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| **Project Title** | Developing an alternative UK industrial crop Artemisia annua, for the extraction of Artemisinin to treat multi-drug resistant malaria. (Council funding cross sector) |
| **Project number:** | LK0822/CP 44 |
| **Project leader:** | Ian Flockhart [Botanical Developments Ltd] |
| **Report:** | Second Annual report, 2008 |
| **Previous report** | First year report 2007 |
| **Key staff:** | Colin Hill - Commercial extraction<br>Trevor Robinson – Crop agronomy, Frontier<br>Nigel Dungey – Seed production, Humber VHB<br>Steven Bentley – Parent breeding, NIAB<br>Christopher Atkinson – Plant physiology, EMR<br>Jack Woolley – Analytical analysis |
| **Location of project:** | NIAB; East Malling Research; De Montfort University; Frontier Agriculture; Humber VHB. |
| **Project coordinator:** | David Hand, Humber VHB |
| **Date project commenced:** | 1st April 2006 |
| **Date completion due:** | 1st April 2010 |
| **Key words:** | *Artemisia annua*, seed production, agronomy, breeding, variety trials, yield, nutrient effects, fertiliser. |

The results and conclusions in this report are based on a series of experiments conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, the biological nature of the work dictates that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

**AUTHENTICATION**

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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# Grower Summary

## Headline

This project has identified that crossing two parent plants with high artemisinin contents can produce progeny with high artemisinin and the seed collected from the progeny can be used to grow field crops. Further work is needed to optimise yields, artemesinin levels herbicide application and harvesting methods.

## Background and expected deliverables

This project is investigating *Artemisia annua* for its potential use in the treatment of malaria. This is achieved by the extraction of the active pharmaceutical ingredient (API) artemisinin from the leaves and young stems of the plant. The API cannot currently be economically synthesised using bulk fermentation or chemical synthesis. Extraction from plants therefore remains the most economically feasible source of this medicine for the foreseeable future.

This 4 year LINK project follows on from a successful one-year, Defra funded project NF0613 which indicated that certain *Artemisia annua* lines grew well under the UK weather conditions (summer 2005) and a few of the better adapted lines also had high levels of artemisinin. However, there is a need for significant work to better characterise this material for UK conditions and develop cost affective planting and harvesting techniques.

The success of major crops within the UK is dependent on an understanding of the many interacting variables affecting productivity. These may have been developed and refined over many years of crop development. It will not be possible to perfect our understanding of this crop and its agronomy in a four year study, but significant progress should be possible.

The Defra work concluded that a fully integrated UK production chain was possible but it would be imperative to have a secured supply of fully characterised germplasm. The germplasm tested in 2005 indicated that this could be achieved and that it would be necessary to develop well controlled experiments and analytical techniques to characterise the germplasm.

## Summary of the project and main conclusions

### Confirmation of improved analytical techniques

As reported in last year's annual report, the standard technique for the quantification of artemisinin in herbal material, or in extracts from herbal material were found to be unreliable. A new technique has been developed, which has facilitated faster and more reproducible results in the past year.

### Evaluation of the best material collected in 2006-7

The parent line 1015 was the best material in both 2005 and 2006. Although the percentage artemisinin content of the samples in 2007 were lower than those reported in 2006, the large variation previously observed between the sample results was no longer seen due to methodology development described above. With the replication of all samples and consistency between the samples analysed, there is now a greater level of confidence in the 2007 results. The best result for the line 1015 was achieved from the harvest made on the 30$^{th}$ August and was 1.2% artemisinin. Selection of new parent material has been successful; giving Artemisinin levels as good as, or better than the original material. Single plant selections made in 2007, which will be re-tested in replicated trials in 2008, indicate that levels of 1.6 to 1.7% have been achieved by this method of selection.

### Evaluation of the crosses made in 2006

Crosses were made at EMR using the best parent material according to analyses made at that time. Analysis of the percentage artemisinin content showed a heritability estimate of 98% on the basis of parent-offspring regression. This was very high and concurs with the estimates in the literature. The results to date indicate that using two parents with high percentage artemisinin give progeny with high percent artemisinin. Therefore, further crosses will employ crossing the best with the best. New parent material selected in 2007 has a higher potential than anything previously used. The best lines; 1053 and 1062, were significantly better than the commercially available line 1001. Frontier Agriculture, therefore commissioned the first UK commercial seed production within this project.

**Optimisation of biomass production in response to nutritional supply**

The consortium concluded that the EMR work should focus mainly on the influence that nitrogen fertigation has on the two main factors contributing to yield of artemisinin from *Artemisia annua,* namely*,* total plant biomass and artemisinin concentration. EMR also carried out a smaller nutritional experiment to determine the effects of potassium on these parameters.

**i) Nitrogen**

Application of nitrogen at approximately 100 mg/l (equating to 3.9 g N/plant) was found to be optimal for *Artemisia annua* growth. Levels above this did not significantly increase growth. Artemisinin concentration, however, was highest at low applications of nitrogen, with its concentration decreasing as nitrogen concentration increases. The total yield of artemisinin on a per-plant basis, increased with increasing nitrogen application up to 106 mg/l. Further increase in yield of artemisinin was not seen above 106 mg/l.

Nitrogen concentration measured in the leaves and total leaf nitrogen of *Artemisia* plants increased with each successive increase in the rate of nitrogen application. Increasing nitrogen concentration application above 106 mg/l did not increase artemisinin yield, plants receiving this concentration of nitrogen have a leaf nitrogen concentration of 5.5% w/w at harvest, this therefore appears adequate for maximising artemisinin yields per plant.

**ii) Potassium**

Potassium concentration in the leaves and total leaf potassium of *Artemisia* plants increased with each successive increase in potassium application. Application of potassium at approximately 155 mg/l (equating to 6.9 g k /plant) was optimal for *Artemisia annua* growth further additions above this level did not increase biomass.

Artemisinin concentration did not change when altering the concentration of potassium supplied. The total yield of artemisinin, on a per plant basis, was slightly higher when potassium application was raised from 53 mg/l to 155 mg/l, although this increase was not statistically significant. A concentration of potassium in leaves of 2.6% w/w at harvest was adequate for maximising artemisinin yield per plant.

**Development of commercial seed production.**

A large-scale commercial trial to produce seed concluded the following:

- Varying the plant density has a direct impact on seed yield; the lowest density having the highest yields both by each plant and by unit area
- At 3 plants/$m^2$, the achieved yield was 6g/plant for the 1015 parent of the ’1053‘ and ’1054‘ crosses, close to the maximum yield achieved in the summer of 2007
- Only the 1015, but not the 1001 parents produced appreciable seed; it is calculated that seed set was around 10% for 1015, but <1% for 1001-01 or 1001-03 parents
- The data suggests that seed on the 1015 is the result of selfing. Altering flowering synchrony, enclosing whole plants in fleece, enclosing branches in paper bags, whether with or without the 1001 parent, all failed to affect seed yield
- 4.3 Kg of the 1015 seed has been harvested. There were 12,000 seeds/g with a 96% germination rate after 3 days of pre-chilling

- A new, promising, but inadequately researched line '1062', yielded nearly 40g/plant from both parents. Only 0.8Kg has been harvested and the seed were smaller, at 20,000 seeds/g, with a lower germination rate of 83% after 3 days of pre-chilling.

**Commercial scale field production**

In the 2007 season, *A. annua* was grown on 4 farms. There was heavy rainfall in the summer of 2007, which delayed planting followed by heavier rain soon after planting, contributing to soil capping and soil wash. Some herbicides that showed promise in 2006 were harsh on the *A. annua* in 2007, with some crop kill.

In 2007, we drilled on a field scale for the first time, using UK seed produced from the commercial seed production trial. This was sown direct as pelleted seed and also as transplants grown in plugs by a commercial plant raiser similar to the style used for a field brassica crop.

**Conclusions from 2007 season with 'Field scale' crops**

- UK produced seed produced a better crop than the commercial Brazilian line '1001'.
- 2007 Pelleted seed produced by our partnership with shallow drilling etc showed promise.
- The herbicide choice and technique employed needs further work.
- Yields of biomass were only a fraction of those obtained in the NIAB plots.
- The percentage artemisinin obtained from the field was lower than plot levels.
- The harvest technique used needs to be improved.

**Financial benefits**

The UK produced seed proved to be of higher quality than that previously imported. In 2007/08, our partnership has been able to produce seed on a commercial scale, based on the testing of the lines produced by EMR. Frontier Agriculture has invested considerable effort into the establishment of a field crop using traditional vegetable transplanting techniques. This will be repeated in 2008.

**Action points for growers**

There are no action points for growers at present, however the significant progress already made suggests that this opportunity could start to provide significant benefits to growers before the end of the project.

# 1 Science Section

Experimental work is described and discussed, according to original project Work Package, in the following sections. Please note that artemisinin is abbreviated to '**Art**.' in both tables and figures throughout the following sections.

## 1.1 Introduction

*Artemisia annua* is a potential new biopharmaceutical crop with no previous history of large scale or even small scale cultivation in the UK outside of botanic and private gardens. It is being investigated here for its potential use in the treatment of malaria. This is achieved by the extraction of the active pharmaceutical ingredient (API) artemisinin from the leaves and young stems of the plant. This is converted into more water soluble derivatives and formulated into oral medication. The active ingredient cannot currently be economically synthesised using bulk fermentation or chemical synthesis. Extraction from plants remains the most economically feasible source of this medicine for the foreseeable future. The need for this medicine is urgent due to the collapse of most currently used medications through development of multiple drug resistance by the malaria parasite.

This 4-year LINK project follows on from a successful one-year, DEFRA funded project NF0613. Significant work has been carried out elsewhere and has been reported in the literature during the last 30 years, but much of this work reported conflicting results with respect to many aspects of cultivation, particularly with respect to plant physiology and API production. A literature search concluded that many of the conflicting results were due to the range of germplasm used and the diverse regions of the world where testing had been conducted. The trial results indicated that certain *Artemisia annua* lines grew well under the UK weather conditions (summer 2005) and a few of the better adapted lines also had high levels of artemisinin. There is a need for significant work to better characterise this material for UK conditions and develop cost affective planting and harvesting techniques.

The success of major crops within the UK is dependent on an understanding of the many interacting variables affecting productivity. These may have been developed and refined over many years of crop development. It will not be possible to perfect our understanding of this crop and its agronomy in a four year study, but considerable progress should be possible.

The work reported in NF0613 concluded that a fully integrated UK production chain was possible, but it would be imperative to have a secured supply of fully characterised germplasm. The germplasm tested in 2005 indicated that this could be achieved and that it would be necessary to develop well controlled experiments and analytical techniques to characterise the germplasm. The key objectives for 2007 were:-

1. To systematically evaluate the best material collected in 2006
2. Confirm its potential using improved analytical techniques
3. Characterise the flowering habit of the material for seed production
4. Make crosses using the best material for evaluation in 2007
5. Develop a robust agronomic guide for growers
6. Improve harvest protocols

Improved material was only available in small amounts for the objectives above, therefore the agronomic objectives 5-6 are still being partly addressed using some imported germplasm and some from a commercial source.

## 2 Materials, Methods and Results according to Work Package

The methodology used is described according to Work Package and is documented below, followed by results relating to that piece of work

### 2.1 Work Package 1; Developing a rapid artemisinin assay – Led by De Montfort University

The aim of this work package is to develop a rapid and accurate analytical technique for the quantification of artemisinin and related compounds. There are two major sub-tasks:

1) rapid and reliable laboratory-based quantification of artemisinin in *A. annua* samples
2) rapid 'field'-based semi-quantification of *A. annua*

The significance of the fluorescent exudates is also being investigated. This phenomenon may not have any impact on artemisinin and as such, will remain a minor task unless it is shown that it will significantly influence growth.

#### 2.1.1 High-Performance Liquid Chromatography-Mass Spectrometry for Artemisinin Quantification

In 2007, it was found that there were problems associated with use of the standard technique for the quantification of artemisinin in herbal material, or in extracts from herbal material. This entailed the use of High-Performance Liquid Chromatography (HPLC), coupled to a diode-array detector, or DAD (essentially a UV lamp). When crude extracts of *A. annua* were analysed by this detection method, the resulting chromatograms were frequently poorly resolved, resulting in unreliable quantification of artemisinin.

Although the resolution of crude extracts has been considerably improved by monitoring column theoretical plates, there was considerable scope for further improvement in rapid analysis of samples, particularly crude extracts, by the use of another type of detection.

**Methods**

To this end, HPLC was coupled not to a DAD lamp, but to a mass spectrometer (MS). The type of MS detector that was used was an ElectroSpray Ionization (ESI) detector. This fires a beam of electrons at the molecule of interest, which then breaks apart (in a process called *fragmentation*) in a characteristic manner, and the resulting charged fragments are recorded by the detector. MS is known to be much more sensitive than DAD, and it has one other great advantage: it is highly selective. A mass spectrometric detector can be asked to record only the fragments generated by a given molecule, and to ignore all others - removing the problem of poor resolution, so that crude extracts can be reliably characterised.

Mass spectrometers can be set to record in either *total ion chromatogram (TIC)* mode, in which case molecular fragments from 50 mass units up to 1000 mass units are recorded, or, for quantitative analysis, in *single ion monitoring (SIM)* mode, in which case only specific fragments are recorded. SIM is the means by which the device can be made to ignore uninteresting molecules, and hence improve sample resolution. For this project, SIM was chosen. Before an HPLC set-up with a mass spectrometer as a detector could be used, a few changes had to be made to the HPLC parameters:

**i) Column set up**

Major changes were made to the flow rate of the mobile phase. Mass spectrometric detectors typically cannot cope with such high solvent flow rates as DADs. Therefore, a smaller column, which requires less solvent, was selected for sample separation. A narrow-bore column was chosen, with an internal diameter of 2.1mm (compared to the standard 4.6mm), and a length of 150mm, (compared to the standard 250mm). The column packing was almost the same as the previous column, using RP-C18 (octadecasilane), but with a pore size of 3µm, compared to the 5µm used previously. When using this column, the flow rate of the mobile phase was reduced to 0.2ml/minute (the recommended maximum for

columns of this internal diameter and pore size), compared to the 1.0ml/min used previously. This reduction in flow rate has the advantage of being much more economical in terms of solvent usage. This smaller column also meant that run time was shortened from 25 minutes to 15 minutes.

**ii) Mobile Phase**

Some improvements were also made to the separation technique by adjusting the solvents used as a mobile phase; a gradient separation method was developed, which was used instead of the previous isocratic separation method. Since the separation achieved by the previously developed isocratic method was reasonable, it was decided to keep the new gradient separation conditions quite gentle and fairly close to the conditions of the isocratic method, whilst trying to eliminate the characteristic problems of isocratic separations: a lot of peaks in the first few minutes, a "dead zone" in the middle of the run where little elutes, and a few wide peaks towards the end. Extreme gradients (from 10% water to 90%, for example) were not ideal for these crude extracts and were eliminated as possibilities early on.

### *2.1.2* Results for High-performance Liquid Chromatography-Mass Spectrometry

After much trial and error, the HPLC gradient method shown in Table 1 was found to give an improved resolution in the analysis of crude extracts: Although, as discussed earlier, MS in single-ion monitoring mode is highly selective and therefore could reliably quantify artemisinin even within a poorly resolved chromatogram, this improved HPLC method means that total-ion chromatogram mode may also be used, and even DAD chromatograms are reasonably well resolved.

**Table 1. Gradient HPLC-method for the separation of crude extracts of *A. annua***

| Time | Solvent A % | Solvent B% | Flow (ml/minute) |
|---|---|---|---|
| 0.00 | 100 | 0 | 0.2 |
| 7.00 | 0 | 100 | 0.2 |
| 10.00 | 50 | 50 | 0.2 |
| 15.00 | 100 | 0 | 0.2 |

Where solvent A: =33% acetonitrile, 66% water - solvent B=66% acetonitrile, 33% water

### 2.1.3 Rapid quantification of artemisinin by SIM MS

The characteristic fragmentation pattern of artemisinin was observed, and these fragments (Figure 2) were input to the MS software. Using this information, the MS detector could then be used to quantify artemisinin, even when present at low levels in crude extracts.

