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:	**LK 0822** **HDC project: CP44**
Project leader:	Ian Flockhart [Botanical Developments Ltd]
Report:	Final report, 2010
Previous report	First year report 2007 Second year report 2008 Third year report 2009
Key staff:	Colin Hill - Commercial extraction Trevor Robinson – Crop agronomy, Frontier Nigel Dungey – Seed production, Humber VHB Steven Bentley – Parent breeding, NIAB Christopher Atkinson – Plant physiology, EMR Jack Woolley – Analytical analysis Corrinne Burns – 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.

Steven Bentley
Technical Leader, Non Food Crops
NIAB

Signature ..... Date .....

Jack Woolley
Professor
De Montfort University

Signature ..... Date .....

Report authorised by:

Chris Atkinson
Plant Physiology
East Malling Research

Signature ..... Date .....

Ian Flockhart
Director
BDL

Signature ..... Date .....

Trevor Robinson
Business Development Manager
Frontier

Signature ..... Date .....

Nigel Dungey
Research Director
Humber VHB

Signature ..... Date .....

Table of Contents

1 Grower Summary

The potential for UK cultivation, testing and supply of Artemisinin from *Artemisia annua* has progressed well. However, it was not be possible to perfect our understanding of this crop and its agronomy in a four year study and commercial field-scale cultivation is yet to be successfully demonstrated.

1.1 Background and expected deliverables

Artemisia annua is being investigated here to demonstrate the potential for UK production of artemisinin used in the treatment of malaria. This is achieved by the extraction of the active pharmaceutical ingredient (API) Artemisinin (ART) from the leaves 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 is a 4 year LINK project (LK0822) and follows on from a successful one-year, DEFRA funded project NF0613. The trial results indicated that certain *A. 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 was a need for significant work to better characterise this material for UK conditions and develop cost effective planting and harvesting techniques.

Previous work concluded that a fully integrated UK production chain was possible and it would be imperative to have a secured supply of fully characterised germplasm. The germplasm tested initially indicated that this could be achieved and analytical techniques to characterise it were developed. Clearly, it was not be possible to perfect our understanding of this crop and its agronomy in a four year study, however significant progress was made.

1.2 Summary of the project and main conclusions

1.2.1 Confirmation of improved analytical techniques

Continuing with the exploration of rapid, accurate and cost-effective means of discerning high-Artemisinin yielding plant material from lower-yielding, work over the last 12 months has considered two new techniques: a) TLC separation of Artemisinin and Deoxyartemisinin and b) Flash column chromatographic separation of Artemisinin and Deoxyartemisinin.

The first of these techniques, TLC densitometry, employs fairly simple, inexpensive means of separating the two compounds, technology which could realistically be adopted in the field. In many chromatographic systems, Deoxyartemisinin co-elutes with Artemisinin – in fact, frequently "Deoxyartemisinin and 'Artemisinin' are not separated at all and therefore 'Artemisinin' is systematically overestimated" in crude plant extracts and semi-pure material (Christen and Veuthey, 2001). Using a two-step column chromatography system, gram-scale separation of Artemisinin and Deoxyartemisinin was achieved – as was the crystallisation to a constant melting range of both compounds.

Additionally, in 2007, a correlation between Artemisinin and chrysosplenetin content was observed in samples of three Cambridge-grown varieties of *A. annua* (1015, 1012-12 and 1046-7). It was suggested that, if the correlation was strong and always present, it could provide an additional means of sample quality assessment. To verify that this 0.1 correlation was always present, the analysis was expanded in 2008/2009 to include eleven varieties of *A. annua* - both before and after desiccation treatment. When eleven varieties were considered, a strong positive correlation existed between levels of "Flavonoid 1" (either eupatin or chrysosplenol-D) and Artemisinin levels – although this correlation was somewhat reduced upon application of the desiccant.

1.2.2 Evaluation of improved germplasm

In 2009 NIAB continued to characterise parent material and evaluate crosses that had been made at Cambridge. Line 1015 was evaluated during the whole summer period as a control. However several varieties have now been evaluated over the duration of the project and established seasonal trends.

The percent of Artemisinin produced in 2009 in the control varieties was about 0.2% higher that produced in 2008. The best material selected in 2007 (from single plants) confirmed their potential in 2009. The highest average Artemisinin yield from four replicates was 2.14% (148% of the 2009 controls) and many of these parents were used to make crosses in 2008 & 9. Additionally, the best material selected in 2008 came from selfed plants and the best line (1001-9) had a 2.19% Artemisinin yield (152% of the controls).

Finally, crosses made in 2009/10 for testing in 2010; the parents were selected to have the highest possible % Artemisinin content and known combining ability. Within the crosses there was a range of genetic distance between the parents (estimated relative to pedigree) which will inform the project further about the effects of inbreeding.

1.2.3 Optimisation of biomass production in response to nutritional supply.

A controlled environment study was undertaken to determine if increasing the supply concentration of boron, above that used in the earlier experiment, would further increase Artemisinin concentration and yield. It was found that increase in boron concentration; a) caused boron concentration in the leaves of plants to be increased which suggested that the uptake of boron at the higher rates of applications was saturated; b) had no significant effect in total plant biomass production; c) had no increase in the Artemisinin concentration in the leaves of *Artemisia*; and finally d) had no increase in the total yield of Artemisinin.

1.2.4 Optimisation of methodology for commercial seed production.

The consortium successfully harvested seeds on large scale (more than 30 g of seed per plant) and additionally confirmed the preliminary yield conjecture that the 7.5 g/plant target could be considerably improved upon. Seeds were again small, but viability was excellent with more than 97% germination. Therefore, the combination of this improved yield, arrangement with fewer plants and exploitation of summer energy economies greatly improved the economic viability of this aspect of the project. This validation step provided the consortium with more than 28 kg of seed (560 million seeds) for exploitation around the world.

1.2.5 Optimisation of methodology for commercial scale field production

Previously (in 2007 and 2008) the establishment of commercial crops was found to be very variable due to considerable risk of the weather conditions at establishment. In 2009, Frontier worked closely with NIAB on the techniques for commercial production, and significant effort was made to establish trials at higher seed rates using irrigation. However, the consortium concluded that material could establish successfully if the planting depth was great enough to allow retention of moisture but not so deep as to prevent establishment. As soil moisture and sowing depth are critical, the establishment of this crop in the spring will always be at risk. However it was found that the crop can be established over winter if the seed is put on the surface of a stale seed bed.

1.3 Financial benefits

The UK-produced seed proved to be of higher quality than that previously imported. During this project, the partnership has been able to produce seed on a commercial scale. Real demand for Artemisinin commenced in 2004 when 30 countries adopted Artemisinin based medicines (ACT's) as the principal treatment for multi-drug resistant malaria. It is now

predicted that 275 million treatments will be required by 2014 (twice those ordered for 2010). It is anticipated that all strategic stocks of Artemisinin in the supply chain will be consumed during 2010 and the 10,000 hectares being grown during 2010 (producing 60 tons of Artemisinin) will be insufficient to meet the demand for treatments in 2011 (requiring 150 tons of Artemisinin). Demand for Artemisinin based medicines is predicted to peak in 2014/5 when the mid prediction of all agencies believe 275 million treatments will be required.

1.4 Action points for growers

The significant progress already made together with the financial predictions for Artemisinin demand over the next few years could provide considerable benefits to growers, but growers should be aware that the crop and its agronomy under UK conditions is not yet fully understood.

2 Science Section

Experimental work is described and discussed in the following sections according to Work Packages.

2.1 Introduction

Artemisia annua is a potential new biopharmaceutical crop with no previous history of large 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 ART 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. However, there was 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 was not be possible to perfect our understanding of this crop and its agronomy in a four year study, but considerable progress was made.

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 it. The key objectives for 2009 were:-

1. Confirmation of appropriate for this project analytical techniques
2. Evaluation of the best material collected in 2008 and production of crosses using the best material for evaluation in 2010
3. Evaluation of Artemisinin yield and plant biomass in relation to boron concentration.
4. Development of the crop establishment techniques for commercial purposes.

Improved material was available for the objectives above; therefore the agronomic objective was addressed using germplasm from Humber VHB.

2.2 Material and Methods

A number of different methodological approaches were used within this project. Separating the methodology from the results section produced a report that was difficult to follow. The results are therefore presented within each section following an explanation of the methodology used.

2.2.1 Work Package 1; developing a rapid Artemisinin assay – Led by De Montfort University

2.2.1.1 Purification of Artemisinin: removal of the inactive contaminant Deoxyartemisinin.

2.2.1.1.1 TLC separation of Artemisinin and Deoxyartemisinin

Although the Artemisinin molecule (Figure 1) is a fairly complex structure, its anti-plasmodial activity resides within a single moiety: the endoperoxide bridge. A change to this bridge – as occurs in the closely related compound Deoxyartemisinin (Figure 2) – renders the molecule useless for the treatment of malaria.

