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Increases in leaf artemisinin concentration in *Artemisia annua* in response to the application of phosphorus and boron

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ABSTRACT

Malaria resurgence particularly in the third world is considerable and exacerbated by the development of multi-drug resistances to chemicals such as chloroquinone. Drug therapies, as recommended by WHO include the use of antimalarial compounds derived from *Artemisia annua* L., i.e. artemisinin-based therapies. This work aims to determine how *A. annua* plant dry matter can be enhanced while maximising artemisinin concentration from understanding the plant's mineral requirements for P and B. Experiments with differing of P, from 5 to 120 mg L^{-1} and B from 0.1 to 0.9 mg L^{-1} were undertaken. Mineral nutrients were supplied in irrigation water to potted plants and after a period of growth, dry matter production and leaf artemisinin concentration were determined. Increases in P application enhanced plant growth and total dry matter production. An optimal application rate, with respect to dry matter, was apparent around 30 mg P L^{-1}. Despite increases in P application having no influence on leaf artemisinin concentration, optimal yields of artemisinin, on a per plant basis, were again achieved at supply rate around 30–60 mg L^{-1}. Increasing B application rate had little influence on dry matter production despite increases in B leaf tissue concentration promoting the total amount of B per plant. Leaf artemisinin concentration significantly increased with B application rate up to 0.6 mg B L^{-1}. The higher artemisinin concentrations when multiplied by total leaf dry matter at the higher B application rates produced an increase in total artemisinin production per plant. There was however no further significant effect on leaf artemisinin concentration when B supply concentrations increased further (0.9 mg L^{-1}). Artemisinin production varied between the two experiments to a greater extent than plant dry matter production and the reasons for this are discussed in relation to growing environments and their possible impacts on artemisinin biosynthesis.

1. Introduction

Efforts to reduce malaria have focused on ways to kill the parasite (drug therapies, i.e. alkaloids) and the mosquito (using DDT) with some successes (Caniato and Puricelli, 2003). However, resistance of the *Plasmodium* (particularly *Plasmodium falciparum,* but emerging suggestions of *Plasmodium vivax* resistance) to cheap and safe drug treatments (chloroquine) has led to its resurgence (Kindermans et al., 2007). Single drug treatment approaches are not now encouraged due to induced resistance (Greenwood et al., 2008), and are being replaced by artemisinin-based combination therapies (ACT) particularly in multi-drug-resistant situations (WHO, 2000). The chemical artemisinin and its derivatives, derived from *Artemisia annua* L. (*qinghao* or sweet wormwood), originally a native of Asia, *Artemisia* now grows wild throughout Europe, North and South America and Australia (Kindermans et al., 2007; Ferreira, 2007; Greenwood et al., 2008), is naturally wind-pollinated and favours outcrossing over selfing (Ferreira et al., 1997). The principal active compound artemisinin or *qinghaosu*, a sesquiterpene lactone (Klayman, 1985) is synthesised predominantly within specialised glandular leaf trichomes along with other terpenoids, at low concentrations (0.1–0.6% dry weight) (Teoh et al., 2006; Putalun et al., 2007; Covello et al., 2007; Towler and Weathers, 2007; Covello, 2008; Maes et al., 2011).

Uses of plant-based artemisinin compounds are not without significant cost, particularly in the third-world where their need is greatest. These costs are in part linked to limitations in the quality of current germplasm (low active concentrations <1%, w/w), the negative impacts of the growing climate, poor agronomy and weak efficacy of post-harvest extraction. Despite the low biomass productivity of *A. annua* (1.5–2 tonnes ha^{-1}) and artemisinin concentrations, production of artemisinin between 6 and 14 kg per hectare is possible (Kindermans et al., 2007).

There are good reasons why we should endeavour to enhance the concentration and yield, per plant, of artemisinin, and there are a number of routes to achieve this. Our aim is to examine the

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agronomic requirements for field growing *A. annua* in temperate regions of Europe. Changes in the production of secondary metabolites are frequently linked to a number of aspects of the plant's interaction with its environment. This notion has been extended to include agronomic factors which influence plant growth rate and development, and the impacts of environmental stress, and how this modifies the accumulation of secondary metabolites (Mahan and Wanjura, 2005; Atkinson et al., 2006; Davies et al., 2009). Recent reports confirm this assertion, with oxidative stress influencing artemisinin production (Aftab et al., 2010).

To maximise artemisinin production, given the naturally low concentrations, we need to understand the relationship between leaf artemisinin concentration and plant biomass yield in order to optimise artemisinin yield (leaf artemisinin concentration × plant dry weight) (Ferreira et al., 2007; Davies et al., 2009). Failure to do so will inevitably lead to reduced field performance of new germplasm and the wastage of resources (fertilisers).

P is essential for plant growth and productivity, being present in DNA, RNA, and ATP, as well as in phospholipids within cell membranes (Rouached et al., 2010). P supply has long been known as a determinant of photosynthetic potential, with deficiency leading to reduced chloroplast carbon fixation. The influence of P deficiency on dry matter production, source:sink activity and the regulation of carbohydrate and protein synthesis is equally well acknowledged. Our current insights into the importance of P nutrition on *A. annua* growth and artemisinin production are however restricted to shoot tissue cultures (Liu et al., 2003). While for B, along with several other micro-nutrients (Cu and Zn), it appears when at low concentrations artemisinin production is dramatically reduced (some 10-fold) (Srivastava and Sharma, 1990).

