration and K3 having the highest. All other macro- and micronutrients were kept constant and were supplied, as liquid feed as described above, with N at 202 mg L
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. The design was a randomized complete block: eight blocks × three treatments; each plot contained three plants, with a total 72 (8 × 3 × 3) plants.

For both the nitrogen and the potassium experiments each pot received the fertigation solution via one 2 L h
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 dripper. Fertigation started in late July. The plants were inspected
3–4 times a week and the amount of irrigation the plants were given was adjusted to ensure the plants were receiving adequate amounts of water. Daily irrigation times were recorded throughout the experiment to calculate the total amount of water and nitrogen applied to each plant.

**Plant measurements**

Main stem heights were measured at 7–12-d intervals from late July to mid-September and finally in early October. To determine plant biomass, artemisinin concentration, artemisinin yield and leaf mineral concentration, plants from both experiments were harvested in October. Leaves and stems from a portion of the main stem of each plant, e.g. one side, were removed from the plant base to the tip. The leaf and stem material was collected from each of the three plants per plot and bulked. The fresh weight of this material was recorded. The material was then dried, in a ventilated oven, at 40–42 °C for at least 48 h, and then passed through a sieve (5-mm mesh) to separate the leaf lamina from pedicles. The dry weights of the two components were recorded. Leaf and stem material was stored, at room temperature, in sealed polythene grip bags to await mineral and artemisinin analysis.

**Plant tissue sampling and analysis of artemisinin by HPLC mass spectrometry**

**Reagents and equipment.** Santonin was purchased from Sigma (St Louis, MO, USA). Electrospray tuning mix was purchased from Agilent Technologies Ltd (West Lothian, UK). Acetonitrile (HPLC gradient grade) and water (mass spectrometry grade) were purchased from Fisher (Loughborough, UK). Reference artemisinin, in bulk, was provided by AECS Quickprep (Bridgend, UK). The compound was crystallized to a constant melting point of 154 °C and a purity of greater than 99 %. Identity of compounds was confirmed by electrospray mass spectrometry and 1H nuclear magnetic resonance on a Bruker 400-mHz Ultrashield.

Artemisinin (3a,5a,9b-tetrahydroxy-3,5a,9-trimethylnaphthol[1,2-b]furan-2,8(3H,4H)-dione; C15H22O5, mol. wt 282.33) was identified and analysed using an Agilent Technologies 1100 Series high-performance liquid chromatography system, with a GraceSmart RP18-HPLC column (150 mm × 2.1 mm; pore size of 3 μm), coupled to an Agilent Technologies G1946 single quadrupole mass spectrometer with electrospray ionization, used in positive ion mode, with single ion monitoring (SIM). HPLC separation was achieved with an isocratic mobile phase of 60 % acetonitrile and 40 % HPLC-grade water at a flow rate of 0.2 cm min⁻¹. The column oven was set at 25 °C. The spray chamber and mass spectrometer parameters were as follows: nitrogen flow at 8 L min⁻¹; drying gas temperature 350 °C; capillary voltage 2800 V in positive mode, 3500 V in negative mode; nebulizer pressure 40 psig; and fragmentor voltage 70 V. The mass spectrometer was
calibrated and, if necessary, tuned daily, using Agilent’s Electrospray Tuning Mix, to ensure peak performance.

Generation of calibration curve. Santonin ([3S]-3a,5,5a,9bβ-
tetrahydro-3a,5aβ,9-trimethylnaphthol[1,2-b]furan-2,8(3H,4H)-
dione; C_{15}H_{18}O_{3}, mol. wt 246.31) was used as an internal
standard, with ions monitored at 173.3 and 247.3. The monitored
artemisinin ions were 283.3 and 209.3. A calibration curve was
prepared in a 2-cm³ crimp vial with 100 µg cm⁻³, and in which santonin, as an internal standard, was
kept constant at 10 µg cm⁻³. All calibration levels were prepared
in triplicate and analysed three times per sample. The ratio of
artemisinin to santonin was calculated for each analysis; linear
regression of the resulting scatter plot was used to establish the
calibration curve. The upper limit of quantification was given as
the highest level of the calibration curve: beyond this, saturation
of response rendered quantification less reliable. The lower
detection limit was taken as five times the standard deviation of
the highest level of the calibration curve, and was calculated to be 0.8 ng.