A calibration curve was generated, see Figure 1, where santonin was used as an internal standard, and the artemisinin level was varied from 0.005mg/ml to 0.04mg/ml. The slope of this curve was obtained, and used to design an Excel spreadsheet that could rapidly calculate the artemisinin level (as % w/w) in any sample, as long as dilution factors and mass of dry herb were input.

**Figure 1. Calibration curve of artemisinin, using santonin as an internal standard**

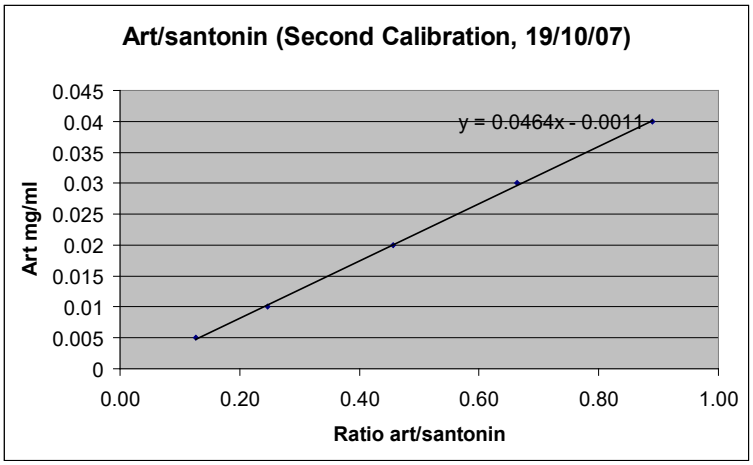
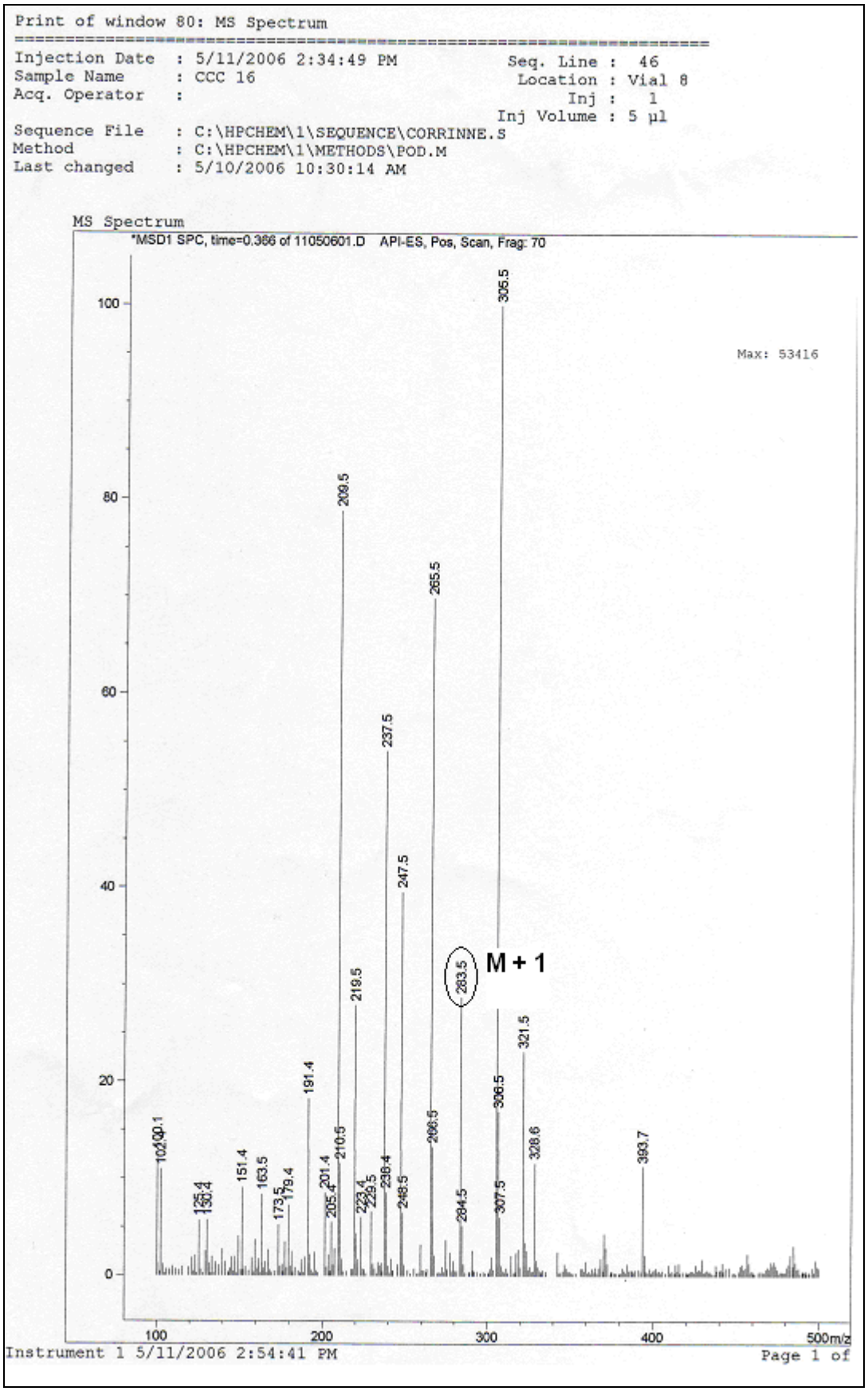


**Figure 2. Mass fragments characteristic of artemisinin.**



**Summary and Actions**

The use of HPLC-MS (ESI) for the reliable artemisinin quantification in large numbers of samples has proved very successful. Using HPLC-MS (ESI) in single-ion monitoring mode, 300 samples per week can be assayed with a high degree of reproducibility in samples. HPLC-MS has the additional advantage of greatly reduced solvent requirements: an 80% reduction. This is now a robust high-throughput analytical method, which is ideal for the analysis of artemisinin.

### 2.1.4 Use of Thin-Layer Chromatography (TLC) for fast analysis

**Background**

As in HPLC-DAD or HPLC-MS, there are two steps to successful artemisinin quantification: separation and detection. After a period of optimisation, separation of artemisinin from a crude extract was obtained by the use of a mobile phase, consisting of 70% diethyl ether and 30% hexane. Artemisinin and related compounds may be visualised using the reagents validated in project NF0613: an acidified alcoholic solution of vanillin, or by iodine vapour. Vanillin spray reagent is preferred, as this form of destructive detection is permanent, unlike iodine detection, and stains artemisinin a clearly visible green. Although TLC is a simple technique requiring minimal analytical equipment, it has not yet been found to be suitable for field-based high-throughput screening for two reasons:

1) It cannot be automated at modest cost; sample extraction, accurate loading onto the plate, and subsequent plate development, take up a lot of time when hundreds of samples are involved;
2) Visual estimation of artemisinin content is not sufficient; it was found that although the poorest samples (below 0.5% w/w artemisinin) could be distinguished from the rest of the samples, high-yield samples could not be distinguished from average samples by eye alone

**Methods**

Using diethyl ether and hexane as a mobile phase, good separation of artemisinin from interfering compounds has been achieved. In addition, whilst considering how to make TLC more suitable for field-based rapid analysis, a new and extremely simple means of rapid sample preparation for TLC was discovered.

Normally, for TLC-based sample analysis, artemisinin (or any other compound of interest) must first be extracted from the fresh or dried leaf material into a suitable solvent, and then a calibrated amount of this solvent extract has to be loaded onto the TLC plate. These time-consuming sample preparation and loading steps may be eliminated. Simply pressing the upper surface of a fresh leaf of *A. annua* onto the TLC plate (Figures 3 and 4) deposits enough artemisinin onto the silica to allow plate development and visual comparison of low-yielding vs. high-yielding *A. annua* cultivars. The surface area of the leaf that is pressed against the plate can be standardised by the use of a cover-sheet in which a series of holes have been punched.

**Figure 3. An alternative method for loading TLC plates**

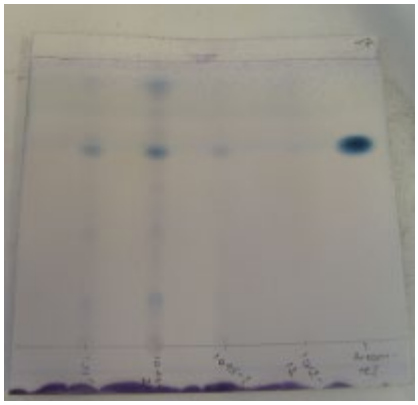
| | |
|---|---|
|  |  |
| a) Coversheet with holes at base, cut to the same dimensions as the TLC plate. | b) Deposition of artemisinin onto the TLC plate, using coversheet to ensure artemisinin is deposited from a reproducible surface area |

### 2.1.5 Results from Thin-Layer Chromatography Method Development

**i) Separation**

Early results indicate that TLC chromatograms produced by the new, simple application method are comparable to those produced by more traditional methods. Figure 4 shows an example:

**Figure 4. TLC chromatogram produced using the new "leaf-spot" technique**



**Key**

**Left to Right: *A annua* lines used were:**

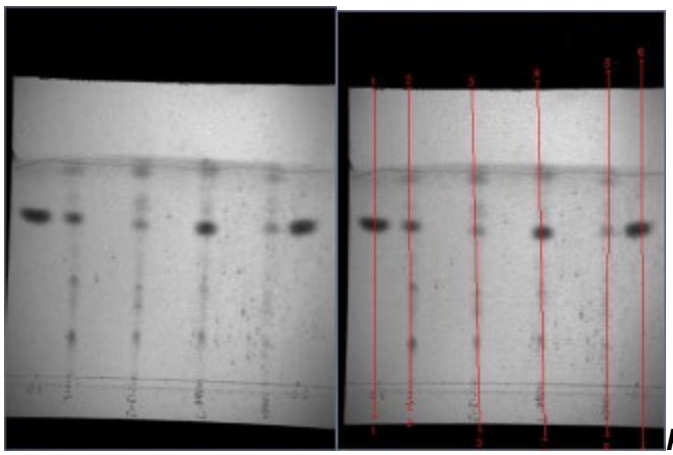
**1015 (1.1% artemisinin w/w DW),**
**1046 (1.3% w/w DW),**
**1045-1 (0.6% w/w DW),**
**1012-12 (0.5% w/w DW);**
**artemisinin reference (1mg/ml)**

Combining this rapid-application separation technique with reliable sample quantification could provide the rapid in-field technique, which forms one of the aims of this Work Package. This rapid spotting technique may be of generic use in the analysis of other plant families, such as cannabis leaf, for example.

**ii) Detection**

The next step was to devise a reliable detection and quantification technique. De Montfort recently acquired Bio-Rad densitometric scanning apparatus and software. This apparatus produces a digital image of a stained TLC-plate, and can quantify the degree of UV-absorbance of the stained areas (separated compounds). This technology is far more accurate than the human eye in the comparison of sample quality and is currently in use in the pharmaceutical industry for the analysis of artemisinin-based drugs (Gabriels and Plaizier-Vercammen 2003). This software has not yet been validated for artemisinin quantification, but it is anticipated that densitometric analysis will play a part in both HT screening and in-field analysis.

**Figure 5. Developed TLC plates examined using densitometric software**



***Key The extreme right and left tracks represent artemisinin standards at 1mg/ml, where 10µl of solution was loaded onto the plate. The four inner tracks represent extracts from four different varieties of* A. annua *ranging from 0.6% w/w (DW) to 1.5% w/w (DW) artemisinin***

Results from initial work are seen in Figure 5 above. Extracts were prepared by immersing 120mg of fresh leaf material, for six seconds, into 1ml of dichloromethane. 10µl of this extract were spotted onto the plate.

**Summary and Actions**

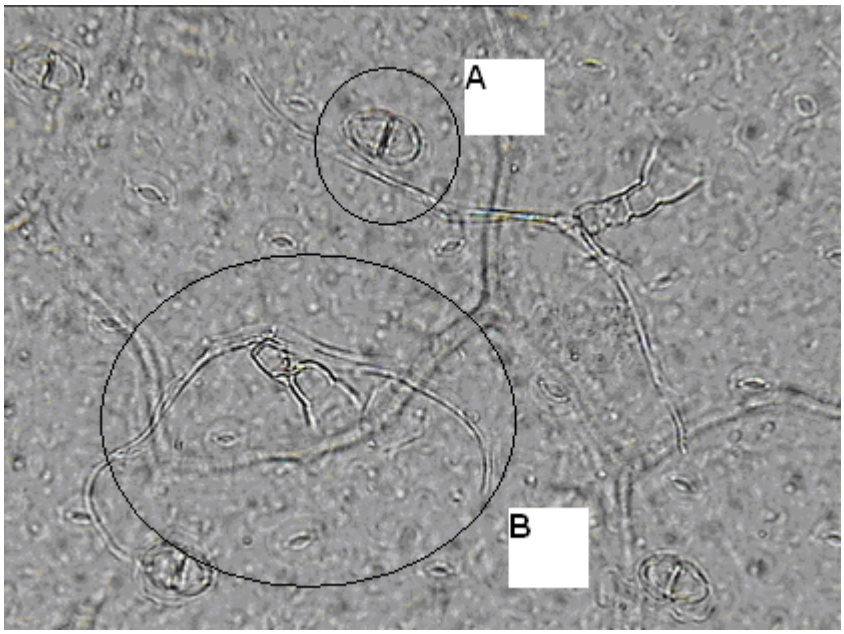
These results show that De Montfort University is close to validating TLC with densitometric scanning analysis, for the accurate quantification of artemisinin and related compounds.

### 2.1.6 Trichome Density

**Background**

Glandular secretory trichomes (GSTs) are the leaf surface structures within which artemisinin is stored (see Figure 6). It was hypothesised that simply counting the number of GSTs per unit leaf area (that is, monitoring the trichome density) would give an indication of potential artemisinin content. The trichome density was monitored on leaves of four new cultivars of *A. annua* across a six-week period of growth. Trichomes per unit area on leaves were counted taken from the top, middle and lower regions of the plants. Correlations between trichome density and artemisinin content were noted.

**Figure 6. Light microscopy image of leaf of A. annua, showing GST (A) and T-trichome (B)**




### 2.1.7 Results of Trichome Density Studies

It was found that artemisinin content was greatest in the middle part of the plant; trichome density was greatest in the top regions of the plant but; trichome density did not clearly correlate with artemisinin content in any cultivar.

Thus the following observations were made:

1) When only the *top leaves* were considered, trichome density did directly correlate, in most cases, to cultivar quality (Table 2).
2) In general, artemisinin content was highest in the middle part of the plant: this is in agreement with other workers (Zhang, 2006) even though trichome density decreases in these older leaves.

**Table 2 Trichome density in four cultivars of *A. annua***

| ***Average Trichome Density (0.7mm$^2$)*** | **1015** | **1012-12 (Lowest artemisinin)** | **1046-7 (Highest artemisinin)** | **1045-1** |
|---|---|---|---|---|
| **Top leaves** | 10 | 7 | 24.5 | 14 |
| **Middle leaves** | 10 | 13.3 | 15.7 | 13.3 |
| **Lower leaves** | 9.7 | 11 | 12.7 | 14.5 |

**Summary and Actions**

The results so far do not yet provide sufficient evidence as to whether trichome density is a reliable indictor of plant quality, although it does appear that when leaves from the top regions of the plants are considered, increased trichome density correlates to eventual increased artemisinin level. The caveat “eventual”, is used here because despite higher trichome density, leaves from the top of the plant do not yet contain high levels of artemisinin. If this trichome-count technique were used in quality analysis, care would have to be taken to assay leaves from the same region of each plant under analysis.

Assays were performed when plants were mature and in one case, flowering. This experiment will be repeated, assaying for an entire growth period, rather than just towards the end of that growth.