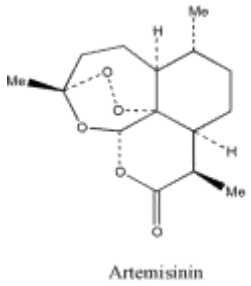

Figure 1. Artemisinin

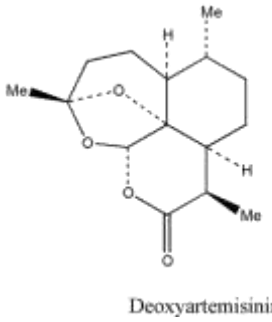

Figure 2. Deoxyartemisinin

Deoxyartemisinin is lacking one of the oxygens of the endoperoxide bridge – creating instead an ether moiety.

The biosynthetic formation of Deoxyartemisinin within the leaf is not fully understood. It has been speculated that the deoxy-form occurs as a result of microbial colonisation of the plant in the field (Srivastava *et al*, 2009). Formation may also be catalysed by the presence of iron within the plant: incubation of Artemisinin with ferrous iron ions showed, in much the same manner as the antimicrobial investigations, a metabolism to Deoxyartemisinin (Zhan *et al*, 2002).

Assays of consortium *A. annua*, grown in the UK, have shown that Deoxyartemisinin is present in dried plant material at levels averaging 5% of the Artemisinin level. African-grown consortium material typically contains Deoxyartemisinin at about 8% of the Artemisinin level – even when the material is of the same genetic stock as UK-grown material. It has been hypothesised that the iron-rich soils of Africa are the cause of this relative increase in Deoxyartemisinin.

But whatever the cause, its presence is a problem. In many chromatographic systems, Deoxyartemisinin co-elutes with Artemisinin – in fact, frequently 'Deoxyartemisinin' and 'Artemisinin' are not separated at all and therefore 'Artemisinin' is systematically overestimated in crude plant extracts and semi-pure material (Christen and Veuthey 2001). This is particularly the case in UV-based high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC).

Although the use of HPLC coupled to mass spectrometric detectors in single ion monitoring (SIM) mode does get around this problem, this is of little help to the many laboratories and field scientists who have no access to such an expensive piece of equipment. Ideally, a simple and inexpensive means of separating the two compounds is needed. The obvious solution is thin layer chromatography (TLC), and to that end a TLC system capable of clearly separating the two compounds was developed.

2.2.1.1.2 Methods, results and discussion

After a great deal of trial and error, it was found that a mixed- solvent system consisting of hexane/ butyl methyl ether/ethyl acetate, at a ratio of 8/1.5/0.5, produced the optimal separation of the two compounds. Using this system, Deoxyartemisinin eluted with a retention factor (R*f*) of 0.41, and Artemisinin moved slightly slower, with an R*f* of 0.33. Additionally, the two compounds respond slightly differently to the vanillin-based visualization reagent: Deoxyartemisinin derivatises to a light green, whilst Artemisinin is dark green or even blue, depending on the freshness of the visualization reagent (Figure 3).

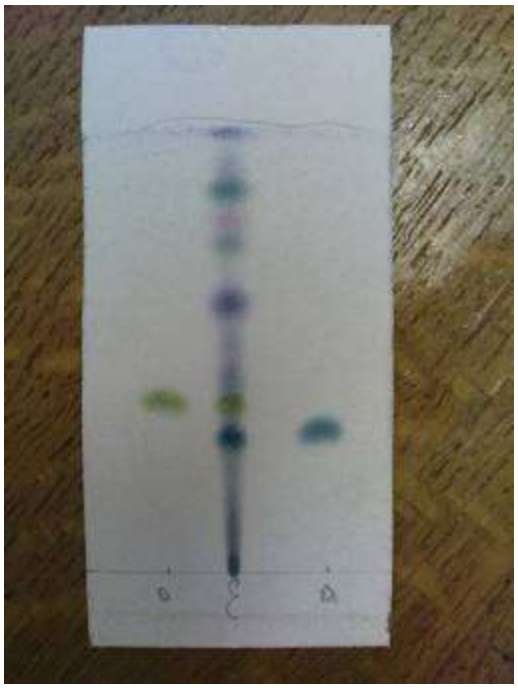

Figure 3. TLC separation of deoxyArtemisinin from Artemisinin. L-R: deoxyArtemisinin, extract from a Tanzanian-grown variety of A. annua, and Artemisinin

Despite the close similarity in structure – with Deoxyartemisinin differing from Artemisinin only by the lack of one oxygen atom – it has been possible to clearly separate the two spots by thin layer chromatography, using standard silica plates and commonplace laboratory solvents. Such a technique should prevent the overestimation of Artemisinin content during TLC-based sample analysis, especially when coupled with the TLC-densitometric system developed within the Consortium in 2009.

2.2.1.2 Flash column chromatographic separation of Artemisinin and Deoxyartemisinin

Having developed a method for the thin-layer chromatography (TLC)-based separation of Artemisinin from its inactive cousin, Deoxyartemisinin, the question remained that, having ascertained that the sample – be it a crude extract, or crystalline matter – does contain a degree of Deoxyartemisinin, how can the unwanted Deoxyartemisinin be removed on a larger scale? Could the TLC method be adapted for column chromatography, allowing the removal of Deoxyartemisinin from gram quantities of impure Artemisinin samples?

Using a two-step column chromatography system, gram-scale separation of Artemisinin and Deoxyartemisinin was achieved – as was the crystallisation to a constant melting range of both compounds.

2.2.1.2.1 Materials and Methods

The first stage involved the use of gravity column chromatography, using silica of 70 – 200 mesh, over which 4g of *A. annua* extract (dried onto 5g silica) containing the two compounds was fractionated using the solvent sequence presented in Table1.

Table 1. Gradient system for fractionation of crude *A. annua* extracts

Solvent Mixture	Volume
Hexane 100%	100 ml
5% ethyl acetate in hexane	100 ml
10% ethyl acetate in hexane	100 ml
20% ethyl acetate in hexane	100 ml
30% ethyl acetate in hexane	100 ml
40% ethyl acetate in hexane	100 ml
50 % ethyl acetate in hexane	200 ml

20 ml fractions were collected. Following TLC of the fractions, those containing Artemisinin and Deoxyartemisinin were pooled and concentrated. Using this system, it was possible to separate Artemisinin and Deoxyartemisinin from the other sample components, but not from each other. Pooled and concentrated fractions containing both these compounds were allowed to stand overnight, and from this liquor, 450 mg of opaque, off-white needles, with a melting range of 125 - 141°C, were obtained. TLC analysis showed that the needles were a mixture of Artemisinin and Deoxyartemisinin, with a small level of other contaminants. This crystalline matter was washed with hexane, re-dissolved in 50% ethyl acetate in hexane, and dried onto 1g of 35 – 70 mesh (Chromatography Grade) silica (Figure 4).

Figure 4. Partially-pure crystals consisting of a mixture of Artemisinin and Deoxyartemisinin

Stage 2 involved the preparation of a second column, using silica with a particle size of 35 – 70 mesh. The silica onto which the semi-pure crystalline matter had been dried was placed onto the top of this column, and the column was run with an isocratic mobile phase of hexane: butyl methyl ether: ethyl acetate (80: 15: 5), under flash conditions. Fractions of 10 ml were collected, and analysed by TLC. Fractions 19 – 23 contained Deoxyartemisinin, and a small amount of another compound, and fractions 24 – 39 contained Artemisinin. Fractions 25-39 were pooled and concentrated on a rotavapor (set to 60°C). Upon concentration to ~ 3 mls, crystals – in the form of opaque white needles - readily formed. 355 mg of crystals were obtained, with a melting range of 152 – 156°C.

Fractions 19 – 23, containing Deoxyartemisinin and another impurity, were reduced in volume on a rotavapor at 60°C. When the volume was ~1 ml, the liquor was allowed to stand. Within 30 minutes, clear white needles had begun to form in the liquor, and within 1 hour no further crystallization was seen. Crystals were dried and weighed. 35 mg of crystals were obtained, with a melting range of 108 - 110°C.

TLC analyses confirmed that the crystals with a melting range on 152 - 156°C were a single compound, with a retention factor comparable to that of an Artemisinin reference. Similarly, the crystals with a melting range of 108 - 110°C had, by TLC, a retention factor comparable to a Deoxyartemisinin reference. The crystalline sample has been submitted for 1H NMR. A very small trace of Artemisinin could be seen (Figure 5).