Sample analysis. Dried leaf material was ground with a pestle
and mortar, and around 100 mg weighed accurately into a
4-cm³ glass vial (Fisher), to which was added 1 cm³ of aceto-
nitrile and the vial capped prior to shaking at around 50 r.p.m.
for 24 h in the dark. This technique has been found to consist-
tently extract >90% of the artemisinin in a herbal sample.
After this maceration 10 µL of liquid was removed and
placed in a 2-cm³ crimp vial with 100 µL of santonin stock
solution (0.1 mg cm⁻³ in acetonitrile) and the sample was
made up to 1 cm³ with HPLC-grade acetonitrile. Samples
were analysed blind, in a programme that periodically included
previously analysed samples to monitor consistency. Precision
and accuracy of analyses were measured by the inclusion of
samples of known artemisinin concentration.

Statistical analysis

Differences between plants receiving different nitrogen con-
centrations and different potassium concentrations were deter-
mined using analysis of variance. Statistical analyses were
performed using Genstat software (v. 9.1, Rothamsted
Experimental Station, Rothamsted, UK). Where appropriate,
least significant differences (l.s.d.) were calculated and dis-
played on the figures.

RESULTS

Nitrogen

Differences in stem height of A. annua grown under the six
different nitrogen treatments were apparent 4 weeks after
the start of the treatment applications (Fig. 1). Significant differ-
ences in stem heights were apparent during September and at
the final harvest in October, with the tallest plants within the
highest N concentration (N6) and the shortest at the lowest
N concentration (N1). This difference in stem height correlated
well with differences in dry matter production in terms of total
plant, leaf and stem biomass at harvest (Fig. 2). Increasing the
applied N concentration from 6 mg L⁻¹ (N1) to 106 mg L⁻¹
(N4) increased total plant biomass eight-fold (from 13.4 to
119.2 g), leaf biomass (from 5.6 to 44.7 g) and stem biomass
(from 7.8 to 74.5 g). Increasing applied N concentration
from 106 mg L⁻¹ (N4) to 306 mg L⁻¹ (N6) did not signifi-
cantly increase total plant or stem biomass any further.

Increasing applied nitrogen from a concentration of 6 mg L⁻¹
(N1) to 206 mg L⁻¹ (N5) increased leaf N concentration from 3
to 6.7% (w/w), while increasing N application above 206 mg
L⁻¹ had no further effect (Fig. 3). Total amount of leaf N (nitro-
gen concentration × leaf dry weight) increased with successive
increases in N application from 171 mg per plant for the N1
treatment to 3727 mg per plant for N6 treatment. The mean

![Fig. 3. (A) Nitrogen concentration in leaves and (B) total leaf nitrogen content (nitrogen concentration × leaf dry weight), at harvest, for Artemisia
annua plants when supplied with differing concentrations of nitrogen. N1 = 6 mg L⁻¹, N2 = 31 mg L⁻¹, N3 = 56 mg L⁻¹, N4 = 106 mg L⁻¹, N5 = 206 mg L⁻¹, N6 = 306 mg L⁻¹. Bars show l.s.d. at 5% level.]
Fig. 4. Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), manganese (Mn), iron (Fe), boron (B), copper (Cu) and zinc (Zn) concentration in leaves at harvest for Artemisia annua plants when supplied with differing concentrations of nitrogen. N1 = 6 mg L\(^{-1}\), N2 = 31 mg L\(^{-1}\), N3 = 56 mg L\(^{-1}\), N4 = 106 mg L\(^{-1}\), N5 = 206 mg L\(^{-1}\), N6 = 306 mg L\(^{-1}\). Bars show l.s.d. at 5% level.

Leaf mineral concentrations of calcium (Ca), manganese (Mn) and copper (Cu) all decreased with increasing concentration of applied N (Fig. 4). Leaf K concentration declined with increasing nitrogen application above 56 mg L\(^{-1}\). Phosphorus (P) and sulphur (S) concentrations were higher in leaves of plants receiving N at concentrations above 56 mg L\(^{-1}\). Boron (B) concentrations were higher in leaves of plants receiving N at concentrations above 31 mg L\(^{-1}\).