### 2.1.8 Potential for the use of Hairy root cultures to study fluorescent exudates

**Background and methods**

Three hairy root cultures of *A. annua* have been developed, by inoculation of sterile seedlings by the bacterium *Agrobacterium rhizogenes*. These cultures will be used to study the biosynthesis of artemisinin. This has potential for application in future plant genetic improvement studies, particularly in the development of higher-yielding varieties.

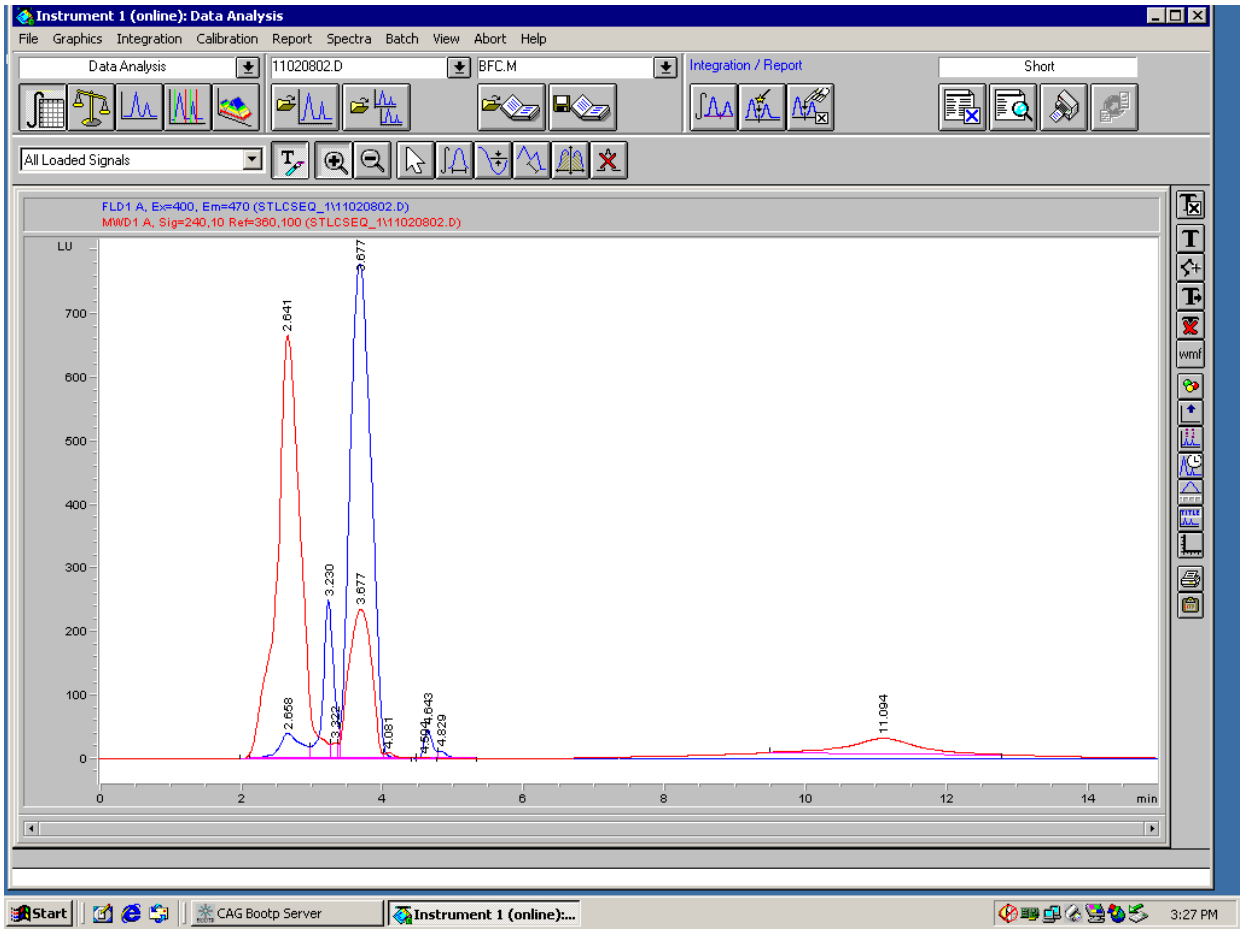
It has been observed previously at De Montfort, that hairy root cultures of *A. annua* are highly fluorescent under UV-light: the fluorescence displays an absorbance maximum at 400nm, and an emission maximum at 470nm. The fluorescent material is exuded into the roots' aqueous nutrient media. Upon discovering this phenomenon, we investigated whether the roots of whole plants also exuded fluorescent compounds into the rhizosphere. Examination of the soil in the pots in which greenhouse-grown *A. annua* plants were growing revealed that fluorescent compounds were being exuded into soil, to a large extent. When water was washed through the pots and collected, the collected water was highly fluorescent.

Excitation and emission scans of a crude aqueous root extract revealed an absorbance maximum at 400nm, and an emission maximum at 470nm. Initial HPLC-fluorescence (FLR) chromatograms showed that crude aqueous hairy root extracts contained three peaks of fluorescence, with the latest eluting peak by far the greatest in magnitude see Figure 7 below. Chromatograms of water taken from the soil surrounding greenhouse-grown *A. annua* showed one fluorescent compound, corresponding to the first peak of hairy root extracts, but little more. It may be that whole plants exude compounds that have different excitation and emission maxima.

Literature reports indicate that other *Artemisia* species can exude autotoxic compounds into the rhizosphere, resulting in weakened plant growth. These compounds have also been reported to have this effect by inhibiting seed germination. A test was designed to determine whether the fluorescent exudates would inhibit the germination of mustard seeds (chosen because they have a very fast germination rate). Some seeds were grown in water only and a second batch in water mixed with a small amount of fluorescent exudate (exudates to water ratio was 1:5 or 1:10).

**Figure 7. HPLC-FLR chromatogram of a methanolic extract of hairy roots of *A. annua*; the blue line shows fluorescence**




### 2.1.9 Results of Hairy root culture Exudate Studies

**i) The impact of fluorescent exudates on the growth of Mustard seeds**

Seeds continued growing for 10 days under both light and dark conditions. In both light and dark conditions, and under both exudate dilutions, the results were the same: although seeds were initially a little slower to germinate, when germination did occur, seedlings were much healthier and less prone to fungal infection when they were grown in the presence of fluorescent exudates see Figures 8 and ( below.

**Figure 8. Mustard seedlings grown in water: *Left* Day 4; *Right* Day 10**

**Figure 9.Mustard seedlings grown in fluorescent root exudates diluted 1:5 with water: *Left* =Day 4; *Right* =Day 10**

**Summary and Actions**

Root fluorescence is quite rare, although it has also been observed in roots of oat, ryegrass and soybean (e.g. Suzuki et al 2006; Møller, and Lundborg 2006; Palmer 2005). Exudation of fluorescent compounds into the soil matrix is even less common.

Currently, efforts are being made to isolate and identify these fluorescent compounds. At the same time, growth curves of hairy root cultures are being obtained. It is of interest here to determine whether the fluorescence is linked to artemisinin production; it has been observed that the root cultures begin to fluoresce at day 10, which is the same day that artemisinin becomes detectable.

### 2.1.10 Overall Summary of Results and Deliverables in Work Package 1

Task A has been completed satisfactorily, although efforts will still be made to ascertain whether further reductions in time and costs can be made. Significant advances have also been made with Task B (field-based semi-quantification) both in TLC semi-quantification, and with considering the possibility of utilising trichome counts as a means of quality analysis.

## 2.2 Work Package 2, Development of improved germplasm – Led by NIAB

In 2007 NIAB continued to characterise parent material and evaluate initial crosses that had been made at EMR. The lines 1015 and 1001 were evaluated during the whole summer period as controls.

### 2.2.1 Choice and quality of parents for new lines

After analysis and selection in 2006, 37 potential parent lines were selected. Re-analysis in the autumn of 2006, indicated that confidence in the selected material needed to be confirmed by further testing. The selected material (plus the control 1015) were therefore maintained by vegetative propagation in the glasshouses over the winter period and multiplied from the stock plants for field propagation in the spring. Cuttings were taken 26/4/07 and transplanted into the field on the 18/05/07. For each parent line there were three plants with four replicates.

Using the standard harvesting method defined in 2006, the material was harvested at three intervals during the summer period.

### 2.2.2 Crosses made during 2007

Some of the parents identified in 2006 were sent to EMR and used to make crosses. The results from that crossing program were reported in 2006. Harvested seed was returned to NIAB for field evaluation in 2007 and this seed was sown in the glasshouse on 20/4/07. The seedlings were pricked out into trays at the two true leaf stage on 9/5/07. These were then grown in a cool glasshouse to produce small plants, which were transplanted into the field on 5/6/07 and 2/7/07, see Figure 10.

*A. annua* seed is produced from out-crossing plants, therefore the progeny may be similar, but they are all genetically unique. Each cross was planted in plots containing 30 plants with four replications.

**Figure 10. Spaced *A annua* plants at NIAB on 17th June 2007**

Using the standard harvesting method defined in 2006 the material was harvested at three intervals during the summer period.

### 2.2.3 Results of new Parent Crosses at NIAB within Work Package 2

The growing conditions in 2007 were very different from 2006. From early June 2007 to mid August it was generally very much wetter and cooler than in 2006. The plants appeared to grow very much more slowly and by the end of August, had not achieved the bulk of plant material that was achieved in 2006. However from the end of August the plants grew very vigorously and achieved a typical size by mid October.

#### i) Choice of parents

The parent line 1015 was the best material in both 2005 and 2006. During 2007 parent lines were harvested at several intervals during the season using the standard harvesting and drying protocols and then analysed by DeMontfort University; see Table 3. The full set of results for all the selected parents are presented in Appendix 1.

**Table 3. Control results for artemisinin at different harvest dates during 2007**

| Parent No | Cross | 1-Aug | 25-Jul | 1-Aug | 30-Aug | 6-Sep | 13-Sep | 27-Sep | 5-Oct |
|---|---|---|---|---|---|---|---|---|---|
| | | Value score | % Artemisinin | | | | | | |
| 1 | 1015 | 5 | 0.6 | / | 1.3 | 1.2 | 1.1 | 1.1 | 1.1 |
| 1 | 1015 | 3 | / | / | 1.1 | 1.0 | 1.2 | 1.3 | .1. |
| 1 | 1015 | 4 | 0.7 | 0.8 | 1.3 | 1.0 | 1.0 | 0.9 | 1.0 |
| 1 | 1015 | 4 | 0.7 | 0.7 | 1.1 | / | 1.2 | 1.2 | 1.2 |
| mean | | **4** | **0.67** | **0.75** | **1.20** | **1.07** | **1.17** | **1.20** | **1.10** |

On 1/08/07, each parent line was evaluated using a visual score from 1 to 5 (5 was considered to be the best agronomic type). Some of the parent material was much more vigorous than others.

Results from samples taken on 25/07/07 showed that the percentage concentrations were circa half those achieved by the end of August. In previous years, it had not been possible to measure this percentage increase during the season. From the end of August there were no further significant increases in the percent Artemisinin content. Generally, this occurred with most of samples from the parent tested and is consistent with the literature.

Although the percentage artemisinin content of the samples were lower than reported in 2006 the large variation previously observed (+ or- 1%) between the sample results was no longer apparent. With the replication of all samples and the consistency between the samples analysed there is now a greater level of confidence in the 2007 results. Table 4 below shows the results for two of the best parent lines selected in 2006. The full set of results for all the selected parents are presented in Appendix 2.

**Table 4. Attributes of the two best *A annua* Parent Line crosses tested in 2007**

| Parent No | Cross | 1-Aug Value score | 25-Jul | 1-Aug | 30-Aug | 6-Sep | 13-Sep | 27-Sep | 5-Oct |
|---|---|---|---|---|---|---|---|---|---|
| | | | % Artemisinin | | | | | | |
| 2 | 1001-9 | 4 | 0.6 | | 0.6 | 1.0 | / | 1.0 | 1.0 |
| 2 | 1001-9 | 4 | 0.8 | 0.9 | 1.0 | 1.1 | / | 1.2 | 1.2 |
| 2 | 1001-9 | 4 | 1.0 | 1.1 | 0.9 | 1.0 | / | 1.3 | 1.1 |
| 2 | 1001-9 | 4 | 0.7 | 0.9 | / | 1.0 | / | 0.9 | |
| **mean** | | **4** | **0.78** | **0.97** | **083** | **1.03** | **/** | **1.10** | **1.10** |
| 322 | 1046-7 | 5 | 0.8 | 1.0 | 1.0 | 1.02 | 1.3 | 1.0 | 1.3 |
| 322 | 1046-7 | 5 | 0.8 | 1.0 | 1.1 | 1.1 | 1.4 | 1.5 | 1.3 |
| 322 | 1046-7 | 5 | 0.8 | 1.1 | / | 1.2 | 1.3 | 1.5 | 1.2 |
| 322 | 1046-7 | 5 | 0.7 | 0.9 | / | 1.2 | 1.3 | 1.3 | 1.1 |
| **mean** | | **5** | **0.75** | **1.0** | **1.05** | **1.18** | **1.33** | **1.33** | **1.23** |

The results show that selection of new parent material has been successful giving Artemisinin levels as-good-as and better than the original material. Single plant selections made in 2007, which will be re-tested in replicated trials in 2008, indicate that levels of 1.6 to 1.7% have already been achieved by this method of selection.

### 2.2.4 Genetic analysis of the crosses

There were two planting timings for the crosses; these were 5/6/07 and 2/7/07. In the artemisinin analysis, samples from plants derived from 'Cross 1' (5/6/07 plantings) showed much less variability than analyses derived from plants in 'Cross 2' (2/7/07 plantings). This may reflect the fact that all the parents used in the analysis were planted at the same time as the first crosses.

It can be seen that the percentage artemisinin increased in both the parents and the crosses as the plants got older or the season changed, see Table 5. Results indicate that the age of the plants was important because the controls in the second planting always expressed

lower artemisinin content than the first. The 1001 Brazilian material, planted in the autumn of 2006 using a seed drill, was also compared with the 1001 controls planted at the same time as 'Cross 1'.

**Table 5 Comparison of artemisinin content with planting date**

| Planting Date | Harvesting Date | % Artemisinin |
|---|---|---|
| 1/10/06 | 20/08/07 | 0.85 |
| 5/6/07 | 20/08/07 | 0.6 |

This suggests that earlier planting will allow the plants to achieve their maximum percentage artemisinin content earlier in the summer and therefore allow earlier harvesting. This will be re-tested in 2008 and if confirmed, could have significant commercial and operational advantages for large producers such as Frontier.

**Analysis of the Heritablility of the Percentage Artemisinin Content**

Relationship with the mid-parent is good in both, however heritability estimates are very high; calculated at 100% for 'Cross 1' and 82% for 'Cross 2' on the basis of parent-offspring regression and 98% on combined data. The results suggest that the best strategy is to select both parents with high percentage artemisinin. This is currently reliably producing progeny with high artemisinin. Therefore future crosses will concentrate generally on crossing the best with the best see Table 6 and Figures 11-13, which show the relationship between artemisinin content of parents compared to final crosses.