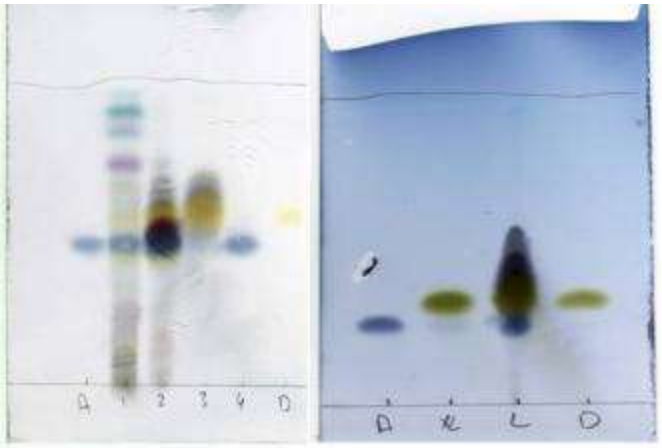

Figure 5. Left image, L-R. A: Artemisinin reference, 1: crude extract prior to chromatography, 2: pooled Artemisinin and Deoxyartemisinin after column 1; 3: pooled Deoxyartemisinin fractions after column 2 (but before sample concentration and crystallization), 4: pooled Artemisinin fractions after column 2 (but before sample concentration and crystallization), D: Deoxyartemisinin reference. Right image. A: Artemisinin reference, XL: Deoxyartemisinin after crystallization; L: mother liquor of Deoxyartemisinin, D: Deoxyartemisinin reference

2.2.1.2.2 Discussion

Using simple laboratory equipment, it has been demonstrated that it is possible to separate Artemisinin from the inactive contaminant Deoxyartemisinin. A two step column chromatography set-up is needed: step 1, to isolate the two compounds from the other constituents of a crude extract, and step 2, to pull the two compounds away from each other. For this last step, two modifications to the standard column chromatography set-up were required:

1. The use of silica with a particle size of 35 – 70 µm (as distinguished from the more usual 70 - 200 µm size);
2. The use of flash chromatography.

Use of silica with a finer particle size - and hence a larger surface area - allows for a greater degree of interaction between solute and stationary phase. Figure 6 graphically illustrates the difference in quality between the standard and chromatography grade silica.

Presumably, the use of flash chromatography - in which the addition of pressure to the column results in a much more rapid elution of compounds than by gravity alone – prevents the dispersion effects that can cause closely eluting compounds to overlap.

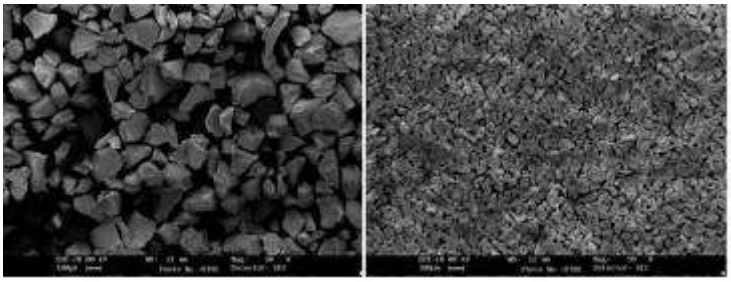

Figure 6. Comparison of particle size and distribution of standard (left) and chromatography grade (right) silicas. Pictures taken at 50 X magnification with a scanning electron microscope.

Although absolute baseline separation was not achieved, it was demonstrated clearly that a high yield of Deoxyartemisinin-free Artemisinin can be produced using simple, inexpensive methods. Whether this technique can be scaled to gram and kilogram levels has yet to be discovered.

2.2.2 Flavonoid profiling of cultivars as an indirect means of Artemisinin quality assessment.

In 2007, a correlation between Artemisinin and chrysosplenetin content was observed in samples of three Cambridge-grown varieties of *A. annua* (1015, 1012-12 and 1046-7). The mean chrysosplenetin: Artemisinin ratio was 0.1 – that is, for every unit of chrysosplenetin, ten units of Artemisinin were detected (for further details, see previous HDC Reports). It was suggested that, if the correlation was strong and always present, it could provide an additional means of sample quality assessment. As flavonoids such as chrysosplenetin are strongly UV-absorbent, they are easily detected by comparatively less expensive laboratory instruments, such as HPLC-DAD (Figure 7). To quantify Artemisinin accurately requires the much more expensive HPLC-mass spectrometer.

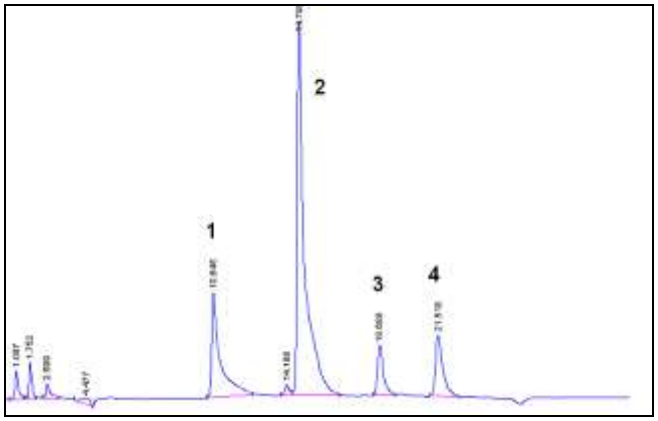

Figure 7. HPLC-DAD chromatogram, recorded at 350 nm, of an extract of A. annua. Four peaks representing flavonoids are observed; Peak 2 represents chrysosplenetin, as identified by 1H NMR. HPLC-MS of the earliest eluting compound showed a base peak of 361 m/z, indicating that it was likely to be either eupatin or chrysosplenol-D. Both compounds have been identified in A. annua (Bilia *et al.,*, 2006)

To verify that this 0.1 correlation was always present, the analysis was expanded in 2008/2009 to include eleven varieties of *A. annua* - both before and after desiccant treatment. The results are presented in Table 2, after which follows a summary. Table 2 shows levels of Artemisinin and four flavonoids (chrysosplenetin, and “Flavs 1, 2 and 3”) in eleven verities of *A. annua* grown in Cambridge in 2008. Each variety was sampled three times: on 31st July, 22nd September, and 22nd September following previous treatment with desiccant.

Table 2. Artemisinin and chrysosplenetin levels in eleven Consortium varieties grown in Cambridge in 2008

Variety	Harvest Date	ART % w/w	Flav 1 % w/w	CAST % w/w	Flav 3 % w/w	Flav 4 % w/w	Ratio CAST/ART
1015	31/7/2008	0.8	0.04	0.11	0.01	0.01	0.14
	22/9/2008	1	0.04	0.11	0.01	0.01	0.11
	22/9/2008 GLY	1.2	0.04	0.11	0.01	0.01	0.09
1046-7	31/7/2008	1	0.1	0.15	0.02	0.01	0.15
	22/9/2008	1.1	0.1	0.16	0.02	0.01	0.15
	22/9/2008 GLY	1.3	0.12	0.19	0.02	0.02	0.15
1012-12	31/7/2008	0.6	0.03	0.11	0.01	0.02	0.18
	22/9/2008	0.7	0.01	0.09	0.01	0.01	0.13
	22/9/2008 GLY	0.8	0.04	0.12	0.01	0.02	0.15
1001-3	31/7/2008	0.7	0.01	0.11	0.02	0.01	0.16
	22/9/2008	0.9	0.02	0.13	0.02	0.01	0.14
	22/9/2008 GLY	1	0.02	0.14	0.03	0.01	0.14
1038-1	31/7/2008	1	0.05	0.12	0.01	0	0.12
	22/9/2008	0.9	0.03	0.1	0.01	0	0.11
	22/9/2008 GLY	1.3	0.11	0.12	0	0	0.09
1045-3	31/7/2009	0.8	0.07	0.18	0.04	0.02	0.23
	22/9/2008	0.9	0.06	0.15	0.04	0.02	0.17
	22/9/2008 GLY	1.2	0.08	0.16	0.03	0.03	0.13
1062-1	31/7/2008	1.2	0.08	0.16	0.01	0	0.13
	22/9/2008	1.4	0.1	0.18	0.01	0	0.13
	22/9/2008 GLY	1.7	0.11	0.22	0.01	0	0.13
1062-4	31/7/2008	1	0.08	0.11	0.01	0	0.11
	22/9/2008	1.2	0.15	0.16	0.01	0.01	0.13
	22/9/2008 GLY	1.5	0.14	0.15	0.01	0.01	0.1
1053-1	31/7/2008	1.4	0.08	0.18	0.02	0.03	0.13
	22/9/2008	0.8	0.05	0.14	0.02	0.03	0.18
	22/9/2008 GLY	1.3	0.07	0.16	0.02	0.03	0.12
1053-2	31/7/2008	0.9	0.05	0.13	0.01	0.02	0.14
	22/9/2008	0.9	0.04	0.13	0.01	0.02	0.14
	22/9/2008 GLY	1.3	0.06	0.16	0.01	0.02	0.12
1053-4	31/7/2008	0.8	0.07	0.08	0.01	0.01	0.1
	22/9/2008	1.2	0.08	0.11	0.01	0.01	0.09
	22/9/2008 GLY	1.1	0.11	0.11	0.01	0.01	0.1

When eleven varieties were considered, a strong positive correlation existed between levels of "Flavonoid 1" (either eupatin or chrysosplenol-D) and Artemisinin levels – although this correlation was somewhat reduced upon application of desiccant (Table 3).

Table 3. Correlation Coefficient between Artemisinin and Flavonoid Level

Harvest dates	Eupatin	Chrysosplenetin
Harvest: 31/7/2008	0.8	0.6
Harvest: 22/9/2008	0.8	0.5
Harvest: 22/9/2008, treated with desiccant	0.7	0.6

The correlation between chrysosplenetin and Artemisinin, however, was less pronounced, both with and without desiccant treatment. This is explained by the fact that the ratios of chrysosplenetin to Artemisinin, when all lines and treatments (i.e. with or without desiccant) are considered together, ranges from 0.09 to 0.23, although the mean (and median) value is 0.13 – close to that observed in the 2007 material.