The concentration of artemisinin in leaves of A. annua was at its highest in plants receiving between 6 and 56 mg L\(^{-1}\) of nitrogen (N1–N3), where it ranged from 0.86 to 0.93 % (w/w; Fig. 5). As nitrogen concentration increased from 56 to 106 mg L\(^{-1}\) the concentration of artemisinin declined to 0.69 % (w/w). A further increase in nitrogen application reduced artemisinin concentration to between 0.53 and 0.56 % (w/w). However, the total yield of artemisinin (artemisinin concentration \times leaf biomass) per plant increased, from 0.053 to 0.31 g per plant, as the concentration of nitrogen supplied to the plants increased from 6 to 106 mg L\(^{-1}\). Increasing nitrogen concentrations from 106 to 306 mg L\(^{-1}\) did not increase artemisinin yields further.

**Discussion**

This experiment shows how nitrogen application enhances plant height and dry matter production in A. annua. Nitrogen fertilization appears to increase the allocation of dry matter more to the stem than to the leaf. However, this enhancement in biomass plateaus with the two highest N treatment applications (N5 and N6) and there was no significant increase in stem height or plant biomass above the N4, 106 mg L\(^{-1}\), treatment. Measurement of leaf nitrogen concentration and total leaf nitrogen content (mg per plant) showed, with the exception of the highest N application (N6), that leaf N concentration was directly proportional to the concentration of the applied solution. That is to say, there was no evidence that uptake or demand had been saturated at the higher nitrogen treatments (N4–N6) and that this provided an explanation for a limitation in further growth enhancement at the highest N treatments. Enhanced dry matter production induced by elevated plant nitrogen is extremely well documented and probably driven by increases in photosynthetic efficiency.

The total amount of N applied to each plant was 0.17, 1.05, 2.1, 3.9, 7.6 and 10.7 g for the N1, N2, N3, N4, N5 and N6 treatments, respectively.

Leaf mineral concentrations of calcium (Ca), manganese (Mn) and copper (Cu) all decreased with increasing concentration of applied N (Fig. 4). Leaf K concentration declined with increasing nitrogen application above 56 mg L\(^{-1}\). Phosphorus (P) and sulphur (S) concentrations were higher in leaves of plants receiving N at concentrations above 56 mg L\(^{-1}\). Boron (B) concentrations were higher in leaves of plants receiving N at concentrations above 31 mg L\(^{-1}\).

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**Potassium**

There were no significant K treatment differences in stem height throughout the experiment (Fig. 6). However, increasing K concentration from 53 to 155 mg L\(^{-1}\) significantly increased total plant biomass (Fig. 7); this difference was due to an increase in stem weight, there being no significant difference between leaf dry weights. Increasing K concentration from 155 to 303 mg L\(^{-1}\) did not increase biomass production further.

Increasing K supply from a concentration of 53 mg L\(^{-1}\) (K1) to 303 mg L\(^{-1}\) (K3) increased leaf K concentration from 1.8 to 3.4 % (w/w; Fig. 8). The total amount of leaf K (leaf K concentration \times leaf dry weight) increased with K application from 706 mg per plant for plants grown with a potassium concentration of 53 mg L\(^{-1}\) (K1) to 1524 mg per plant for those supplied with 303 mg L\(^{-1}\) potassium (K3). The mean total amount of K supplied to each plant was 2.2, 6.9 and 12.4 g for the K1, K2 and K3 treatments, respectively. Leaf mineral concentrations of N, P, Ca, Mg, Mn and B decreased with increasing K application (Fig. 9). There were no significant differences in leaf mineral concentration for Fe, Cu or Zn.

Concentration of artemisinin in leaves did not significantly differ between potassium treatments (Fig. 10). However, this concentration of artemisinin was lower (0.55 %, w/w) than the maximal apparent for the nitrogen treatments. However, leaf nitrogen concentration measured in the potassium-treated plants was around 6 % (w/w), i.e. the concentration achieved for the N5 and N6 treatments. All K-treated plants were supplied in the N5 range, i.e. at 206 mg L\(^{-1}\), which gave a leaf concentration of 5.5 % N, which supports the expectation of N and K treatment comparability. These nitrogen treatments had very comparable artemisinin concentrations at around 0.55 % (w/w) with respect to the potassium treatments. Total yield of artemisinin (leaf artemisinin concentration \times leaf dry weight) was lower for the K1 treatment, but there were no statistically significant differences between treatments (Fig. 10).
tissue S concentration and declines in K, Ca and, to a lesser extent, Mn. This may be due to ‘dilution effects’; for example, as the growth-limiting element, or nutrient, is applied, the relative rate of dry matter accumulation increases more than the rate of uptake of a particular element causing its concentration to decline (see Jarrell and Beverly, 1981).