**Table 6 Mean percentage Artemisinin in all parents and crosses**

| Dates analysed | Mean % artemisinin of all Parents | Mean % Artemisinin in Crosses on 5/6/07 = Cross 1 | Mean % Artemisinin in Crosses on 2/7/07 = Cross2 |
|---|---|---|---|
| 25 /07/ 2007 | 0.5954 | 0.4939 | |
| 01 /08/ 2007 | 0.7533 | | |
| 20 /08/ 2007 | 0.8537 | | |
| 30 /08/ 2007 | 0.8071 | 0.8907 | |
| 06 /09/ 2007 | 0.8253 | | |
| 13 /09/ 2007 | 0.8767 | | 0.687 |
| 27 /09/ 2007 | 0.9281 | 0.9413 | |
| 05 /10/ 2007 | 0.8533 | | |
| 11 /10/ 2007 | 0.8987 | | 0.7137 |

**Figure 11 'Cross 1'; relationship with the mid-parent according to artemisinin content (both axes)**

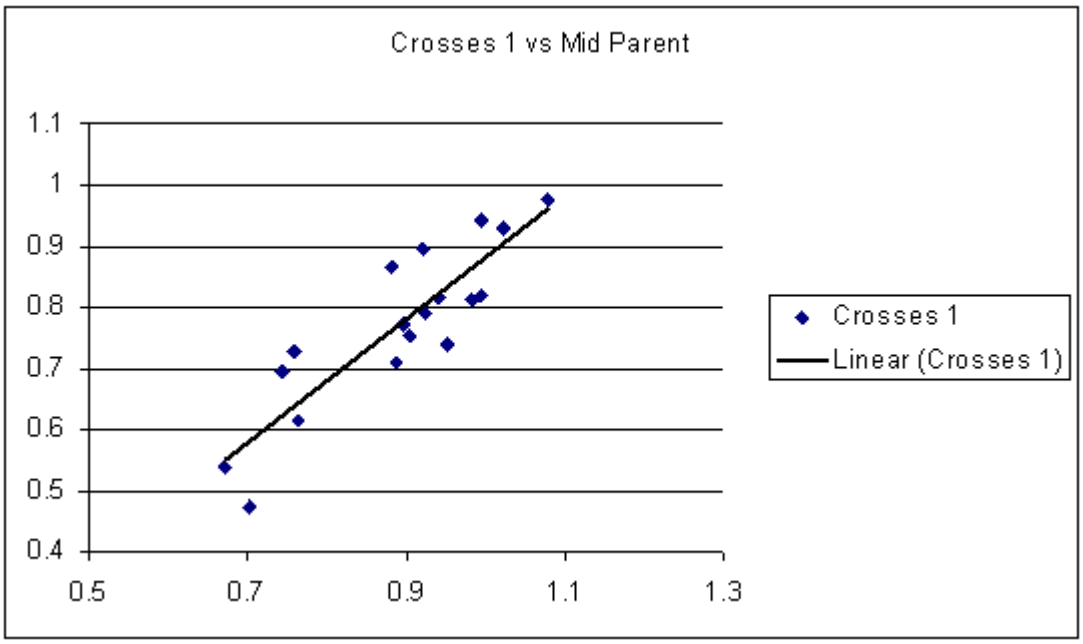



**Figure 12'Cross 2'; relationship with the mid-parent according to artemisinin content (both axes)**

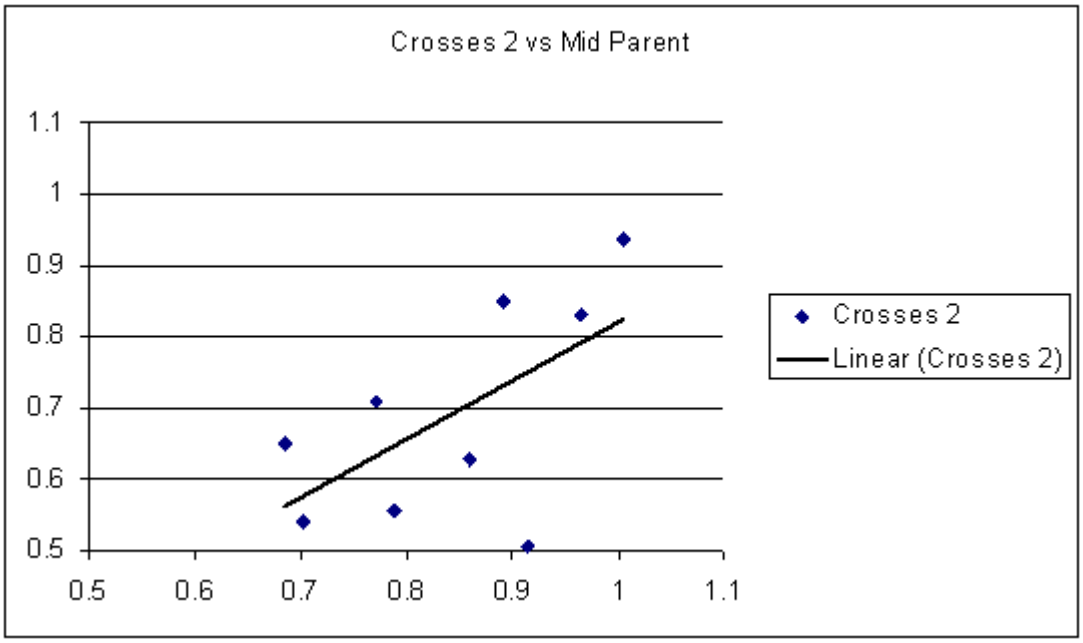


**Figure 13 Relationship with the mid-parent according to artemisinin content (both axes)**

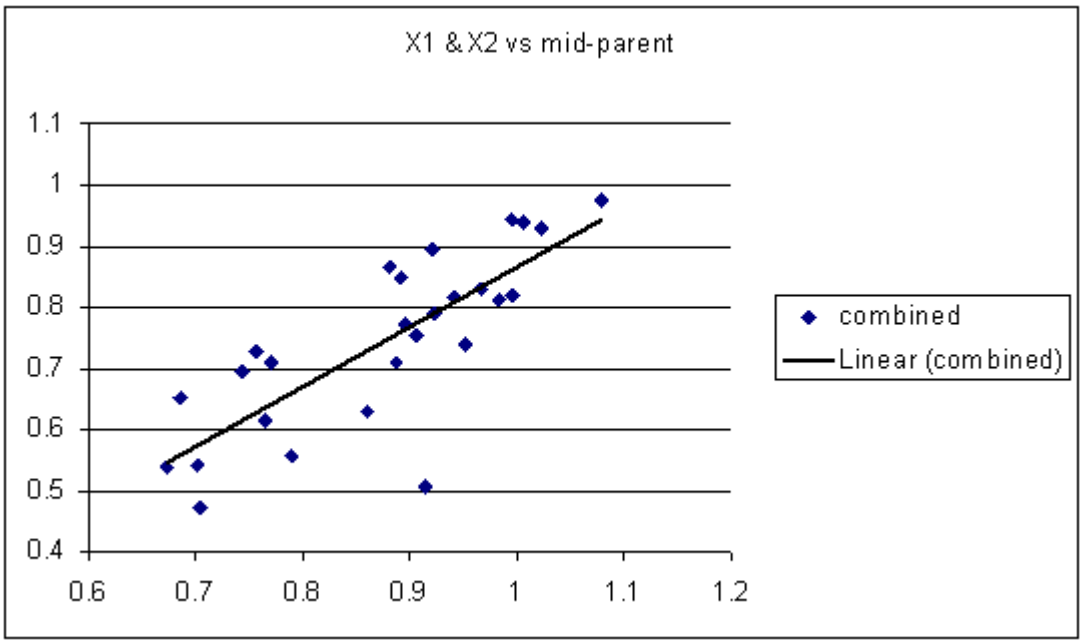


**Table 7 Results from the two best crosses evaluated in 2007. The full set of results can be seed in Appendix 2**

| Cross | 1-Aug Value score | 25-Jul | 30-Aug | 27-Sept |
|---|---|---|---|---|
| | | % Artemisinin | | |
| 1001 | 4 | 0.3 | no result | 1.0 |
| 1001 | 3 | no result | 0.7 | 0.8 |
| 1001 | 4 | 0.4 | 0.5 | 0.9 |
| 1001 | 4 | 0.5 | 0.7 | 0.8 |
| **mean** | | **0.4** | **0.63** | **0.88** |
| 1053 | 5 | 0.7 | 1.2 | 1.1 |
| 1053 | 5 | no result | 1.0 | 1.1 |
| 1053 | 5 | 0.5 | 0.8 | 0.9 |
| 1053 | 5 | 0.6 | 1.3 | 0.9 |
| **mean** | | **0.6** | **1.08** | **1.0** |
| 1062 | 5 | 0.6 | no result | 1.4 |
| 1062 | 4 | no result | no result | 1.2 |
| 1062 | 5 | 0.6 | 1.1 | 1.0 |
| 1062 | 4 | 0.8 | 1.0 | 1.2 |
| **mean** | | **0.67** | **1.05** | **1.2** |

Table 7 shows that the best crosses from this programme so far, are significantly better than the commercially available line 1001.

**Summary**

Significant progress has been made in the characterisation of the material, this has been due in part to the good progress and repeatability made in the sample analysis. As a result the following main aims have been addressed:

- Selected improved parents
- Developed and tested better crosses, which are now being produced under commercial conditions in 2008.
- Understand the seasonal variation in artemisinin content of *A. annua* under UK growing conditions.

## 2.3 Work Package 3, Agronomy and seed production

This work package includes work undertaken at a commercial site, East Malling Research and Frontier agriculture

### 2.3.1 Optimisation of biomass production and artemisinin yield in response to variation in nutritional supply for pot grown plants at EMR

**Aims**

Following the annual partners meeting at EMR on the 5th of June it was decided that for future work in years 2 and 3, EMR should concentrate on the optimisation of biomass production and artemisinin yield in response to variation in nutritional supply for pot grown plants (Fig 14). The consortium agreed that the EMR work should focus mainly on the influence that nitrogen fertigation has on plant biomass and artemisinin concentration and subsequently yield of artemisinin for *A. annua*. EMR also carried out a smaller nutritional experiment to determine the effects of potassium on the same growth parameters.

Six hundred individual seedlings of *A. annua* plants, each in a module of 2.5 cm x 2.5 cm and 3 cm deep, were supplied by Frontier on 21 June 2007. On the 27 June, 400 of the seedlings were potted up into 7.5 litre pots using Klassman medium Irish graded peat without added N, P and K, to which 1.9 g of $CaCO_3$/l 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 30, <0.6 and 5.3 mg/l of compost. The plants were placed into an unheated glasshouse for three weeks to allow them to establish, before placing them out onto a gravel bed, on 19th July 2007, in 15 rows (4.8 m wide) and 0.8 m apart, the pots were staggered along the row to allow 20 pots to a row. At the end of each row there was a guard plant. Two rows to the south of the plot and one row to the north of the plot acted as guard plants.

**Figure 14. A view of the *Artemisia annua* pot experiment At EMR where plants are being given different nutritional regimes via fertigation; nitrogen experiment**

**i) Nitrogen experiment; six treatments were applied**

Stock solutions were made up that provided nitrogen at concentrations of 0 mg/l, 25 mg/l, 50 mg/l, 100 mg/l, 200 mg/l, and 300 mg/l when diluted with water in a ratio of 1:100 (Fig 15). The irrigation water contained an additional 6 mg/l of nitrogen, the concentrations of N applied to the plants were therefore 6 mg/l (N1), 31 mg/l (N2), 56 mg/l (N3), 106 mg/l (N4), 206 mg/l (N5), 306 mg/l (N6). Ammonium nitrate was used to adjust the concentration of N. The stock solution provided, P at a concentration of 40 mg/l, K at 156 mg/l, Ca at 80 mg/l, Na at 33 mg/l, Zn at 0.1 mg/l, B at 0.3 mg/l, Cu at 0.1 mg/l, S at 112 mg/l, Fe at 2.8 mg/l and Cl at 3.5 mg/l, and was applied at these concentrations to all the plants within the experiment.

The experimental design was that of a randomised complete block: 6 treatments x 8 block, each plot contained 3 plants. Total number of plants was 144 (6 x 8 x 3). Each pot received the fertigation solution via one 2 l/h dripper. Fertigation started on the 19 July 2007. To avoid

infiltration of rain into the pots, the top of the pots were individually covered with a plastic sheet taped to the sides, a 3-4 $cm^2$ hole in the centre through which each plant was passed. The pots were checked on a regular basis (3-4 times a week), by lifting all the pots in a row to determine whether they were heavy or light, if heavy the irrigation was reduced and if light the irrigation was increased. Daily irrigation times were recorded throughout the experiment.

Plant heights were measured at intervals from the start to the end of the experiment. The plants were finally harvested on the 10 October 2007 to determine fresh and dry weights and to produce samples for chemical analysis (mineral and artemisinin). The protocol used for sampling the *A. annua* plants at harvest was as follows:

Leaves and stems from a portion of one side of the main stem of each plant were removed, from the base to the top of the plant. This plant material was collected from each of the 3 plants in a plot and bulked together. The fresh weight of this portion of the plant was recorded. The plant material was then placed into drying trays and placed into a drying oven at 40-42 $^0$C and dried for at least 48 hours. The leaves and stems that remained on the 3 plants in a plot were removed from the leader stem and a bulk fresh weight of this material was taken. The fresh weight of the main leader stems of the 3 plants in a plot was bulked together and the fresh weight recorded. The bulked main stems were placed into an oven at 80 $^0$C for 48 hours and a record of the dry weight taken.

The material that was placed in low temperature oven, when dry, was rubbed through a sieve of 2-3 mm mesh size, removing the leaf material from the stalk. The dry weight of stalk and leaf material was recorded. The leaf and stalks were bagged up separately into poly grip bags. Leaf material samples from each experimental plot (72 in total; 48 from the nitrogen experiment and 24 from the potassium experiment) were sent to De-Montford University for Artemisinin analysis and another set of samples was used for complete mineral analysis (Natural Resources Management Limited). The stalks from one plot of each of the 6 nitrogen treatments and 3 potassium treatments were also sent off to NRM for complete mineral analysis to give some idea of the mineral concentrations within the stalk material.

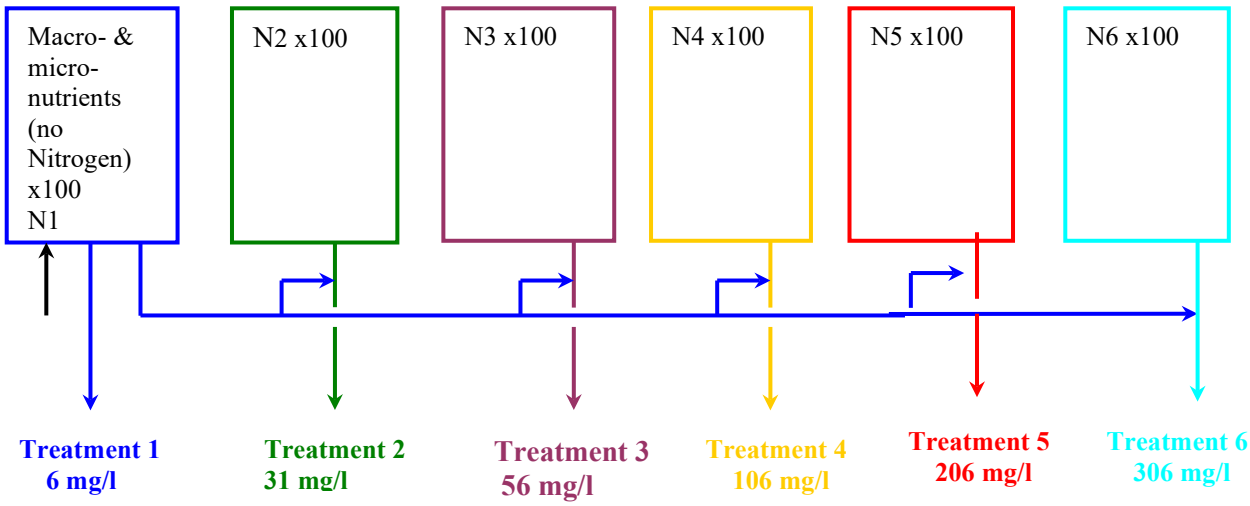
**ii) Potassium experiment; three treatments were applied**

Stock solutions were made up that provided potassium at concentrations of 51 mg/l (K1), 153 mg/l (K2), and 301 mg/l (K3), when diluted with water in a ratio of 1:100. The irrigation water contained an additional 2 mg/l of potassium; the concentrations of K applied to the plants were therefore 53 mg/l (K1), 155 mg/l (K2), and 303 mg/l (K3). Different concentrations of potassium sulphate were used to alter the concentration of K. The stock solution provided N at a concentration of 202 mg/l, P at 40 mg/l, K at 156 mg/l, Ca at 80 mg/l, Na at 33 mg/l, Zn at 0.1 mg/l, B at 0.3 mg/l, Cu at 0.1 mg/l, Fe at 2.8 mg/l and Cl at 3.5 mg/l, and was applied at these concentrations to all the plants within the experiment. Sulphur was applied at a concentration of between 69-170 mg/l, with K1 plants having the lowest concentration and K3 having the highest.