Use of desiccant; in four of the eleven varieties assayed, desiccant treatment did not affect the chrysosplenetin to Artemisinin ratio. In three of these cases (1046-7, 1001-3 and 1062-1), a correlated increase in both chrysosplenetin and Artemisinin was observed. In the fourth case (1053-4), Artemisinin level actually dropped, whilst that of chrysosplenetin increased slightly.

However, in the other seven lines, the ratio was affected by desiccant application. In four of these (1012-12, 1045-3, 1053-1 and 1053-2), a small increase in chrysosplenetin was observed, but to a lesser magnitude than the increase in Artemisinin. In two cases (1015, 1038-1), Artemisinin increased whilst chrysosplenetin level did not change. In the final line, 1062-4, Artemisinin increased whilst chrysosplenetin level dropped.

2.2.2.1 Comparison with 2007 material

1015 showed a different flavonoid profile in 2008 as compared to 2007. In 2008, the levels of "Flavonoid 1" (either eupatin or chrysosplenol-D) were much lower than the levels of chrysosplenetin, whereas in 2007 the levels had been nearly equal. 1012-12 and 1046-7, however, appeared very similar in flavonoid profile in 2008 compared to 2007. In both 2007and 2008, 1015 produced a maximum chrysosplenetin content of 0.11% w/w.

In 2008, 1046-7 contained slightly more chrysosplenetin compared to 2007 (0.19 % w/w and 0.18% w/w respectively). 1012-12, though, showed quite a large increase in chrysosplenetin in 2008 compared to 2007 (0.12% w/w and 0.07 % w/w respectively). This increase was not reflected in the

Artemisinin levels, which were slightly lower in all three varieties in 2008 compared to 2007. Such year-to-year variations - small as they are - in the chrysosplenetin: Artemisinin ratio, within the same germplasm, show that environmental conditions are able to override genetic similarity, and reduce the reliability of this indirect means of Artemisinin quality assessment. To further explore the effect of environmental variation on the chemical profiles, Consortium-developed material grown in areas other than Cambridge (Surrey, Lincoln and Morocco) were assayed. The table 4 below shows the results.

Across a growth season outside of Cambridge, a wide range of chrysosplenetin: Artemisinin ratios ranged from 0.06 to 0.27 (Table 4). The case of 1046-7 is revealing: in Cambridge, it was 0.1 to 0.15 – but in Surrey, the ratio dropped to 0.08. The difference can be explained by the fact that in both locations, the chrysosplenetin levels were the same as in Cambridge, but Artemisinin levels in the Surrey-grown material were higher.

Table 4. Artemisinin and Chrysosplenetin in Consortium material grown outside Cambridge.

Sample	Details	Artemisinin % w/w	Flavonoid 1	Chrysosplenetin % w/w	Ratio Chrysosplenetin/ Artemisinin
8Z	Harvested mid-September 2008	0.3 (0.038)		0.08 (0.002)	0.27
8Y		0.3 (0.040)		0.08 (0.0005)	0.27
8X	Harvested late September 2008	0.6 (0.01)		0.06 (0.0002)	0.1
8V		0.5 (0.02)		0.03 (0.0001)	0.06
8S	Harvested late October 2008	0.4 (0.002)		0.03 (0.004)	0.075
8Q		0.5 (0.003)		0.03 (0.002)	0.12
8P		0.4 (0.002)		0.03 (0.004)	0.075
1053 X	Harvested early October 2008, in Surrey	0.6 (0.07)		0.03 (0.001)	0.05
1046-7 Vegetative	Harvested early August 2008 (? Surrey)	1.8 (0.07)	0.1	0.14	0.08
1046-7 Flowering		1.0 (0.03)	0.06 *(0.0009)*	0.08	0.08
1001-9 Vegetative		1.8 (0.05)	0.03 *(0.001)*	0.09 (0.002)	0.05
1001-9 Flowering		1.4 (0.16)	0.1 *(0.0001)*	0.06 (0.0005)	0.04
Moroccan I (1053)		0.5 (0.015)	0.012	0.058 (0.002)	0.12 (0.06)
Moroccan A (1053)		0.4 (0.02)	0.017	0.074 (0.003)	0.19 (0.008)
	Correlation Coefficient Chrysosplenetin Artemisinin				
Frontier		-0.5			
Humber VHB		0.6			

Additionally, the Consortium-developed but Moroccan grown material showed a ratio that differed according to drying methods. Both Moroccan samples were of cross 1053, but the sun-dried sample (Moroccan I) showed both a decrease in Artemisinin and an increase in chrysosplenetin, compared to the “fast-dried” ample (Moroccan A).

Both cases, though, showed much less Artemisinin compared to Cambridge-grown material, and also much less chrysosplenetin. Finally, having examined the effects of different locations on Consortium-material, the ratio of chrysosplenetin: Artemisinin in non-Consortium material was investigated. The table 5 below shows the results of four non-Consortium samples.

In non-Consortium material, chrysosplenetin : Artemisinin ratios range from 0.07 to 0.19, with the lowest ratio was seen in the Anamed sample, and the highest in the Ghanaian (Table 5).

Table 5. Artemisinin and flavonoid levels in non-Consortium material

Sample	**ART % w/w**	**Peak 1**		**Peak 2 (Chrysosplenetin)**	
		% w/w	**Ratio FLAV: ART**	**% w/w**	**Ratio FLAV: ART**
Rutland, UK	0.5 (0.001)	0.01 (0)	0.02 (0)	0.059 (0.002)	0.12 (0.004)
Anamed (Swiss)	0.8 (0.014)	0.04 (0)	0.05 (0.0007)	0.057 (0.0007)	0.07 (0)
Kenyan	0.9 (0.07)	0.06 (0.006)	0.07	0.1 (0.06)	0.11
Ghana	0.5 (0.001)	0.037 (0)	0.07 (0.004)	0.091 (0.0007)	0.19 (0.002)

2.2.2.2 Summary and Discussion

This exploration of flavonoid profiles in *Artemisia annua* began in 2007. Previous research had demonstrated the anti-cancer potential of methoxylated flavonoids (Kobayakawa *et al*, 2004), and it was thought that the presence of chrysosplenetin could add value to *A. annua* as a commercial crop – hence the interest in quantifying this compound in Consortium-grown material. It was during this quantification that the correlation with Artemisinin was noticed.

HPLC-DAD separation of extracts of *A. annua* showed a consistent pattern of four flavonoid-type compounds (Figure 7), of which the peak eluting at 14.7 minutes was demonstrated, by HPLC-MS and NMR comparison with a reference standard, to be chrysosplenetin. An earlier eluting peak (“Flavonoid 1”) was hypothesized to be either eupatin or chrysosplenol-D, based on LCMS analysis, but insufficient sample was available to allow for NMR analysis of this compound.

After examining the ratio of chrysosplenetin to Artemisinin over two years, in Consortium and non-Consortium material, and of Consortium material grown in varying locations, the conclusion is that

the chrysosplenetin: Artemisinin ratio, whilst averaging at about 0.1, shows too much temporal, geographical and genetic variation to provide a consistent, universal means of indirect Artemisinin assessment. Chrysosplenetin levels could not be used to identify Artemisinin levels in an unknown sample.

However, the results do show that chrysosplenetin: Artemisinin ratios are constant within batches (e.g., in material grown in one location during one summer, where material is harvested and dried in the same manner). In this case, the grower would have to determine the ratio for that batch early in the season – which could then be reliably presumed to remain constant. But the usefulness of such a system is fairly limited, and it is reasonable to suppose that the TLC densitometry system of Artemisinin quantification (described in HDC Year 3 Report) is a better means of quality assessment for those without access to LCMS.

2.3 Work Package 2; Development of improved germplasm – Led by NIAB

In 2009 NIAB continued to characterise parent material and evaluate crosses that had been made at NIAB. The line 1015 was evaluated during the whole summer period as a control. However several varieties have now been evaluated over the duration of the project which shows some seasonal trends.

2.3.1 Parents selected in previous years.

These were grown as spaced plants, shown below in Figure 8. Each parent was vegetatively propagated with three plants per plot and four replications.

Figure 8. Parent and cross material on 5th June 2009

The growing conditions seemed very favourable in 2009 and the plants established very rapidly. Figure 9 shows some of the parent material one month later.

Figure 9. Parent material on the 5th Jul 2009

Table 6 shows the parent material that was maintained and retested in 2009. This was material that had already shown potential in existing crosses and has been used as a seasonal control.

Table 6. Control varieties grown in 2009

Parent	Value	Untreated			Treated	
		27/09/2007	22/09/2008	26/08/2009	22/09/2008	26/09/2009
	1 – 5	2007	2008	2009	2008	2009
1015	4	1.2	1	1.17	1.20	1.51
1001-9	3	1.1	1.05	1.3	1.25	1.41
1046-7	5	1.33	1.1	1.26	1.40	1.58
1001-3	4	1	0.9	1.12	1.00	1.32
1001-1	4	0.95		1.06	1.20	1.40
Average		1.116	1.0125	1.182	1.21	1.44
		100%	91%	106%		

The results showed similar trends to the parents tested in 2007 & 8. The percent of Artemisinin produced in 2009 in the control varieties was about 0.2% higher that produced in 2008. Though the results in 2009 seem very much better it is more a reflection of the poor growing season in 2008. However the average result for 2009 appears to be 6% better than in 2007, though the general control 1015 was slightly lower.