Although there was acidification of the compost to pH 4.8, which might be in response to the application of the ammonium nitrate treatment, this effect would have been much the same across all treatments. There did not appear to be any suggestion that either toxicity or deficiency, in regard to S or K, Ca and Mn, respectively, could explain limitations in further nitrogen-induced growth enhancement. We therefore conclude, with respect to growth, that nitrogen application at approximately 100 mg L⁻¹, and a leaf concentration of approximately 5.7 % (w/w), was optimal for A. annua growth.

Measurements of leaf artemisinin concentration were highest (around 0.9 %) at the low application rates of nitrogen (N1). As the treatment rate of nitrogen increased, artemisinin concentration declined significantly and was lowest at around 0.5 % for the N5 and N6 treatments. One suggestion is that the increase plant nitrogen status stimulates growth, which dilutes the artemisinin concentration. The interlinking of carbon and nitrogen metabolism beyond the requirements for growth and development are well known (Van Dam et al., 1996; Fritz et al., 2006 and references within). For example, the glucose/fructose ratio may also have a greater stimulatory influence on artemisinin synthesis relative to that of photo-synthetically derived sucrose (Wang and Weathers, 2007).

Enhanced nitrogen application is well documented in encouraging cell expansion, increases in leaf lamina area and canopy development. Vacuolar sequestration of nitrate appears to be the most likely mechanism driving leaf expansion (Millard, 1988). With monocots (e.g. Festuca arundinacea) the increase in leaf elongation rate induced by increased nitrogen supply extends the cell division phase, giving rise to a greater increase in leaf mesophyll cell number compared with adjacent epidermal cells. This was accompanied by a ‘compensatory’ increase in epidermal cell length (MacAdam et al., 1989). The situation with dicots (e.g. Ricinus communis) is, not surprisingly, dependent on the timing of nitrogen enhancement in relation to leaf development stage. Once the full cellular complement of a leaf has been achieved (predetermined) nitrogen status only influences leaf cell size and not cell number (Roggatz et al., 1999).

The influence that nitrogen supply can have on leaf growth provides some insight into a possible mechanism, at least indirectly, by which the observed decline in leaf artemisinin concentration came about. However, it should be noted that a high nitrogen concentration was also apparent in potassium-treated plants, where no increase in leaf biomass occurred. Therefore, simple nitrogen dilution of the leaf artemisinin content may not be the only explanation of the observed
nitrogen effects. As yet, however, we can only speculate as to how the biosynthesis of sesquiterpenes, such as artemisinin derived from acetyl coenzyme A and glycolytic intermediates, are linked to both nitrogen and carbon supplies.

Artemisinin is found sequestered in the upper sub-cuticular spaces of glandular trichomes, predominantly on leaves and to a lesser extent on flowers (Duke and Paul, 1993; Duke et al., 1994). Glandular trichomes, in general, have been shown to accumulate large quantities of potentially toxic, secondary metabolites (Wagner, 1991; Iijima et al., 2004). Trichomes accumulate and store these compounds, in specific cellular locations, outside metabolically active compartments of the plant; the diverse nature of their chemistry fuels a multi-functional understanding of plant defence options. It is clear that these glands contain cells with metabolically functional chloroplasts containing starch, which in some specific cases are shown to be involved in terpenoid production (Wagner, 1991; Duke and Paul, 1993). This is supported by more recent evidence of terpenoid biosynthetic in situ gland enzyme activity, which has developed our understanding of metabolite accumulation in relation to gene transcript levels of various terpene synthases (Iijima et al., 2004).