The experimental design was that of a randomised complete block: 3 treatments x 8 block, each plot contained 3 plants. Total number of plants was 72 (3 x 8 x 3). Each pot received the fertigation solution via one 2 l/h dripper. Fertigation started on the 19 July 2007. To avoid infiltration of rain into the pots, the top of the pots were individually covered with a plastic sheet taped to the sides, a 3-4$cm^2$ hole in the centre through which each plant was passed. The pots were checked on a regular basis (3-4 times a week), by lifting all the pots in a row to determine whether they were heavy or light, if heavy the irrigation was reduced and if light the irrigation was increased. Daily irrigation times were recorded.

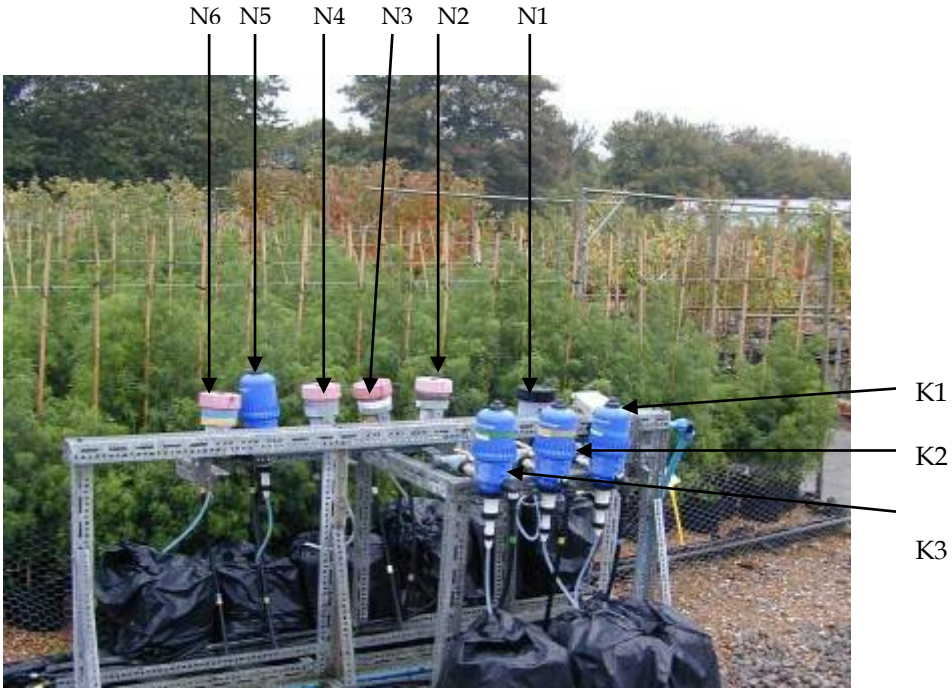
Plant heights were measured at intervals from the start to the end of the experiment. The plants were harvested on 18 /10/ 2007 to determine fresh and dry weights and to produce samples for mineral and artemisinin analysis. The protocol for sampling is the same as for the nitrogen experiment (see above).

**Figure 15. Diagram showing how the stock solution of macro- and micro-nutrients was mixed with incoming mains water**




*Note: This provided the fertigation for treatment 1 (no nitrogen). In addition, increasing concentrations of ammonium nitrate solutions were diluted 100 x into the macro- and micro-nutrient fertigation solution to provide the fertigation solutions for treatments 2, 3, 4, 5 or 6*

**Figure 16. Structure of the dosatron arrangement supplying different concentrations of nitrogen to the plants in the N-experiment**




(N1=18 mg/l, N2=43 mg/l, N3=68 mg/l, N4=118 mg/l, N5=218 mg/l, N6=318 mg/l) and different concentrations of potassium to the plants in the K-experiment (K1=53 mg/l, K2=155 mg/l, K3=303 mg/l)

### 2.3.2 Results of Nitrogen Fertigation Experiments at East Malling Research

Differences in growth of *A. annua* in the 6 treatments became apparent 4 weeks after the start of the application of the treatments (Figure 17). The differences between the plants in the different treatments becoming more apparent as the experiment continued and by the 10th October the tallest plants were to be found in N6 and the smallest in N1. This difference in growth translates to differences in biomass between the plants in terms of total biomass, leaf biomass and stem biomass at harvest (Figure 18). Increasing N concentration in the irrigation from 6 mg/l (N1) to 106 mg/l (N4) increases total biomass 8 fold (from 13.4 g to 119.2 g), leaf biomass (from 5.6 g to 44.7 g) and stem biomass (from 7.8 g to 74.5 g). Increasing N concentration from 106 mg/l (N4) to 306 mg/l (N6) does not significantly increase total or stem biomass any further, however leaf dry weight is increased further when raising levels of N from 203 mg/l to 303 mg/l.

Pictures taken at the time of harvest clearly show the effect that low nitrogen treatments have on plant size of *A. annua* (Fig 19), plants supplied with 6 and 56 mg/l of nitrogen do not have the bushiness of the plants supplied with concentrations above 106 mg/l.

**Figure 17. Stem growth of *Artemisia annua* when supplied with differing concentrations of nitrogen (N1=6 mg/l, N2=31 mg/l, N3=56 mg/l, N4=106 mg/l, N5=206 mg/l, N6=306 mg/l)**

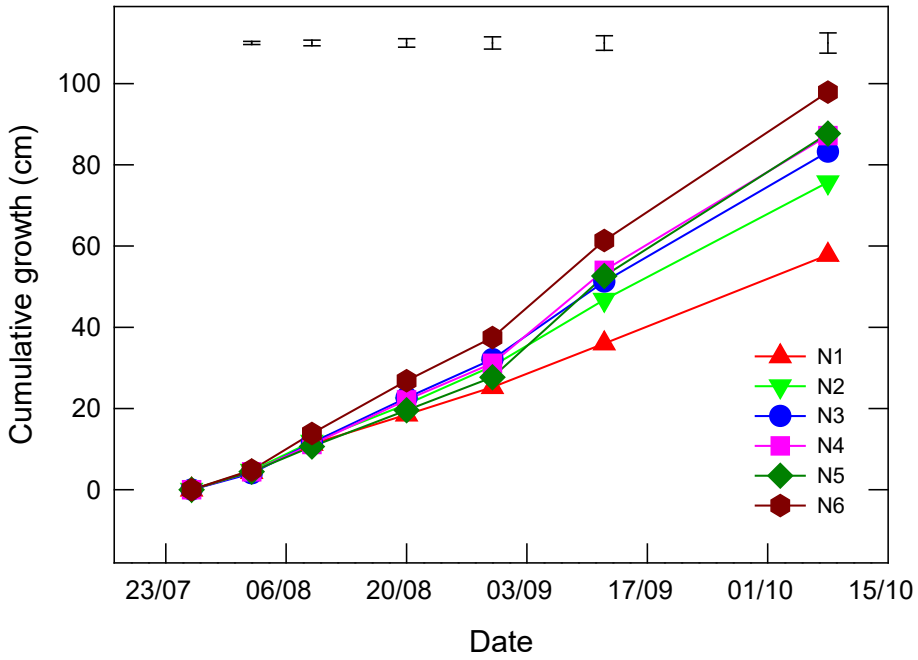


**Figure 18. Total plant dry weight, leaf dry weight and stem dry weight of *Artemisia annua* plants when supplied with differing levels of Nitrogen (N1=6 mg/l, N2=31 mg/l, N3=56 mg/l, N4=106 mg/l, N5=206 mg/l, N6=306 mg/l)**

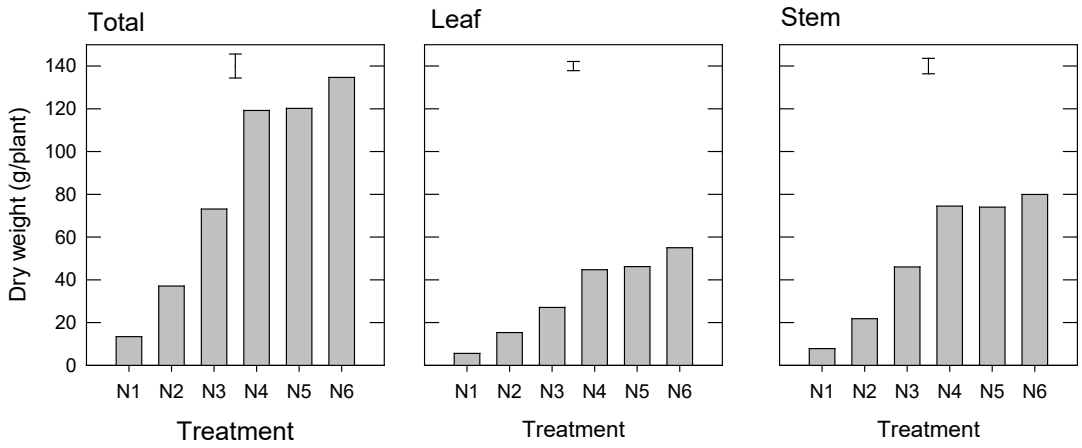
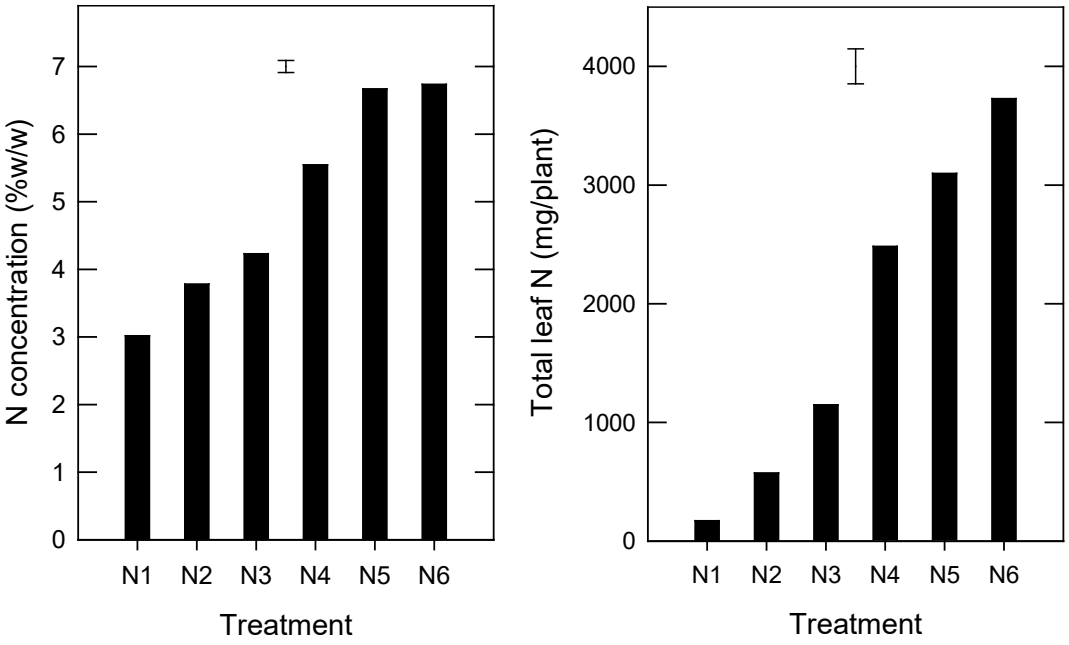



**Figure 19. Plants showing the effects of application of different concentrations of nitrogen on growth of *Artemisia annua* (N1=6 mg/l, N2=31 mg/l, N3=56 mg/l, N4=106 mg/l, N5=206 mg/l, N6=306 mg/l)**

**Figure 20. Nitrogen concentration in leaves and total leaf nitrogen content (nitrogen concentration x leaf dry weight) at harvest for *Artemisia annua* plants when supplied with differing concentrations of nitrogen** (N1=6 mg/l, N2=31 mg/l, N3=56 mg/l, N4=106 mg/l, N5=206 mg/l, N6=306 mg/l)

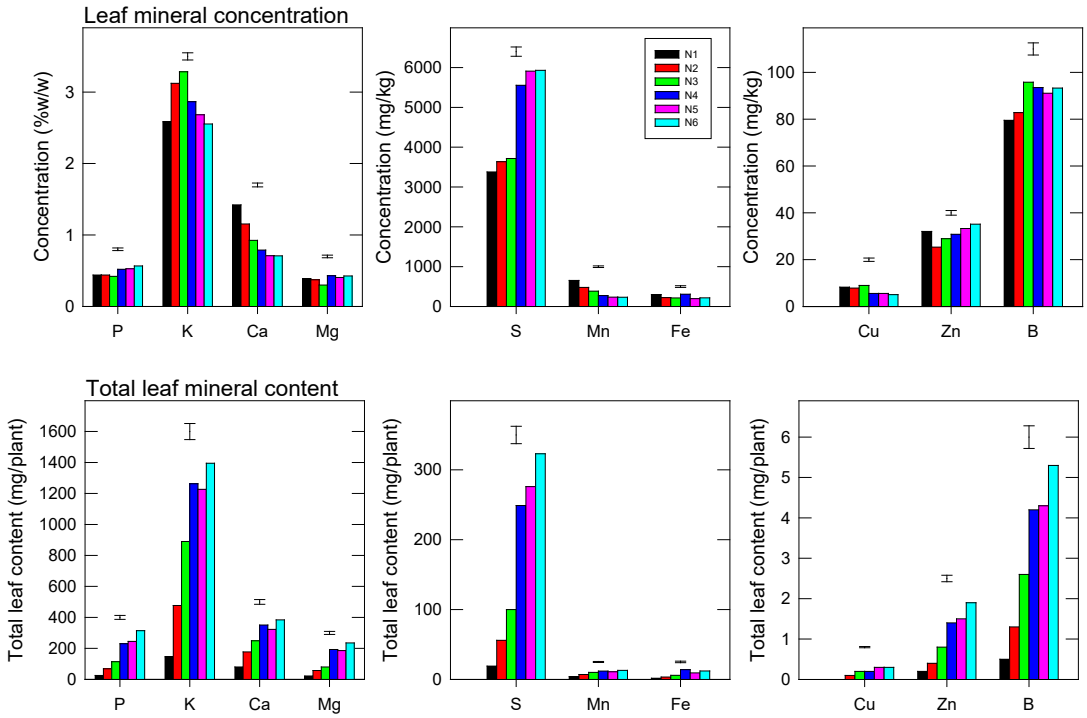



Increasing the nitrogen supplied to *A. annua* plants from a concentration of 6 mg/l (N1) to 206 mg/l (N5) increases the N concentration of the leaves from 3% w/w to 6.7% w/w, increasing N application from 206 mg/l (N5) to 306 mg/l (N6) did not alter leaf mineral concentration any further (Fig 20). The total amount of nitrogen (nitrogen concentration x leaf dry weight) found in the plant leaf biomass of *A. annua* increased with each successive increase in nitrogen application from a low of 171 mg/plant found in plants supplied with nitrogen at a concentration of 6 mg/l (N1) to a high of 3727 mg/plant for those supplied with nitrogen at 306 mg/l (N6). Estimating the total amount of nitrogen in the stem of plants for each of the treatments gives values of 61, 357, 592, 1751, 1213 and 2246 mg/plant for N1, N2, N3, N4, N5, and N6 respectively.

These values are only there as a guide as stalk mineral analysis were only done on one set of samples per treatment and therefore variability has not been accounted for. The total amount of nitrogen (amount of N in stock solution + irrigation water) supplied to each plant in each of the treatments was 0.17, 1.05, 2.1, 3.9, 7.6, 10.7 g for N1, N2, N3, N4, N5 and N6 respectively.