The best material selected in 2007 (from single plants – result not replicated) confirmed its potential in 2009 (Table 7). The highest average yield from four replicates was 2.14% (148% of the 2009 controls) Artemisinin and many of these parents were used to make crosses in 2008 & 9.

Table 7. The best material selected in 2007

Parent	Value	Cross number and % of ART	Untreated			Treated		% of Art Controls in 2009
			27/09/2007	22/09/2008	26/08/2009	22/09/2008	26/09/2009	
	1 – 5		2007	2008	2009	2008	2009	
1062-1	3-4	1001-9 x 1046-7 1.41 x 1.58	1.65	1.5	1.69	1.75	2.13	
1062-2	3		1.6	1.4	1.59	1.65	2.14	148%
1062-3	5		1.55	1.5	1.73	1.65	2.12	
1062-4	5		1.5	1.25	1.44	1.60	1.94	
Average			1.58	1.41	1.61	1.66	2.08	

The best material selected in 2008 came from selfed plants (result not replicated). Their potential was confirmed in 2009 (replicated trial) see Table 8. The best line had 2.19% Artemisinin (152% of the controls).

Table 8. The best material selected in 2008 from selfed plants

Parent	Value	Breeding line code and %of ART from 2009	Untreated		Treated	% of ART Controls in 2009
			22/09/2008	26/08/2009	26/09/2009	
	1 - 5		2008	2009	2009	
708	3	1046-7 1.58%	1.4	1.53	2.16	
182	3	1015 1.51%	1.2	1.58	2.1	
585	2	1001-9 1.41%	1.3	1.61	2	
589	3	1001-9 1.41%	1.3	1.56	2.19	152%

The genetic gain was not much different from the material selected in 2007 and the value of the types was lower. However we believe that some of these may make very good parents for future crosses as their combining ability is already proven, i.e. cross 1062.

The full statistical analysis of these and other parent lines tested in 2009 are shown in Appendix 1.

In 2009 new parents were selected from individually tested plants from within the best crosses. This had proven to be very successful in 2007. All 240 plants within the 3 best crosses tested in 2009 were sampled. After an initial analysis in early August the best plants were sampled a further 2 times to confirm the results. Table 9 shows the final result from late September. These results are therefore not replicated. The same technique, used in previous years, has also been a good predictor of future performance in replicated trials.

Table 9. New parents selected from the best crosses

Parent	Value	Breeding line code	Untreated		Treated	% of ART from control in 2009
			% of ART and codes for parents used		26/08/2009	
	1 - 5			2009	2009	
137	4	1107	1.51 x 2.12	1.61	2.1	
141	4		1015 x 1062-3	1.44	2.02	
213	5			1.65	2.01	
215	3-4			1.5	2.12	
322	4-5	1105	1.41 x 1.8	1.9	2.36	
507	3		1001-9 x 1053-1	1.5	2.19	
508	4			1.55	2.11	
551	3	1118	1.8 x 2.13	1.8	2.55	177%
671	4		1053-1 x 1062-1	1.73	2.08	
678	2			2.03	2.46	
745	3			1.9	1.96	

Since we started the project in 2006 we have been able to consistently increase the Artemisinin content of parent material each year. Clearly there is a seasonal effect but the genetic gain of our best material appears to have been greater. Figure 10 illustrates this gain since the start of the project.

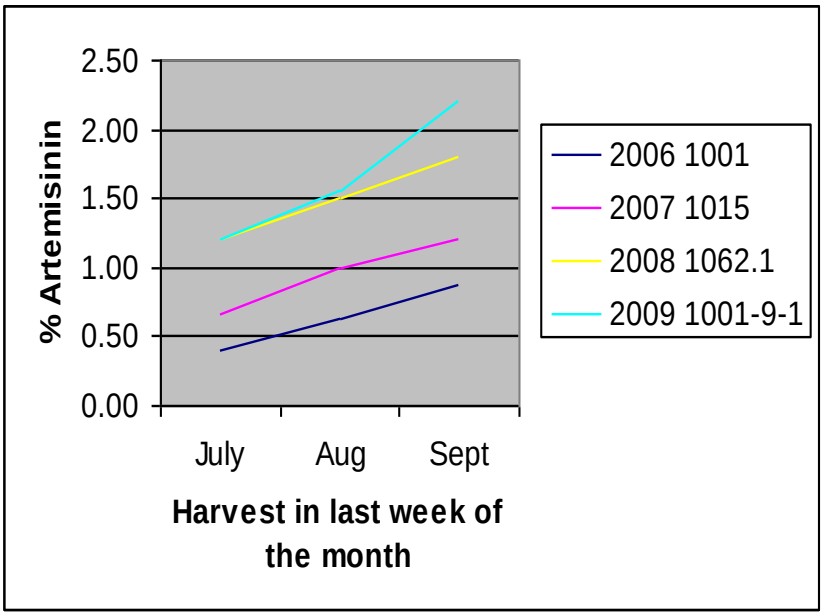

Figure 10. Gain since the beginning of the project.

We also have evidence to suggest that the % artemisinin observed in plants can be affected by conditions after harvest (Ferreira *et al*., 2010). Table 10 shows the affect of light on the artemisinin content of plants after harvest. This was only a small unreplicated trial but the affect was very large and appears to support some recently published work. We are not clear as to how this affect might be exploited commercially but it would seem appropriate to confirm these results in a fully replicated trial in 2010.

Table 10. The effect of post harvest storage of material after cutting

Harvest	Sampling	Breeding Code	Light/Dark	% Artemisinin
08/09/2009	30/09/2009	1015	Dark	1.34
08/09/2009	30/09/2009	1062-1	Dark	2.27
08/09/2009	30/09/2009	1015	Light	1.57
08/09/2009	30/09/2009	1062-1	Light	2.55

The success of this breeding programme has resulted in the need to maintain large numbers of parent lines. This can be expensive on both time and space. A micro-prop system was developed to maintain our best material (Figure 11). This has the advantage of providing security for our material and significant cost savings as this only requires propagating once a year.

Figure 11. Micro-prop Artemisia annua

2.3.2 Crosses evaluated in 2009

The crosses tested in 2009 were the eight best lines selected from crosses made at NIAB in the summer of 2008.

The control (1062) was the best cross tested in 2008. This material was grown from seed and transplanted on June 1st 2009. These plots can be seen in Figure 12 on the 16th of July and Figure 13 on the 7th of September. Each plot contained 60 plants and was replicated 4 times.

Figure 12. Replicated plots of crosses tested in 2009 – 16th July.

Figure 13. Replicated plots of crosses tested in 2009 – 7th Sept.

During the growing season all the plots were sampled to determine the potential of the crosses. Notes were also taken for the uniformity of the plants in the plots. Uniformity was better than had

been observed for many of the plots in 2007 and 2008. We believe this was better because only the crosses with good seed sets on both the parents had been selected for testing. This suggests that these were true crosses and the few off types were probably selfed plants. The plots were commercially harvested as shown in Figure 14, weighed and sampled. Only 6 of the 9 crosses tested were taken to full harvest. The remaining three lines were felt to be to variable and therefore possibly not true crosses.

Figure 14. Harvest of crossing trials

The cross 1062 performed better than in 2008 which was to be expected relative to the other parent results. The new crosses were better than cross 1062 but the genetic gain was not as great as we had hoped especially from cross 1118. Cross 1062 performed better than either of it's two parents. There appeared to be better combining ability between those two lines. Therefore, we are optimistic that the same cross made with the selfed parents (585 & 708) could also show good combining ability in 2010.

Leaf yield from the plot was evaluated as in previous years to make an estimate of the whole plot yield. However this was still found to be an over estimate of the total yield due to sampling error. Commercial yields have always been significantly lower than our estimates. Some difference was expected as controlled trials are always better than farm yields but there was clearly an overestimate in the trials.

Therefore for the single cross 1062 the whole plot was evaluated rather than using a sampling method. This indicated that the plot yield was 4.5kg/plot dried leaf rather than 7.28kg/plot. As we expected sampling by hand did not take sufficient of the large woody material and shredding the whole plot before sampling caused a high proportion of sample contamination with the woody material. Therefore further work is required to find a better estimate of total leaf yield or a proxy measure. However the current methods still allows a comparison between the crosses evaluated though we have not done any statistical analysis on these. The higher % Artemisinin crosses

appear to have lower leaf yields (Table 11) yet the visual scores for the plots were the same. Clearly the economic value of any new varieties will be a balance between leaf yield and % content.