B acts as an essential micro-nutrient which limits plant growth both when deficient and at elevated, toxic, concentrations; for which the margin between B deficiency, adequacy and toxicity is small (Marschner, 1995; Nable et al., 1997; Roessner et al., 2006; Yau and Ryan, 2008). B is somewhat unique, as an essential element, in that species differ, mechanistically, in their ability to transport B outside the transpirational xylem flow (Nable, 1988). Co-transport of B occurring through bonding with sorbitol within the phloem, e.g. the Rosaceae (Brown and Shelp, 1997). This alters the potential for growth limitations due to B-mobility restrictions for species not solely dependent on the transpirational flux for supplying organs with low transpiration rates i.e. developing fruits (Brown and Shelp, 1997). Deficiency of B in soils is often linked with evidence for its involvement in reproductive development and fruiting, and cell expansion particularly in roots (Shorrocks, 1997; Dell and Huang, 1997). Structurally it is used in cell walls and metabolism in relation to development associated with these organs and the regulation of endogenous hormones, while specific membrane transport proteins are now recognised i.e. BOR 1 (Dell and Huang, 1997; Power and Woods, 1997; Takano et al., 2005, 2006).

We hypothesize that the availability of phosphorus (P) and boron (B) will determine plant dry matter production and that of the secondary metabolite artemisinin. Recent evidence supports the validation of this hypothesis and links plant nutrition and environmental stress with artemisinin production (Davies et al., 2009; Aftab et al., 2010).

The objectives of the experimental work carried out on *A. annua* at EMR were to optimise the concentration of plant nutrients to maximise plant biomass and artemisinin yield and to develop a field system for growing the crop in the UK.

2. Materials and methods

2.1. Plant material and experimental setup

Seeds of *A. annua*, accession number NIAB 1053, a hybrid of two heterogenous populations (1001-1 × 1015, with seed from the 1015 parent only), were supplied to East Malling Research by Humber VHB (Chichester, UK). The seed was sown in April 2008 thinly into trays of potting compost, watered and covered with glass to retain moisture and kept at temperatures of 15 °C. In early May, approximately 1000 seedlings were pricked out and potted into trays of modules, each module being 2.5 cm × 2.5 cm and 3 cm deep. Within 20 d, 300 of the seedlings were potted up into 7.5 L pots using Klassman medium Irish graded peat with no added N, P and K, to which 1.9 g of $CaCO_3$ L^{-1} was added to raise the pH between 5.8 and 6. Plants were placed into an unheated glasshouse for 10 days to establish, before placing them out onto a well drained gravel bed, in June, in 12 rows (4.8 m wide) and 0.8 m apart, the pots were staggered along the row with 20 pots per row. The first and last rows were guarded by additional row on non-experimental plants. Rain infiltration was excluded from reaching the compost by covering the top of each pot with horticultural plastic, approximately ×1.5 the diameter of the pot, taped to the outside of the pot, a small hole was made in the centre of the plastic through which the plants could grow. 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.

2.2. Experimental treatments

Phosphorus experiment. P was supplied to the plants, via a fertigation system, at six concentrations; 0 mg L^{-1} (P1), 5 mg L^{-1} (P2), 15 mg L^{-1} (P3), 30 mg L^{-1} (P4), 60 mg L^{-1} (P5), and 120 mg L^{-1} (P6). Potassium dihydrogen phosphate was used to adjust the P concentration and K_2SO_4 was used to balance the K supplied in each of the treatments. Other macro- and micro-nutrients were kept constant and supplied at the following concentrations: N 106 mg L^{-1}, K 155 mg L^{-1} (found to be the optimal Davies et al., 2009), Ca 80 mg L^{-1}, Na 33 mg L^{-1}, Zn 0.1 mg L^{-1}, B 0.3 mg L^{-1}, Cu 0.1 mg L^{-1}, Fe 2.8 mg L^{-1} and Cl 3.5 mg L^{-1}, to all the plants. S was applied at a concentration of between 48 and 107 mg L^{-1}, with P1 plants having the highest concentration and P6 having the lowest concentration. The design was a randomised complete block: 6 treatments × 8 blocks, each plot contained three plants. Total number of plants was 144 (6 × 8 × 3).

Boron experiment. B was supplied to the plants, via a fertigation system, at three concentrations; 0.1 mg L^{-1} (B1), 0.3 mg L^{-1} (B2) and 0.6 mg L^{-1} (B3). Different concentrations of $B(OH)_3$ were used and all other macro- and micro-nutrients were kept constant and supplied as described above (with the exception that S was supplied at 112 mg L^{-1}). The experimental design was that of a randomised complete block: 3 treatments × 8 blocks, each plot contained three plants. Total number of plants was 72 (3 × 8 × 3). Our initial experiment did not conclusively determine the optimal concentration of applied B (~0.6 mg L^{-1}) which saturated B leaf tissue demand, either with respect to dry matter production, or B tissue concentration. We therefore repeated the experiment increasing the applied B concentration by 50%. Seed was sown, in mid July and pricked out into similar peat media and allowed to establish as described above. In September, 72 similar plants were selected (above ground uniformity) and placed onto two free-draining benches within the GroDome facility (Unigro Limited, Fawkham, Kent) at EMR. This state-of-the-art controlled environment (containment) facility provides optimised radiation capture and temperature control. The specifications of the controlled environment facility have been described (Colgan et al., 2010). Plants were spaced 50 cm apart and each bench had 12 rows of plants with 3 plants per row. Day time temperature (06:00–21:00 h) within the compartment was set at 22 °C and night time temperature (21:00–06:00 h) was set at 17 °C. In October, supplementary lighting, provided by 8 sodium lights (SONTI 400 W), was switched on for 4 h d^{-1} as daylight extension,