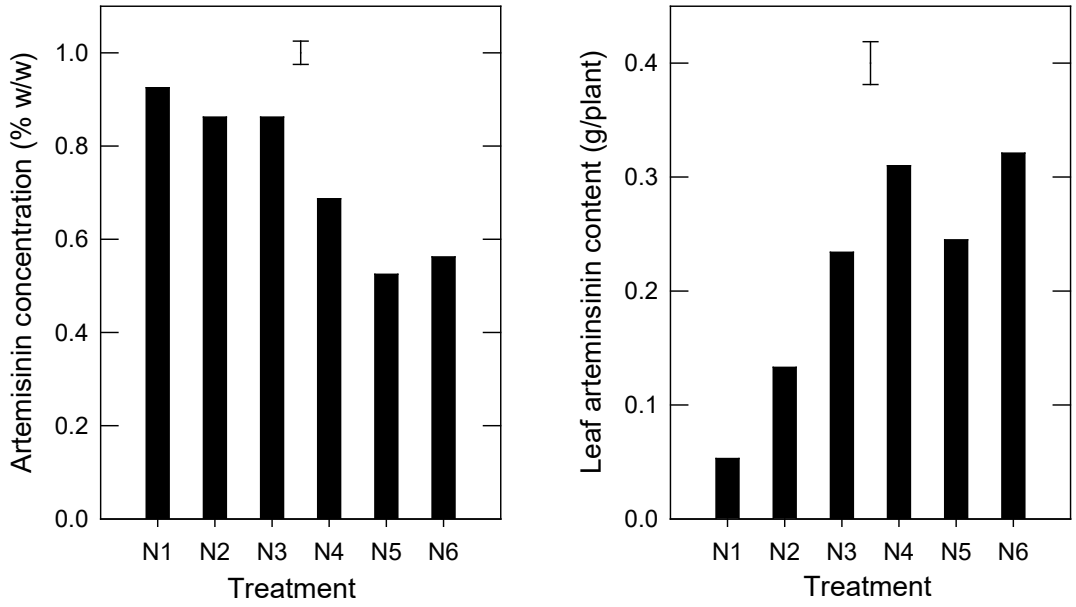
**Figure 21. Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn) and boron (B) concentration in leaves and total leaf content (mineral concentration x leaf dry weight) at harvest for *Artemisia annua* plants when supplied with differing levels of nitrogen (N1=6 mg/l, N2=31 mg/l, N3=56 mg/l, N4=106 mg/l, N5=206 mg/l, N6=306 mg/l)**




Leaf mineral concentrations of calcium (Ca), manganese (Mn) and copper (Cu) decreased with increasing nitrogen application concentration (Fig 21). Apart from in the N1 treatment, which has the lowest leaf concentration of potassium (K), the leaf mineral concentration of K also decreases with increasing nitrogen application. Phosphorus (P), sulphur (S) and boron (B) are found in higher concentrations in leaves of those plants that have received the higher levels of nitrogen. However, when the mineral content of the leaves of the plant is expressed in terms of total amount of mineral in the leaf material per plant, it increased for all minerals as nitrogen concentrations increased from 6 mg/l to 106 mg/l, with further increases from 106 mg/l to 306 mg/l being less marked. This reflects the pattern in the amount of biomass produced by the plants in each treatment.

**Figure 22. Artemisinin concentration in leaves and total artemisinin content of leaves (mineral concentration x leaf dry weight) at harvest for *Artemisia annua* plants when supplied with differing concentrations of nitrogen (N1=6 mg/l, N2=31 mg/l, N3=56 mg/l, N4=106 mg/l, N5=206 mg/l, N6=306 mg/l)**




The concentration of artemisinin in leaves of *A. annua* was at its highest in plants receiving between 6-56 mg/l of nitrogen (N1-N3), where concentrations of artemisinin were in the range of 0.86-0.93% w/w (Figure 22). The concentration of nitrogen increased from 56-106 mg/l, while the concentration of artemisinin decreases to 0.687% 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 x leaf biomass) per plant increased as the concentration of nitrogen supplied to the plants increased from 6 mg/l to 106 mg/l (Figure 22). Increasing nitrogen concentrations from 106 mg/l to 306 mg/l did not increase artemisinin yields any further.

### 2.3.3 Results of Potassium Fertigation Experiments at East Malling Research

There were no differences in cumulative growth of *Artemisia annua* in terms of height between the 3 treatments (Figure 23) over the time period of the application of the treatments. However increasing potassium from 53 mg/l to 155 mg/l significantly increases total biomass of a plant (Fig 24); this difference is mainly due to an increase in stem weight, there being no significant difference between the dry weights of leaves. Increasing potassium concentration from 155 mg/l to 303 mg/l does not increase biomass of the plant further.

Pictures taken at the time of harvest clearly show that plants receiving 53 mg/l were of similar height, but are not multi-stemmed or as bushy as plants receiving more than 155 mg/l of potassium (Figure 25).

**Figure 23. Stem growth of *Artemisia annua* when supplied with differing concentrations of potassium (K1=53 mg/l, K2=155 mg/l, K3=303 mg/l)**

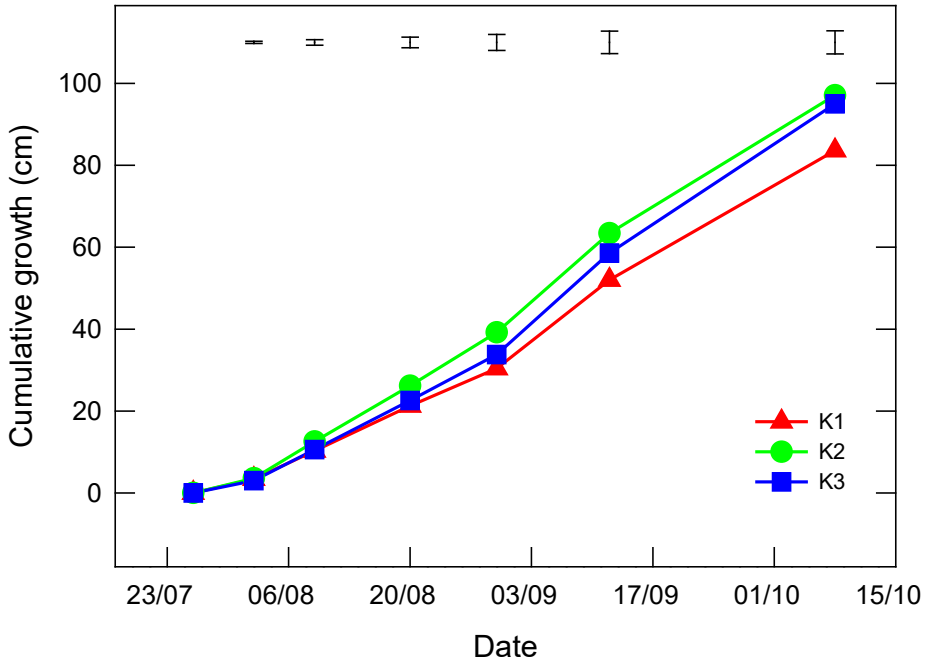
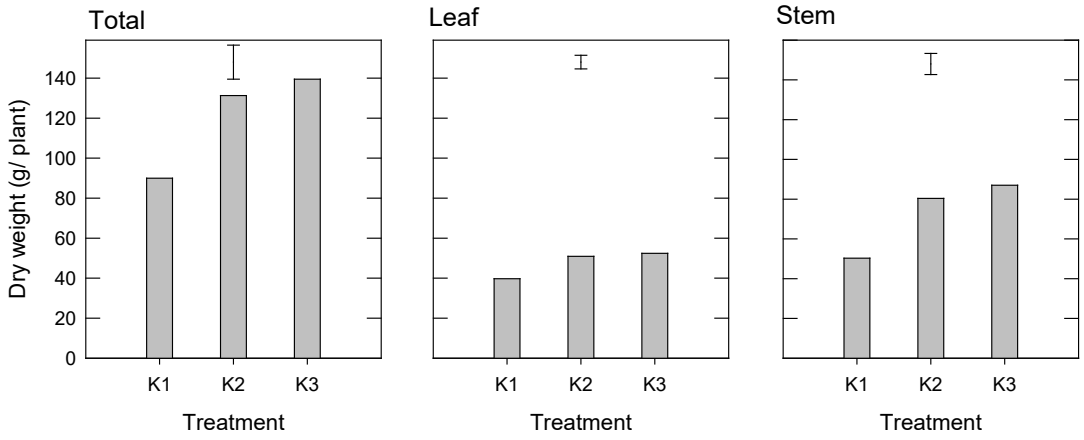



**Figure 24. Total plant dry weight, leaf dry weight and stem dry weight of *Artemisia annua* plants when supplied with differing concentrations of potassium (K1=53 mg/l, K2=155 mg/l, K3=303 mg/l)**




Increasing the potassium supplied to *A. annua* plants from a concentration of 53 mg/l (K1) to 303 mg/l (K3) increased the K concentration of the leaves from 1.8 % w/w to 3.4 % w/w (Figure 26). The total amount of potassium (potassium concentration x leaf dry weight) found in the plant leaf biomass of *A. annua* increases with each successive increase in potassium application from a low of 706 mg/plant found in plants supplied with potassium at a concentration of 53 mg/l (K1) to a high of 1524 mg/plant for those supplied with potassium at 303 mg/l (K3). Estimating the total amount of potassium in the stem of plants for each of the treatments gives values of 1540, 2590, 3011 mg/plant for K1, K2 and K3 respectively.

These values are only there as a guide, because stalk mineral analysis were only done on one set of samples per treatment and therefore variability has not been accounted for. The total amount of potassium (amount of K in stock solution + irrigation water) supplied to each plant in each of the treatments was 2.2, 6.9 and 12.4g for K1, K2, K3 respectively.

**Figure 25. Pictures showing the effects of application of different concentrations of potassium on growth of *Artemisia annua* plants (K1=53 mg/l, K2=155 mg/l, K3=303 mg/l)**


Leaf mineral concentrations of nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), manganese (Mn) and boron (B) decreases with increasing potassium application (Figure 27). There were no differences in leaf mineral concentrations of iron (Fe), copper (Cu) or zinc (Zn) (Figure 27). The total amount of each mineral taken up into the leaves of the plant (mineral concentration x leaf dry weight) does not differ between the plants receiving different amounts of potassium for any of these minerals.

The concentration of artemisinin in leaves of *A. annua* plants does not differ between those receiving different concentrations of potassium (Figure 28). The total yield of artemisinin (artemisinin concentration x leaf biomass) is lowest in those plants receiving 53 mg/l of potassium (K1), with those receiving 155 mg/l and 303 mg/l of potassium having a higher yield; however this difference is not statistically significant.

**Figure 26. Potassium concentration in leaves and total leaf potassium content (potassium concentration x leaf dry weight) at harvest for *Artemisia annua* plants supplied with differing levels of potassium (K1=53 mg/l, K2=155 mg/l, K3=303 mg/l)**

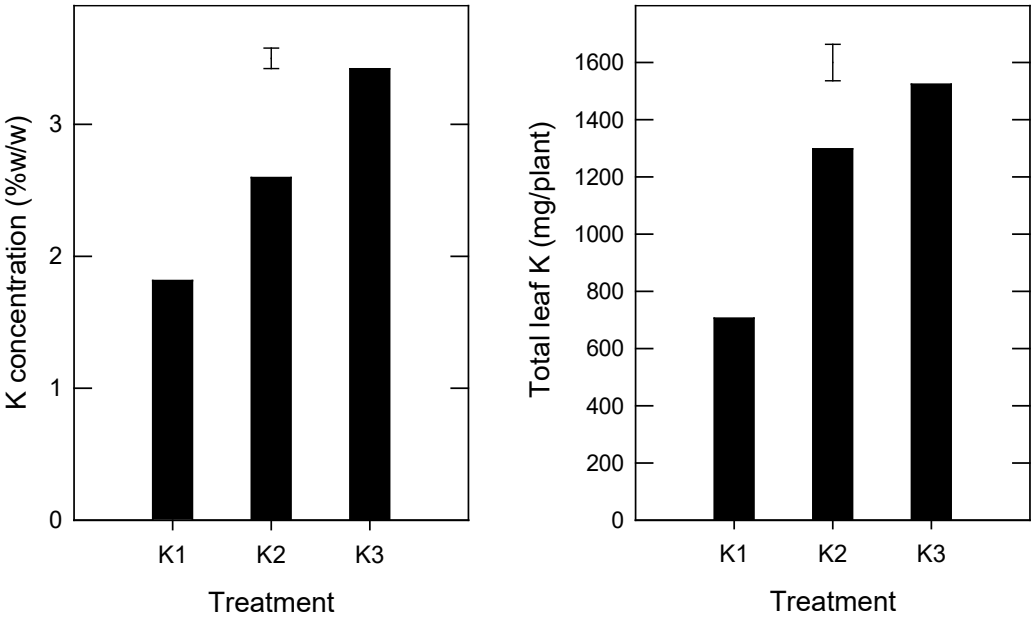



**Figure 27. Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn) and boron (B) concentration in leaves and total leaf content (mineral concentration x leaf dry weight) at harvest for *Artemisia annua* plants when supplied with differing levels of potassium (K1=53 mg/l, K2=155 mg/l, K3=303 mg/l)**

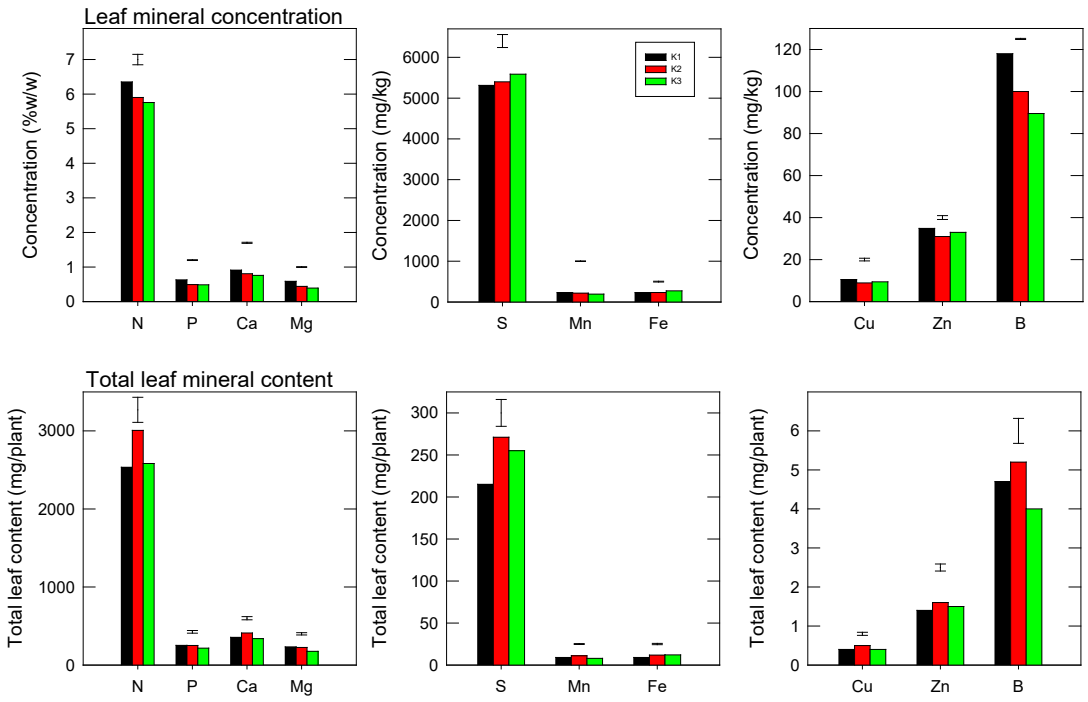
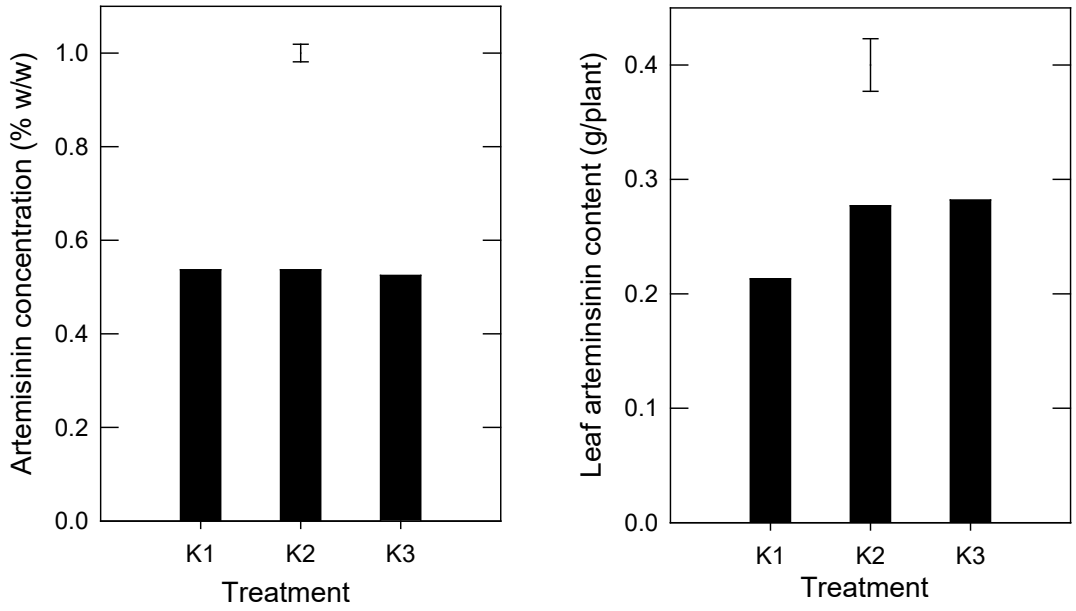