Table 11. Leaf yield and % Artemisinin results from the crosses tested in 2009

Cross	Parents	Leaf yield kg/plot	Mean % Artemisinin
1062	1001-9 x 1046-7	7.28	1.66
	1.432 x 1.542		
1104	1001-9 x 1062-4	6.85	1.75
	1.432 x 1.971		
1105	1001-9 x 1053-1	7.42	1.65
	1.432 x 1.766		
1111	1046-5 x 1062-4	6.54	1.73
	1.542 x 1.971		
1114	1046-7 x 1053-4	6.89	1.72
	1.542 x 1.534		
1118	1053-1 x 1062-1	6.87	1.72
	1.766 x 2.118		

If it is assumed that the lower leaf yield of 4.5 kg/plot would equate to 2.5 t/ha. The leaf yields 1.66% Artemisinin and therefore this would be 41.5 kg/ha. The extraction percentage is currently approximately 50% which would deliver 20.75 kg/ha of commercial product. Currently the price of atremisinin is between $200 (2009) & $380 (2010) which would give a potential value of $4150/ha to $7885/ha. Even at 1t/ha of dried leaf the value would be $1660 and due to the projected world shortages the price per kg has been steadily increasing.

2.3.2.1 New crosses being made in 2009/10 for testing in 2010

The parents (Table 12) were selected to have the highest possible % Artemisinin content and known combining ability. Within the crosses there is a range of genetic distance between the parents (estimated relative to pedigree) and it was hoped this will inform the project further about the effects of inbreeding.

Table 12. Crosses being made at Humber VHB in 2009/10

Cross No	Parents		Parents
1	**1118-1**	**x**	**1105-3**
	2.55 % Art		2.36 % Art
2	**1118-1**	**x**	**1062-3**
	2.55 % Art		2.15 % Art
3	**1118-1**	**x**	**1118-5**
	2.55 % Art		2.08 % Art
4	**1118-1**	**x**	**1107-5**
	2.55 % Art		2.01 % Art
5	**1105-3**	**x**	**1062-3**
	2.36 % Art		2.15 % Art
6	**1105-3**	**x**	**1105-8**
	2.36 % Art		2.11 % Art
7	**1105-3**	**x**	**1107-5**
	2.36 % Art		2.01 % Art
8	**1046-7-3**	**x**	**1001-9-3**
	2.14 % Art		2.22 % Art
9	**1001-9-3**	**x**	**1015-1**
	2.22 % Art		2.09 % Art

2.3.3 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 achieved.

The best method for the selection of new parents has been further verified. Parent material, with greater than 2% Artemisinin content, has been developed for the first time. The new parents have been crossed for evaluation in 2010.

2.4 Work package 3; Agronomy and seed production led by Humber VHB

2.4.1 Optimisation of biomass production and Artemisinin yield in response to variation in nutritional supply for pot grown plants (EMR)

In years 2 and 3, the consortium concluded that EMR work should focus mainly on the optimisation of biomass production and Artemisinin yield in response to variation in nutritional supply for pot grown plants.

In 2007, we concentrated on the effect that nitrogen and potassium has on the biomass and Artemisinin yield in *A. annua* (see annual report 2007). In 2008, the effect that phosphorus and boron had on plant biomass and Artemisinin yield been assessed (see annual report 2008). Boron supplied, in the 2008 study, at the highest concentration (0.6 mg L^{-1}) was found to increase Artemisinin concentration and yield in leaves of *A. annua* plants, but the effect of increasing boron above this concentration was not tested. In 2009 due to the predicted decline in allocated income, to EMR, no experiments were costed, or planed. However, as with earlier years (i.e. plant breeding) EMR undertook an additional objective not covered within the project (and therefore no associated project milestone). A controlled environment study was undertaken to determine if increasing the supply concentration of boron, above that used in the earlier experiment, would further increase Artemisinin concentration and yield. Due to the delay and late supply of seed material from the partners this experiment was not started until late summer when natural radiation levels would have been on the decline. To avoid low radiation levels, air temperatures, and growth rates it was decided by EMR that the most satisfactory approach, despite the issue of not being able to compare the results easily with the previous experiment, to undertake a nutrient growth study within a controlled environment, rather than under natural summer growth conditions outside. This new objective was carried out within EMR GroDome (Unigro Ltd.) containment facilities. This is a state-of-the-art controlled environment (containment) facility which offers the means to optimise radiation capture and subsequent plant growth rates (temperature control). Despite the different location, the experimental format was generally similar to previous nutrition experiments carried out at EMR within this project.

2.4.1.1 Materials and methods

Seeds of *A. annua*, accession number NIAB 1053 (cross of 1001-1 × 1015, with seed from the 1015 parent only), were supplied to EMR by Humber VHB. The seed was sown, immediately, on 16 July 2009 thinly into trays of potting compost, watered and covered with glass to retain moisture. On 27 July 2009, approximately 400 seedlings were pricked out and potted into trays of modules, each

module being 2.5 cm × 2.5 cm and 3 cm deep. On 26 August, 100 of the seedlings were potted up into 7.5 litre pots using Sinclair medium Irish graded peat with no added nutrients, to which 1.9 g of CaCO3 per litre (peat) was added to raise the pH to approximately 5.7. Plants were placed into an unheated glasshouse for 9 days to establish. On 4 September 2009, 72 of the best plants were selected (above ground uniformity) and placed onto two free-draining benches within the GroDome facility at EMR. Plants were spaced 50 cm apart and each bench had 12 rows of plants with 3 plants per row (Figure15). Day time temperature (06:00 -21:00 h) within the compartment was set at 22 ºC and night time temperature (21:00 – 06:00 h) was set at 17 ºC. On 30 September, supplementary lighting, provided by 8 sodium lights (400 Watts), was switched on for 4 hours per day to extend daylight hours, to ensure they received 14 hours of light per day.

a) b)

Figure 15. A view of part of the Artemisia annua in the gro-dome at EMR in 2009 a) at the start and b) at the end of the experiment.

Three treatments were applied as follows: Stock solutions were made up that provided B at concentrations of 0.1 mg/L (B1), 0.6 mg/L (B2) and 0.9 mg/L (B3), when diluted with water in a ratio of 1:100. The interim '0.6 mg/L treatment' in this experiment was the highest concentration used in the previous experiment reported in 2009. Different concentrations of boric acid were used to alter the concentration of B. The stock solution provided N at a concentration of 101 mg/L and K at 156 mg/L (found to be the optimal levels from 2007 experiment), P at 40 mg/L, Ca at 80 mg/L, Na at 33 mg/L, Zn at 0.1 mg/L, S at 113 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.

The experimental design was that of a randomised complete block: three treatments × 8 blocks, each plot contained three plants. Total number of plants was 72 (3 × 8 × 3). Each pot received the fertigation solution via one 2 L/h dripper. Fertigation started on the 4 September 2009. The pots were checked on a regular basis (weekly), by randomly lifting a number of pots 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 in detail. Plant heights were measured fortnightly from the start to the end of the experiment. The plants were harvested on the 9 November 2009. All leaves and side shoots were removed from the main stem of each of the three plants in a plot and the plant material bulked together. The fresh weight of this portion of the plant was recorded. The main leader stems of the three plants in a plot were also bulked together and fresh weight recorded. The plant material was then placed into drying trays and placed into a drying oven at 40-42 °C and dried for at least 48 h.

The leaf and side shoot plant material, when dry, was rubbed through a sieve of 5 mm mesh size, thereby removing the leaf (lamina) material from the stem. The dry weight of main stem, side shoots and leaf material were recorded separately. The leaf and stems were bagged up separately into poly grip bags and samples of the leaf material (24 in total) were sent to De Montford University for Artemisinin analysis and Natural Resources Management Limited, NRM, for leaf B mineral analysis.

2.4.1.2 Results

Plants of *A. annua* grew well within the GroDome environment particularly given the time of year and natural radiations levels. There were no differences in cumulative growth of *A. annua* in terms of height between the three treatments (Figure 16) over the time period of the application of the treatments. Plants receiving boron at concentrations of 0.1 mg/L had a slightly higher leaf biomass than the other treatments, whilst stem biomass and the total biomass did not differ between the three treatments (Figure 17). Pictures taken at the time of harvest show that plants receiving boron at different levels are similar in height and bushiness (branching and lamina development) across all treatments (Figure 18).

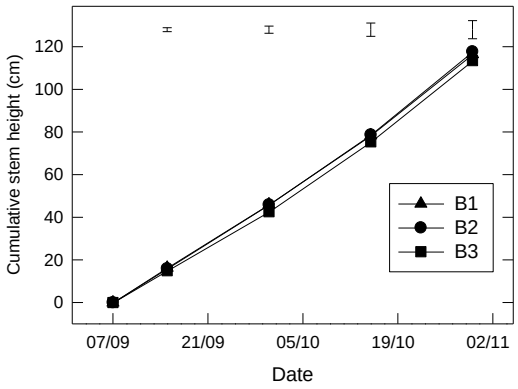

Figure 16. Stem growth of Artemisia annua when supplied with differing concentrations of boron (B1=0.1 mg/L, B2=0.6 mg/L, B3=0.9 mg/L)

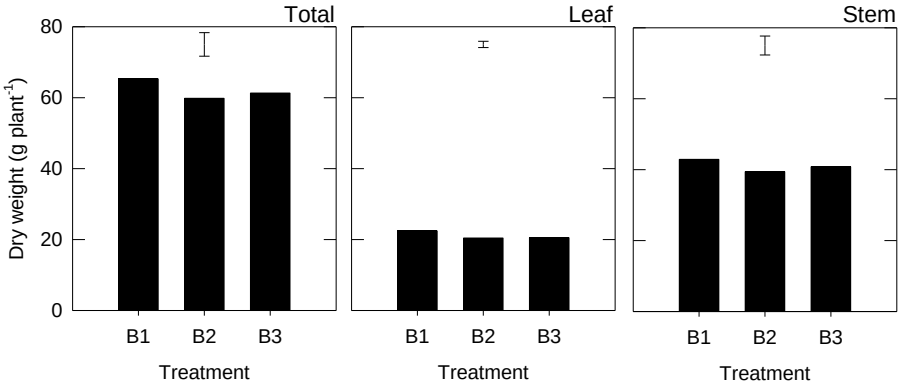

Figure 17. Total plant dry weight, leaf dry weight and stem dry weight of Artemisia annua plants when supplied with differing concentrations of boron (B1=0.1 mg/L, B2=0.6 mg/L, B3=0.9 mg/L)

Figure 18. Pictures showing the effects of application of different concentrations of boron on growth of Artemisia annua plants boron (B1=0.1 mg/L, B2=0.6 mg/L, B3=0.9 mg/L).