There are important questions regarding how glandular trichome number might be controlled or what opportunities exist to enhance their number. Trichome morphogenesis is well dissected in Arabidopsis thaliana mutants with the role of 40 plus regulatory genes identified (Folkers et al., 1997; Schwab et al., 2000; Ishida et al., 2008) with complementary molecular links to root hair development (see review of Wagner et al., 2004 and references within). Again, it is more recent work with Arabidopsis which suggests that epidermal cells produce trichomes in a random fashion (Scheres, 2002). But despite this apparent random origin they subsequently inhibit neighbouring epidermal cells from also producing trichomes. At least for some species (e.g. Betula spp.) it is suggested that trichome development begins at a very early stage of leaf development; they become apparent at the leaf primordia stage (Valkama et al., 2004). Again in some species (e.g. Plectranthus, Lamiaceae) this means that final trichome number is fixed early in development (Ascensao and Pais, 1987), while in others cases (e.g. Mentha spp.), the ceasing of gland initiation can be directly linked to termination of
leaf expansion (Maffei et al., 1989; Turner et al., 2000). In this species there were also clear differences in gland density with leaf node position, early leaves showing much lower densities than those at higher nodes. In *Artemisia* it appears that these glands are present on flowers and leaves at an early stage of development, with leaf primordia being densely covered (Ferreira and Janick, 1996). During later development the early capitulate glands become less obvious as filamentous trichomes develop, with the adaxial leaf surfaces showing rows of trichomes and glands (Duke and Paul, 1993). Conversely, the abaxial surface showed a random pattern of glands. A further complication to understanding the relationship between gland density, development stage and metabolite concentration is the suggestion that artemisinin accumulates predominantly at organ physiological maturity during anthesis (Ferreira and Janick, 1996). How nitrogen nutrition might influence *Artemisia* leaf trichome density is still unclear, but warrants further investigation.

In a practical sense we are particularly interested in optimizing artemisinin yield irrespective of whether this be on a per-plant or per-land area basis. One way to achieve this requires nitrogen optimization based on artemisinin yield per plant, and this can be calculated from the multiplication of leaf artemisinin concentration by leaf dry matter yield. From this the total yield of artemisinin, on a per-plant basis, increased with increasing nitrogen application up to 106 mg L\(^{-1}\). Increasing nitrogen concentration application above 106 mg L\(^{-1}\) did not increase artemisinin yield. At this concentration of nitrogen, the plant had a leaf nitrogen concentration of 5.5 % (w/w) at the time of harvest, which was adequate for maximizing artemisinin yields.

![Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), manganese (Mn), iron (Fe), boron (B), copper (Cu) and zinc (Zn) concentration in leaves at harvest for *Artemisia annua* plants when supplied with differing concentrations of potassium. K1 = 53 mg L\(^{-1}\), K2 = 155 mg L\(^{-1}\), K3 = 303 mg L\(^{-1}\). Bars show l.s.d. at 5 % level.](image1)

![Artemisinin concentration in leaves and total artemisinin content of leaves (mineral concentration/leaf dry weight) at harvest for *Artemisia annua* plants when supplied with differing concentration of potassium. K1 = 53 mg L\(^{-1}\), K2 = 155 mg L\(^{-1}\), K3 = 303 mg L\(^{-1}\). Bars show l.s.d. at 5 % level.](image2)

In practical sense we are particularly interested in optimizing artemisinin yield irrespective of whether this be on a per-plant or per-land area basis. One way to achieve this requires nitrogen optimization based on artemisinin yield per plant, and this can be calculated from the multiplication of leaf artemisinin concentration by leaf dry matter yield. From this the total yield of artemisinin, on a per-plant basis, increased with increasing nitrogen application up to 106 mg L\(^{-1}\). Increasing nitrogen concentration application above 106 mg L\(^{-1}\) did not increase artemisinin yield. At this concentration of nitrogen, the plant had a leaf nitrogen concentration of 5.5 % (w/w) at the time of harvest, which was adequate for maximizing artemisinin yields.

*Artemisia annua* plants treated with different concentrations of potassium showed increased concentrations in their leaves and total amounts of potassium per plant. Optimal *A. annua* growth occurred with the application of potassium at approximately 155 mg L\(^{-1}\) (equating to 6.9 g K per plant). Potassium concentrations above this did not increase dry matter biomass, and nor was there any change in leaf artemisinin concentration. However, the total yield of artemisinin, per plant, was slightly higher when potassium application was raised from 53 to 155 mg L\(^{-1}\), although this increase was not statistically significant. A concentration of potassium in leaves of 2.6 % (w/w) at
harvest was adequate for maximizing artemisinin yield per plant.

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