**Figure 28. Artemisinin concentration in leaves and total artemisinin content of leaves (mineral concentration x leaf dry weight) at harvest for *Artemisia annua* plants when supplied with differing levels of potassium (K1=53 mg/l, K2=155 mg/l, K3=303 mg/l)**




### 2.3.4 Main Conclusions from the Nitrogen and Potassium Fertigation Experiments

The main conclusions for nitrogen fertigation experiments were as follows:

- Application of nitrogen at approximately 100 mg/l (equating to 3.9 g N/plant) was optimal for *Artemisia annua* growth. Levels above this do not significantly increase growth
- Artemisinin concentration was highest at low applications of nitrogen, with its concentration decreasing as nitrogen concentration increases
- The total yield of artemisinin on a per plant basis, increased with increasing nitrogen application up to 106 mg/l. Further increase in yield of artemisinin was not seen above 106 mg/l
- Nitrogen concentration in the leaves and total leaf nitrogen of *Artemisia* plants increased with each successive increase in rate of nitrogen application
- Increasing nitrogen concentration application above 106 mg/l did not increase artemisinin yield, plants receiving this concentration of nitrogen have a leaf nitrogen concentration of 5.5% w/w at harvest, this therefore appears adequate for maximising artemisinin yields per plant

The main conclusions for potassium fertigation experiments were as follows:

- Potassium concentration in the leaves and total leaf potassium of *Artemisia* plants increased with each successive increases in potassium application
- Application of potassium at approximately 155 mg/l (equating to 6.9 g k /plant) was optimal for *Artemisia annua* growth. Concentration above this did not increase biomass
- Artemisinin concentration did not change when altering the concentration of potassium supplied
- The total yield of artemisinin, on a per plant basis, was slightly higher when potassium application was raised from 53 mg/l to 155 mg/l, although this increase was not statistically significant
- A concentration of potassium in leaves of 2.6% w/w at harvest was adequate for maximising artemisinin yield per plant

### 2.3.5 Seed production

Relatively small scale commercial winter seed production was undertaken for several promising lines during the period 2007 to 2008 under controlled glasshouse conditions.

**i) Lines "1053" and "1054" in 2007/08**

The winter programme/trial was designed to answer a number of questions including:

- Can improved synchrony of flowering increase yield especially in the 1001 parent?
- Can increased winter light improve winter yield?
- Is the seed obtained from the 1015 parent the result of selfing?
- Can adequate quantities be produced to support Frontiers full efforts & for sale?

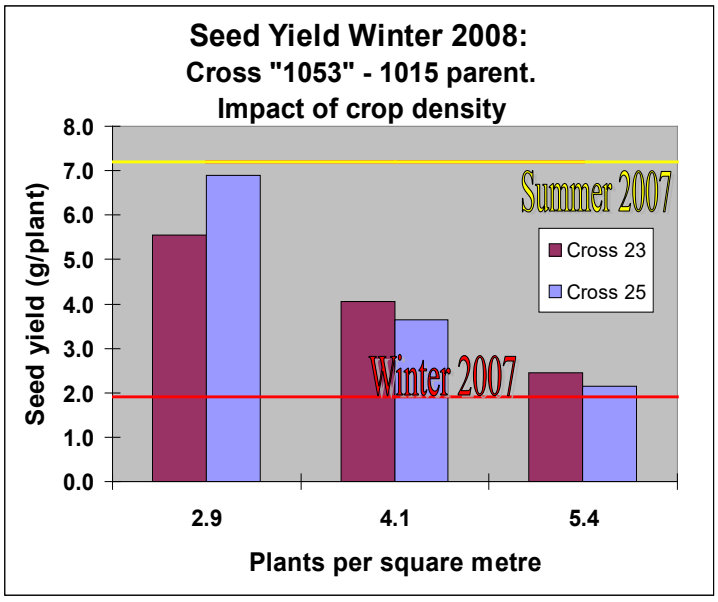
Two tranches of Mother Plants were set up in weeks 32 and 33 of 2007, with production plant cuttings taken over weeks 39 to 41. Plants were entered into Short Days (SDs) over the period week 44 to 47 with the 1015 parents entered a week later than the equivalent aged 1001 plants. Nevertheless the 1015 still flowered a further week earlier than 1001 with the result that only two tranches of crosses were set up in weeks 49 and 50.

The opportunity was taken to devise a replicated crop density trial whereby each of three density treatments for the "1053" cross occupied 6 benches (60m2), and for the "1054" cross 2 benches. Both crosses were duplicated with the second tranche of plants. The treatment blocks were sufficiently large to ensure minimum impact of side effects and guarding was considered unnecessary. In total >2000 plants were included in the trial.

Pollination was encouraged by knocking the plants three times a week and seed harvested in weeks 11 and 12 of 2008. Parent plants were separated into their clonal lines prior to collection of the seed. Seed lost in this process was considered negligible. A detailed winnowing process was followed to ensure that seed yields recorded at the end were comparable with contaminants anticipated to comprise >5% of actual seed weight.

### 2.3.6 Results of winter seed production in lines 1053 and 1054

**Figure 29 Impact of crop density on seed production in lines 1053 and 1054**




i) *Impact of crop density*. The seed yield of the 1015 parent increased from circa 2g/plant at 5.4 plants/m2 to circa 6g/plant at the lowest density of approximately 3 plants/m2. In this way the high density plants resembled the winter crop of 06/07, while the low density resembled summer conditions. This result was replicated over both batches of the "1053" cross. Exactly the same trend was seen on the "1054" cross. This responsiveness to lower density meant that yield per unit area was actually promoted by having fewer plants. See Figure 29.

ii) *Yields obtained from each parent.* The 1015 parent, at the lowest density, yielded 5.6g and 7.7g/plant for the "1053" and "1054" lines respectively; however, the comparable 1001 parent (either -01 or -03) yielded only 0.5 and 0.4g/plant. The seed yield was too low for

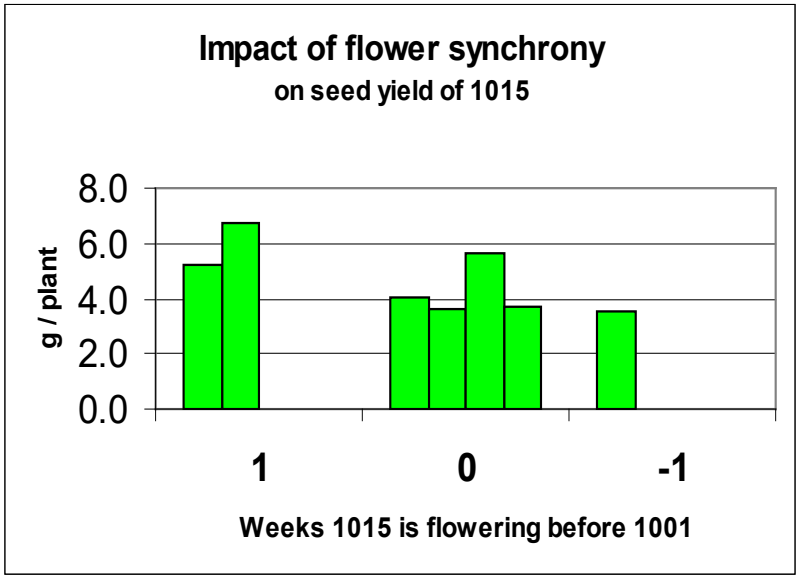
 

satisfactory winnowing and wholesale seed harvest from this parent was abandoned. The failure of the 1001 parent confirms similar observations on the previous productions, and hastens suspicions that these parents are not cross-fertilising.

iii) *Potential selfing of line 1015 due to synchrony of flowering*
Small, single bench trials were set up whereby the synchrony of flowering was altered with the 1015 either advanced or retarded in its pollen drop in relation to the 1001. However, altering the timing of pollen drop had little impact on seed set.

In order to test the selfing hypothesis further, plants were placed in increasing isolation; first by enclosing within a bench of solely 1015 plants, secondly by enclosing within fleece and thirdly by enclosing branches within paper bags. In the latter case one plant ("-intra") or two plants ("inter"-) were included in the bags where the second plant might be also 1015 or alternatively 1001. While enclosing within a fleece might seem to slight decrease yield per plant and also % content of seed in the plant matter recovered prior to winnowing (% of sieved weight), all bagged samples had similar % content of seed to the control. Significantly also, inclusion of the 1001 parent had absolutely no positive impact on either g/branch or % seed content See figure 30.

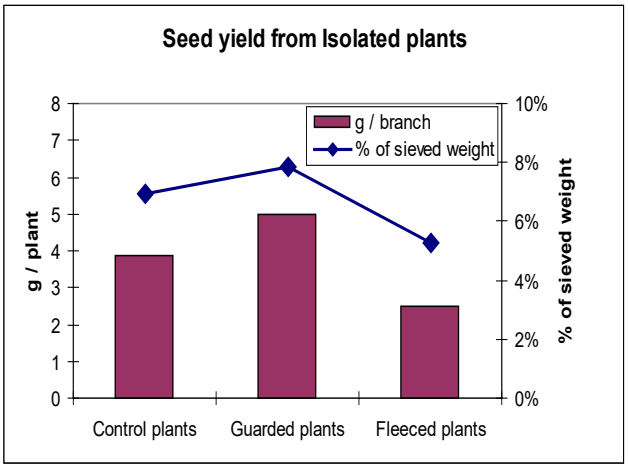
**Figure 30, The impact of Flower synchrony on seed yield for *A annua* plants grown under green house conditions**

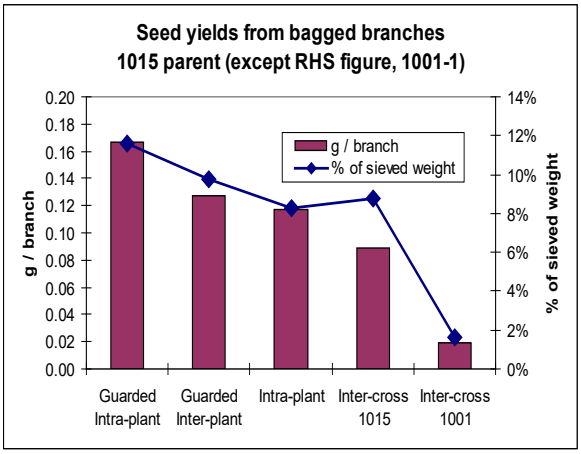



This is compelling evidence that cross-pollination is not occurring. Finally, when the numbers of seed recovered per plant are compared with the high yielding "1062" cross, it becomes apparent that the 1015 achieves at maximum 10% of seed set, while the 1001 achieves only 0.7% of potential, which is completely in line with published levels of selfing in this particular material.

**Figure 31 (a) and (b) The impact of isolation on *A. annua* plants on seed yield and potential for 'selfing' under greenhouse conditions**




iv) *Recovered yields and quality*

There is strong evidence here to suggest that the 1015 is 'selfing', and if this is true, then it may be assumed that all material recovered from these plants is comparable despite their designated cross code. Thus in total 4.34 Kg of seed was recovered. Results from Frontier have indicated 96% germination of these seeds after three days of pre-chilling and that there were approximately 12,000 seeds per gram. The yield thus represents 52 million seeds.

**ii) Winter seed production programme of line '1062', 2007/08**

This new cross of line 1062, highly promising for its artemisinin content, is composed of two new parental lines coded at HVHB as 17 and 18. Only 12 plants were received from NIAB in time to enter the flower initiation sequence, with 4 of each parent entered into SDs over 3 weeks. Fortuitously, pollen drop was simultaneous between the parents, so all plants could be used. Plants were held in a separate zone to the "1053" & "1054" crosses, but this also meant that they were at a lower temperature (20°C) and were exposed to different light levels – only 10klux, but with no guard plants and at no more than 2.4 plants/m2. Maturation was concluded at the higher temperature and light conditions along with the other *A. annua* plants.

### 2.3.7 Results of seed production from line 1062

i) *Yields from each parent*

Yields from each of the three entry dates into Short Days were identical. Parent 17 yielded 36.6g/plant while parent 18 yielded 40.5g/plant. This 6 fold increase in yield per plant over the 1015 parent in the "1053" & "1054" crosses, which translates into an effective 12 times greater yield per unit area, is very encouraging against a background of 15g/plant for the highest previously recorded seed recovery. Nonetheless, given the differing conditions, particularly light received by each plant in this cross, it might be prudent to budget a more modest 20g/plant under scale-up conditions see Figure 8 below.

**Table 8. Details of seed yield from the different *A. annua* line crosses**

| Cross | Batch | Yield (Kg) |
|---|---|---|
| 1053 - 1015 | 23 | 1.42 |
| 1053 - 1015 | 25 | 1.44 |
| 1054 - 1015 | 24 | 0.68 |
| 1054 - 1015 | 26 | 0.53 |
| 1015 various | 27,28,29 | 0.27 |
| **Total** | | **4.34** |

**ii) *Seed Quality***

The seeds, were assessed by Frontier and were found to be considerably smaller (20,000 seeds/g); this smaller seed may be at a disadvantage for pelleting and seedling establishment in the field. Germination was also slightly poorer (83% after 3 days in pre-chilling) but of course might have improved with a longer chilling period. In this trial, the plants were physiologically younger (having been cooler) but treated similarly to the first cross of "1053" for seed maturation and allowed to dry out relatively early. The plants were also harvested a week earlier, along with the selfing trial samples, to prevent any chance of confusion with the other material. It is possible that a longer, more appropriate maturation period may improve seed size.

***iii) Recovered Yields***

From the initial 24 plants, only 21 survived to harvest and in total 0.81 Kg was recovered. Given that this cross has not been tested adequately in the field, particularly in regard to seedling establishment, it would be premature to present this material on an equal footing to "1053" as a commercial line.