Increasing the boron supplied to *A. annua* plants from a concentration of 0.1 mg/L (B1) to 0.6 mg/L (B2) increased the B concentration of the leaves from 112 to 133 mg/kg, increasing the concentration of boron supplied to the plants to 0.9 mg/L (B3), only increased the concentration in the leaves to 139 mg/kg, which was not significantly higher than those receiving 0.6 mg/l (Figure 19). The total amount of boron (boron concentration × leaf dry weight) found in the plant leaf biomass of *A. annua* increased with each successive increase in boron application from a low of 2.50 mg/plant found in plants supplied with boron at a concentration of 0.1 mg/L (B1) to a high of 2.85 mg/plant for those supplied with boron at 0.9 mg/L (B3), however, this effect was not quite statistically significant (F.pr = 0.052). The total amount of boron supplied to each plant via irrigation was 2.09, 12.54, 18.81 mg for B1, B2, and B3 respectively.

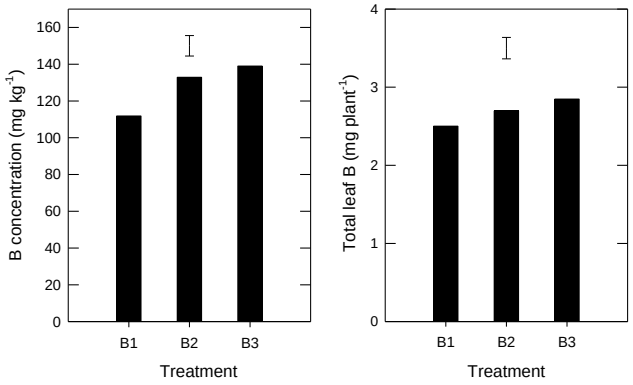

Figure 19. Boron concentration in leaves and total leaf boron amount per plant (potassium concentration × leaf dry weight) at harvest for Artemisia annua plants when supplied with differing levels of boron (B1=0.1 mg/L, B2=0.3 mg/L, B3=0.6 mg/L)

Artemisinin concentration in leaves of the *A. annua* plants was highest for plants receiving B at concentrations of 0.6 and 0.9 mg/L, with Artemisinin values of 0.88 and 0.89 % w/w respectively (Figure 20). Those receiving the lower level of B (0.1 mg/L), had a reduced Artemisinin concentration of 0.78 % w/w, however, statistically this was not significantly lower than the other treatments (P=0.095). The total yield of Artemisinin (Artemisinin concentration × leaf biomass) did not increase significantly with increasing boron application.

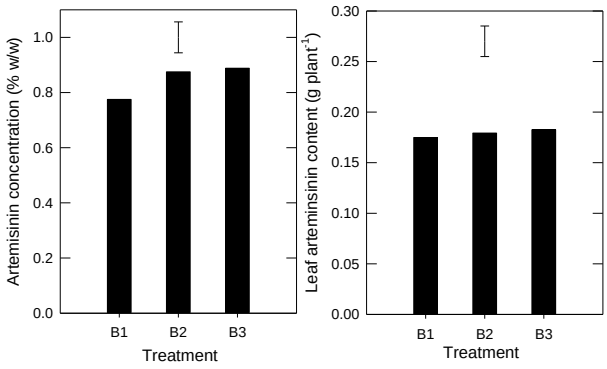

Figure 20. Artemisinin concentration in leaves and total Artemisinin content of leaves (mineral concentration × leaf dry weight) at harvest for Artemisia annua plants when supplied with differing levels of boron (B1=0.1 mg/L, B2=0.6 mg/L, B3=0.9 mg/L)

2.4.1.3 Conclusions

Boron concentration in the leaves of plants was increased as boron supplied increased from 0.1 mg/L to 0.6 mg/L, increasing the concentration supplied to the plants above this did not increase boron concentration in the leaves any further. This suggests that the uptake/demand for boron at the higher rates of applications (0.6 and 0.9 mg/L) was saturated.

- Application of boron across the range evaluated had no significant effect on *A. annua* growth rate or total plant biomass production.
- Artemisinin concentration in the leaves of *A. annua* did not increase when boron application increased above 0.6 mg/L (although there was an increase in Artemisinin concentration between plants receiving 0.1 and 0.6 mg/L, this was just outside the 5% significance level).

The total yield of Artemisinin was not increased when boron application was raised above 0.6 mg/L
Increasing the concentration of boron in irrigation water above 0.6 mg/L does not have any effect on Artemisinin concentration or Artemisinin yield.

2.4.2 Comparison with 2008 experiment

As already suggested above, the experiment conducted in 2009 was a compromise with respect to the extent with which it could be compared with work undertaken in 2008. Clearly, the growing environments were very different, despite the plant material coming from the same supply source. The follow points outline what comparisons could and could not be made.

Whilst plants reached a similar height in the 2008 and 2009 experiment, those in 2008 had total plant dry weight more than double from those in 2009. Also, the proportion of leaf to stem biomass was reduced in 2009 (leaf:stem ratio 1:1.95) compared to 2008 (1:1.3).

- Total amount of boron in leaves of plants were only 2.5 and 2.7 mg/plant (0.1 and 0.6 mg/L respectively) in 2009, compared to 4.8 and 8 in 2009. This is likely due to the reduction in leaf dry matter at harvest date in 2009.
- The amount of boron supplied to the plants in the treatments receiving 0.1 mg/L and 0.6 mg/L was about 2.5 x that of the previous year. Boron concentration in the leaves of plants receiving 0.6 mg/L was similar in both years (136 mg/kg to 133 mg/kg in 2008 and 2009 respectively). Those receiving B at 0.1 mg/L had higher boron concentrations in leaves (111 mg/kg) in the 2009 experiment than compared to the 2008 experiment (88 mg/kg).
- Leaf Artemisinin concentrations increased in 2009 compared to 2008, for 0.1 mg/L in 2009 the concentration was 0.78 % (w/w) compared to 0.65 in 2008 and for 0.6 mg/L in 2009 the concentration was 0.88 % (w/w) compared to 0.78 in 2008. Due to lower leaf dry matter allocation per plant, the Artemisinin yield in 2009 was however lower for 0.1 mg/L and 0.6 mg/L plants (0.17 and 0.18 g/plant respectively) than for the plants in 2008 (0.36 and 0.46 g/plant respectively). Different pattern in dry matter allocation are often apparent when plants are grown under even partial artificial illumination. So this type of change would have been predicted from the GroDome experiment.

2.4.3 Seed production – led by Humber VHB

Validation of the growing protocol previously developed for “1053” was tested on this new line in 2008 in a pilot seed production unit (Figure 21). Eight-week-old mother plants were used to supply cuttings which in turn were ready for entry into short-days after 5 weeks under 10klux lighting. Alternate rows of each parent were placed on benches. Flowering occurred after 4 weeks each clone within a few days of the other. Plants were then shaken regularly to disperse pollen. After a period of drying out, the plants were harvested after a further 11 weeks. The process from mothers to harvest took 28 weeks.

On this larger scale more than 30 g of seed per plant was harvested and confirmed the preliminary yield conjecture that the 7.5 g/plant target could be considerably improved upon. Seeds were again small, but viability was excellent with more than 97% germination. The combination of this improved yield, arrangement with fewer plants and exploitation of summer energy economies greatly improves the economic viability of this aspect of the project. This validation step provided more than 28 kg of seed (560 million seeds) for exploitation.

Figure 21. Pilot seed production unit.

2.4.3.1 Tertiary Breeding Material

Clonal material from 2009 field trials was received in December. These nine clones were available only in low numbers but will be tested for timing of flowering and compatibility within selected crosses in the spring of 2010. It is hoped that crops from resulting seed, to be field tested in 2010, will be capable of delivering in excess of 2% Artemisinin.

2.4.3.2 Library Plants

A library of clones has been maintained since 2006. As new material included, the older lines deemed no longer to have potential within the breeding programme have been discarded. As of December 2009, the library consisted of 16 clones maintained in 24h and low temperatures. Plants are periodically trimmed with regeneration as cuttings 2-3 times a year.