**Summary**

A large-scale research production was arranged in the 20klux Veg Zone (900m2) of the R&D Unit. The key findings of this work included:

- Varying the plant density has a direct impact on seed yield; the lowest density having the highest yields both by each plant and by unit area
- At 3 plants/m2, the achieved yield was 6g/plant for the 1015 parent of the "1053" and "1054" crosses, close to the maximum yield achieved in the summer of 2007
- Only the 1015, but not the 1001 parents produced appreciable seed; it is calculated that seed set was around 10% for 1015, but <1% for 1001-01 or 1001-03 parents
- The data suggests that seed on the 1015 is the result of selfing. Altering flowering synchrony, enclosing whole plants in fleece, enclosing branches in paper bags, whether with or without the 1001 parent, all failed to affect seed yield.
- 4.3 Kg of this 1015 seed has been harvested and been found to be 12,000 seeds/g and have 96% germination after 3 days of pre-chilling
- A new, promising, but inadequately researched line "1062" yielded nearly 40g/plant from both parents. 0.8Kg has been harvested and seed found to be smaller, at 20,000 seeds/g, with a lower germination rate of 83% after 3 days of pre-chilling

## 2.4 Work Package 4. Harvesting, product stabilisation and secondary products; led by Frontier Agriculture

### Introduction

In 2006, the first commercial scale field cropping, mechanised in an 'Arable Style' was undertaken by Frontier. Three growers took part and they were situated in Lincolnshire, Norfolk and Surrey. These gave some encouraging results, but were planted using only imported Brazilian seed. There were four main problems noted after the end of this harvest that needed attention:

- Poor quality seed in terms of size and germination rate
- Poor establishment – very low plant populations.
- Weed control and problems associated with herbicide use
- Harvesting technique.

### 2.4.1 Field planting of *A. annua* in 2007 by Frontier

In the summer of 2007 *A. annua* was grown on four farms, repeating the three from 2006 and adding a new farm situated in Kent. The summer of 2007 saw very heavy rainfall, delaying planting and even heavier rain within 48 hours after planting, contributing to soil capping and soil wash. Certain herbicides that had showed promise in 2006, were harsh on the *A. annua* in 2007, leading to some crop kill.

The seed was drilled on a field-scale for the first time UK using seed from a commercial production source of crosses made at NIAB. This was sown direct as pelleted seed and also as transplants, which were grown in plugs by a commercial plant raiser along the style of a field brassica crop.

Based on experience from the 2006 plantings, adjustments were made to drilling, seed bed preparation and herbicide choice and this resulted in better plant populations and uniformity with both Brazilian seed and UK seed at some sites. The growth of weeds was causing problems and control through the use of herbicides was insufficient to adequately control their growth. There was a small scale screening test at one site to look at several herbicide active ingredients. This will be repeated in 2008, but at a larger scale and with better replication.

### 2.4.2 Results of cropping at different sites

The Kent crop had severe soil capping with very poor emergence and was abandoned soon after planting. The Lincolnshire crop also had poor emergence, particularly from heavy rains, bad weed control and was monitored but abandoned without harvesting. Three of the five Norfolk fields were abandoned, two soon after planting due to very severe soil wash and flooding and one before harvest due to poor crop population and weeds. One out of the four fields at the Kent location had severe crop kill from herbicide.

Two fields were finally harvested in Norfolk, totalling about 10 ha and three fields in Kent totalling about 12.5 ha. Therefore a total of 22.5 ha were machine harvested and dried successfully. The UK pelleted direct sown seed produced a fair crop that was clearly better than Brazilian seed in neighbouring fields. It was, in comparison with Brazilian seed, showing more uniform plants, better germination, a similar percentage artemisinin content but higher fresh yield.

Some improvements were made to the machine harvester, but it is still not as efficient as it should be. 'pre-harvest' treatment before harvesting was used on all crops and worked well. Drying of the crop was achieved through the use of two types of hot air dryers (according to what was available locally) and both of these worked well. Summary results are shown below in Table 9.

**Table 9. Artemisinin percentage in *A. annua* plants from a Commercially grown Farm Crop according to pre-harvest treatment and seed source**

| CROP REF. | 20 SEPT | 11 OCT Pre-harvest treatment 1 week | 19 OCT Pre-harvest treatment 2 weeks | HARVEST Cut sample ex Trailer |
|---|---|---|---|---|
| Surrey UK seed | 0.5 | 0.6 | 0.8 | |
| Surrey Brazil seed | 0.7 | 0.6 | 0.9 | 0.7 |
| Surrey Transplants | 0.6 | 0.5 | 0.8 | |
| Norfolk Brazil West | 0.7 | 0.5 | 0.7 | 0.6 |
| Norfolk Brazil East | 0.7 | 0.6 | 0.6 | 0.5 |

**Table 10. Harvested Yields of *A. annua* biomass according to site and seed source**

| Crop Ref. | Wet weight, Tonne /ha | Dried and bagged |
|---|---|---|
| Surrey Field 1 Brazil | 1.65 | Total for all 3 fields = 7.602 tonnes. This is equivalent to 0.61 tonne /ha |
| Surrey Field 2 Brazil | 1.77 | |
| Surrey Field 3 UK | 1.92 | |
| Norfolk 2 fields Brazil | 1.25 | 3.34 ton total = 0.34 tonne /ha |

There was a marked difference between the yield from the UK project lines and those obtained from commercial sources in Brazil. The results in Table 10 show that if only the site in Surrey is considered, Brazilian lines averaged over two fields gave a wet weight of 1.70 tonne /ha. In contrast, the UK seed yielded 1.92 tonne /ha. See also figures 32, 33 and 34.

**Conclusions from Work Package 4; 2007 season with 'Field scale' crops**

- UK seed produced a better crop than commercially available Brazilian seed
- UK seed pelleted for use in 2008, prepared for shallow drilling etc showed promise
- Herbicide techniques and choice still needs much work
- Field grown yields are only at a fraction of NIAB plot yields
- Field artemisinin percentage is also lower than NIAB plot levels
- Use of pre harvest treatments are useful
- Harvest techniques need to be improved (see also Figure 32)

**Figure 32 Norfolk crop of *A. annua* harvested into a trailer in 2007**

**Figure 33 A weedy crop of *A. annua* in 2008 at a site in Norfolk**

**Figure 34 A good quality crop at a Surrey using UK seed, 2008**

## 3 Summary of Results and Discussion

The detailed results are given sequentially for each individual Work Package, which was necessary in order to follow specific descriptions of many different methodological approaches, through to the results associated with them. This section will therefore only cover overarching results which impact across several work packages.

### 3.1 Artemisinin analysis and plant breeding approaches

The development of the HPLC-MS (ESI) analytical system will facilitate progress in the plant breeding work with a greater degree of confidence. Considerable progress has already been made in this area, including the choice and development of both parents and crosses. The initial focus for development of robust systems was to drive the plant genetic improvement, however, future work in field cultivation will require fast efficient and cost effective analytical systems to ensure confidence when commercial crops are ready for harvest and subsequent processing.

### 3.2 Seed Production and Parentage

There is uncertainty about the parentage of certain crosses, which as been highlighted during the seed production process. In particular, the amount of selfing that is possible and/ or desirable is unclear. Other workers have clearly stated that inbreeding suppression in this species will result in very low levels of seed set. Furthermore, seeds produced from selfing will often produce plants that are lacking in vigour, both at the establishment stage and subsequently. With this in mind, several aspects of the work in 2008 will help to clarify the extent of crossing and or selfing within the seed production lines. Screening of the 1053 and 1062 populations in 2007 produced a significant range of results for the 120 plants tested and it was from these crosses that the new parent lines containing 1.7% artemisinin have been selected.

### 3.3 Commercial cropping of *A. annua* in the UK

Commercial crop development was problematic in 2007, but the weather conditions were very adverse and quite unusual. One major development since the previous reporting period was the use of the UK produced seed from UK lines. This produced significantly better plant stands through better germination and establishment. In 2008 further improvements in the seed will be combined with higher seed rates and improved planting techniques to achieve plant stands that will be closer to that needed for an economically viable crop. Significant effort will be expended on the development of weed control protocols in 2008. This will be less critical if the germplasm facilitates early more vigorous establishment, since a well established crop produces significant weed competition.

## 4 Conclusions

- Significant development of the artemisinin analytical techniques has been possible and will make the effective selection in 2008 of improved material for UK production more accurate
- Further replicated trials in 2008 of both new and selected crosses and the parent material will give a greater confidence in the results
- New agronomically acceptable plant material with high artemisinin content was identified in 2007 and will be used to produce seed in 2008 for UK testing in 2009
- High quality UK seed production is feasible and with experience, the efficiency of production, will be improved further. This will allow the economic use of higher seed rates, combined with the increased vigour of UK produced seed for better field establishment.
- Acceptable methods for the field establishment of commercial crops has as yet to be proven

## 5 Technology transfer

There remains at least one vital step that needs to be in place before technology transfer can be envisaged, namely commercial scale field cultivation. Other facets of the work are also progressing well, but are not yet fully complete or operational. Considerable effort however, is being made, particularly Frontier Agriculture to tackle this aspect of the business. There is also invaluable input throughout from farmers and vegetable growers.

## 6 Glossary

| | |
|---|---|
| Chromophore: | light-absorbing moiety |
| Gradient: | Of chromatography: when a solvent or solvent mixture is gradually changed over time to elute a number of compounds from a column (e.g. starting with 100% water at Time 0, and finishing with 100% methanol at Time 1). |
| HPLC-DAD: | high-performance liquid chromatography-diode array detection |
| HPLC-MS (APCI): | high-performance liquid chromatography-mass spectrometry (atmospheric pressure chemical ionization) |
| HPLC-MS (ESI): | high-performance liquid chromatography-mass spectrometry (electrospray ionization) |
| IAA: | Indoleacetic acid |
| Isocratic: | Of chromatography: when a single solvent (e.g. 100%) methanol) or solvent mixture (e.g. 60% methanol, 40% water) is used to elute a number of compounds from a column |
| Maceration: | The extraction of herbal material by steeping in solvent |
| *N*: | theoretical plates (a measure of a chromatographic column's performance) |
| TLC: | thin layer chromatography |

## 7 References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# 8 Appendices

## Appendix 1. Parent material tested in 2007

| Trial Code | Code | % Art 2006 | 01-Aug Value score | 01-Aug | 06-Sep | 05-Oct |
|---|---|---|---|---|---|---|
| | | | | % Artemisinin | | |
| | | | | | | |
| 1 | 1015 | 1.95 | 5.00 | 0.75 | 1.07 | 1.10 |
| 2 | 1001-9 | 1.66 | 4.00 | 0.97 | 1.03 | 1.23 |
| 46 | 1001-10 | 1.95 | 4.75 | 0.80 | 0.77 | 0.88 |
| 81 | 1001-11 | 1.30 | 4.50 | 0.80 | 0.93 | 0.95 |
| 85 | 1001-13 | 1.20 | 3.75 | 0.85 | 0.90 | 0.9 |
| 301 | 1046-1 | 1.24 | 5.00 | 0.88 | 0.85 | 0.85 |
| 304 | 1046-4 | 1.42 | 5.00 | 0.80 | 0.95 | 0.98 |
| 322 | 1046-7 | 1.57 | 5.00 | 1.00 | 1.18 | 1.23 |
| 324 | 1026-1 | 0.95 | 3.75 | 0.75 | 0.73 | 0.63 |
| 352 | 1038-4 | 1.30 | 3.75 | 0.70 | 1.07 | 1.05 |
| 446 | 1043-1 | 1.22 | 4.25 | 0.78 | 0.70 | 0.75 |
| 494 | 1001-1 | 1.06 | 3.75 | 0.60 | 0.93 | 0.90 |
| 497 | 1012-10 | 1.27 | 3.75 | 1.03 | 0.88 | 0.90 |
| 501 | 1012-11 | 1.28 | 3.75 | 0.63 | 0.73 | 0.75 |
| 505 | 1001-3 | 1.55 | 4.00 | 0.67 | 0.77 | 0.93 |
| 508 | 1012-12 | 0.95 | 3.00 | 0.40 | 0.60 | 0.53 |
| 521 | 1012-16 | 0.97 | 4.00 | 0.53 | 0.75 | 0.75 |
| 578 | 1045-1 | 0.77 | 4.00 | 0.37 | 0.63 | 0.58 |
| 579 | 1045-2 | 0.76 | 2.25 | 0.35 | 0.50 | 0.40 |
| 583 | 1045-3 | 1.18 | 4.75 | 0.67 | 0.98 | 0.98 |
| 604 | 1030-1 | 1.26 | 4.50 | 0.50 | 0.57 | 0.80 |
| 682 | 1036-1 | 1.24 | 4.00 | 0.90 | 0.90 | 0.85 |
| 747 | 1035 | / | 3.25 | 0.90 | 0.83 | 0.95 |
| 798 | 1001-14 | / | 4 | 0.7 | 0.8 | 0.75 |
| 909 | 1001-15 | / | 2.5 | 1.05 | 0.93 | 1.00 |
| 931 | 1001-16 | / | 4.75 | / | 0.75 | 0.85 |
| 942 | 1001-17 | / | 3 | 0.93 | 0.80 | 0.68 |
| 947 | 1001-18 | / | 4.25 | 0.63 | 0.63 | 0.75 |
| 958 | 1001-19 | / | 3.5 | 0.90 | 0.87 | 0.90 |
| 961 | 1001-20 | / | 4.75 | 0.63 | 0.88 | 0.88 |
| 997 | 1001-21 | / | 4 | 0.68 | 0.85 | 0.95 |
| 998 | 1001-22 | / | 5 | 0.65 | 0.67 | 0.68 |

 

**Appendix 2 Crosses tested in 2007**

| Trial Code | Breeding Code | Pedigree | 1-Aug Value score Average | 25-Jul % Artem Cross 1 | 30-Aug % Artem Cross 1 | 27-Sep % Artem Cross 1 |
|---|---|---|---|---|---|---|
| 1001 | 1001 | Unknown | 3.75 | 0.40 | 0.63 | 0.88 |
| 1 | 1015 | Unknown | 4.00 | 0.67 | 1.20 | 1.10 |
| 43 | 1053 | 1015 x 1001-1 | 5.00 | 0.60 | 1.08 | 1.00 |
| 44 | 1054 | 1015 x 1001-3 | 4.50 | 0.57 | 0.93 | 0.98 |
| 45 | 1055 | 1015 x 1012-10 | 4.75 | 0.57 | 0.90 | 0.95 |
| 46 | 1056 | 1015 x 1012-11 | 3.75 | 0.40 | 0.80 | 0.93 |
| 2 | 1060 | 1001-10 x 1012-12 | 2.50 | 0.40 | 0.55 | 0.70 |
| 3 | 1061 | 1015 x 1043-1 | 4.25 | 0.40 | 0.88 | 0.95 |
| 4 | 1062 | 1001-9 x 1046-7 | 4.25 | 0.67 | 1.05 | 1.20 |
| 5 | 1063 | 1046-4 x 1001-9 | 3.50 | 0.57 | 1.13 | 1.13 |
| 6 | 1064 | 1046-4 x 1001-9 | 3.00 | 0.55 | 0.93 | 0.98 |
| 7 | 1065 | 1046-4 x 1001-10 | 4.50 | 0.50 | 0.93 | 0.93 |
| 8 | 1066 | 1015 x 1001-9 | 4.50 | 0.55 | 1.05 | 1.13 |
| 9 | 1067 | 1001-9 x 1012-12 | 4.25 | 0.40 | 0.70 | 0.93 |
| 10 | 1068 | 1001-10 x 1045-3 | 4.00 | 0.50 | 1.00 | 1.08 |
| 11 | 1069 | 1001-11 x 1045-3 | 3.50 | 0.50 | 0.90 | 0.90 |
| 15 | 1073 | 1015 x 1012-12 | 4.25 | 0.45 | 0.83 | 0.93 |
| 17 | 1075 | 1001-3 x 1012-16 | 4.25 | 0.40 | 0.73 | 0.75 |
| 18 | 1076 | 1001-10 x 1015 | 5.00 | 0.50 | 0.93 | 0.83 |
| 19 | 1077 | 1001-10 x 1045-1 | 3.25 | 0.30 | 0.55 | 0.60 |

 