2.4.3.3 Exploitation

A sample of seed was grown on an associate farm in Morocco in 2008. The plants were planted as modules at 1m spacing in January and given fertigation until harvested in May. The large plot yielded the equivalent of 5 tonne per ha of dried stripped foliage. Despite it having a low Artemisinin content (0.5%) the Artemisinin yield equivalent was 25 kg/ha. This low content was presumably related to total crop bulk and not to excess nitrogen as N-content (3.5%) was not high in comparison to concentrations found in the EMR nutrition studies. With higher content lines on the horizon there is potential to exploit the intermediary (“1062”) material world-wide. To this end samples have been despatched to major players in Africa and Canada in spring of 2009 and to China, Madagascar, India and Africa in autumn 2009. It is not known how this material will perform in differing environments although the first indications have been favourable. It is also anticipated that

considerable trialling may be necessary for these producers to build confidence in the consortium as a seed supplier.

2.5 Work package 4; Harvesting, product stabilisation and secondary products; led by Frontier Agriculture

2.5.1 Field planting of A. annua *in 2009 by Frontier*

In 2007 and 2008 the establishment of commercial crops was found to be very variable. There was considerable risk due to the weather conditions at establishment. It was therefore concluded that in 2009 further plot development was required. Therefore Frontier worked closely in 2009 with NIAB on the techniques for commercial production.

Significant effort was made to establish trials at higher seed rates (x1, x2, & x4) at NIAB in Cambridge using irrigation (Figure 22 and Figure 23). An additional trial was also established at Seale Hayne in Devon early in the year but with no irrigation. It was hoped that both the higher seed rate and irrigation would overcome the establishment problems previously experienced.

Figure 22. Crop drilled 31 March, photograph taken on18 June.

Figure 23. Crop drilled on 18 May 2009, photograph taken on the 18 June.

However all of the trials failed to establish. Only a few plants were able to establish even though the seed was known to have been of high quality. The dry spring conditions of 2009 resulted in the worst establishment we have seen throughout this project despite irrigation.

Soil moisture and sowing depth are known to be critical which means that establishment of this crop in the spring will always be a risk. However we have found that the crop can be established over winter if the seed is put on the surface of a stale seed bed. Figure 24 and Figure 25 show seedlings establishing from a sowing on the 24/12/2008. It can be seen from Figure 24 that the soil surface is very wet and generally remains wet during the winter months.

Figure 24. Hand sown on 24 December, photograph taken 16 January

Figure 25. Hand sown 24 December, picture taken 22 April.

These early established plants have the advantage of achieving significant amount of biomass by mid June at a time when most of our experimental material was only just beginning to get established.

2.5.1.1 Summary

Establishment is possible in May and June but for farmers there is an unacceptably high risk of failure. However reliable establishment is possible during the winter period and has the added benefit of allowing an earlier harvest.

3 Commercial context (Botanical Developments Limited: Colin Hill)

3.1 Artemisinin Market and the impact of new high yielding *Artemisia annua* hybrids

The Artemisinin market is a relatively new one with only ten years of history. It has developed both a spot market and a contract market. Figure 26 below shows how the price on the spot market has fluctuated over the past ten years with a high price of US$1100/kg in 2006 and a low of US200/kg in 2009 reflecting the changes in the supply and demand. The contract market has been more stable and has ranged only from US$300/kg to US$450/kg. Current spot market price (February 2010) was US380/kg and long term contract price is US$350/kg.

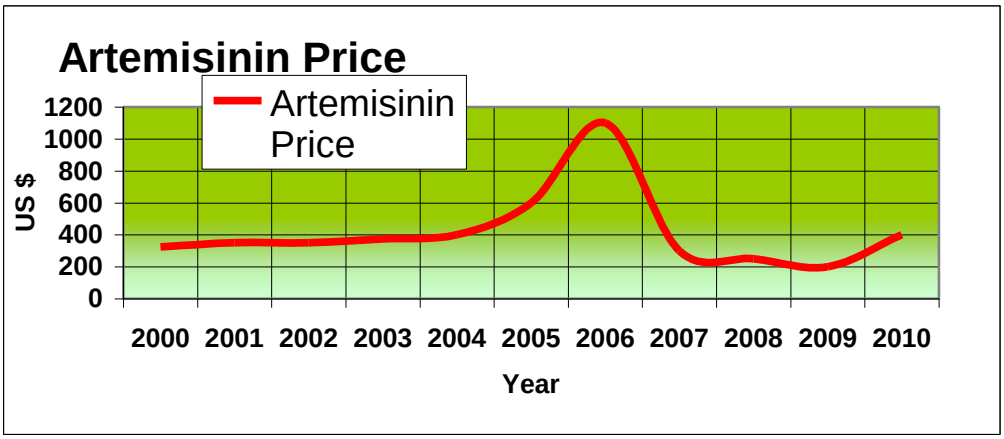

Figure 26. Artemisinin Spot price 2000-2010

Real demand for Artemisinin commenced in 2004 when 30 countries adopted Artemisinin based medicines (ACT's) as the mainline treatment for multi drug resistant malaria. This rapid increase in demand resulted in the very high spot price and also in a rapid expansion of Artemisia growing particular in China. This increase in growing led to a surplus of Artemisinin in the supply chain and prices fell quite quickly to a low in 2009. During this period another 70 countries have adopted Artemisinin based medicines as their mainline treatment and demand has continued to increase. It is now predicted 275 million treatments will be required by 2014 (twice those ordered for 2010) (Figure 27). It is anticipated all strategic stocks of Artemisinin in the supply chain will be consumed during 2010 and the 10,000 hectares being grown during 2010 (producing 60 tons of Artemisinin) will be insufficient to meet the demand for treatments in 2011 (requiring 150 tons of Artemisinin).

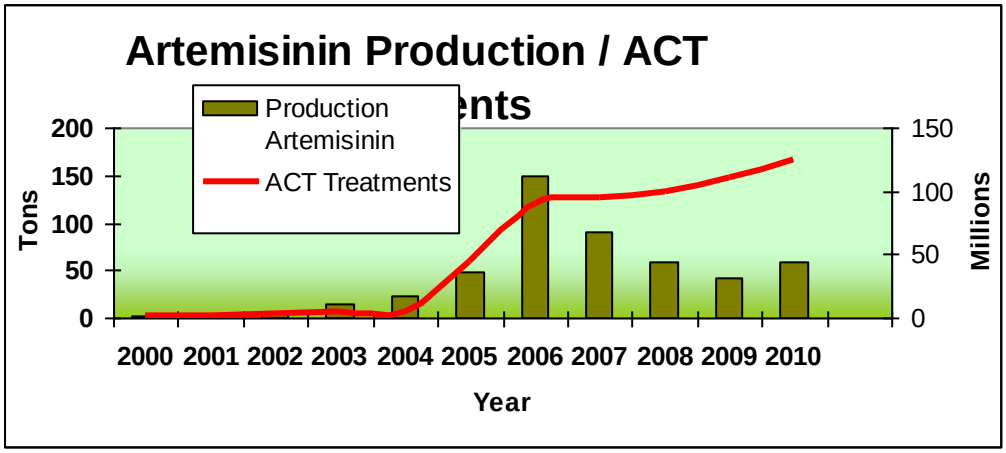

Figure 27. Artemisinin production (tons) and number of Artemisinin treatments (millions) 2000-2010

Demand for Artemisinin based medicines is predicted to peak in 2014/5 (Figure 27) when the mid prediction of all agencies believes 275 million treatments will be required to be supplied. After 2015 demand is expected to fall slightly as a result of parallel policies of supplying bed nets and room spraying beginning to become increasingly effective.

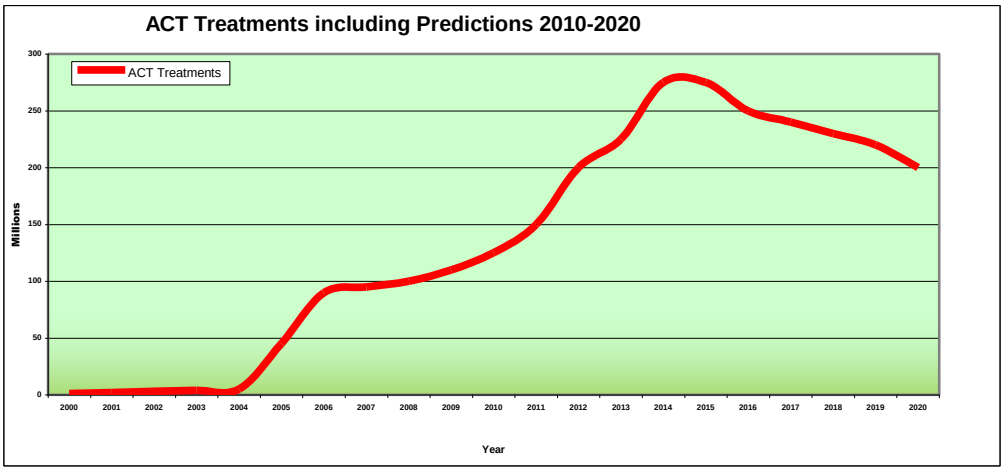

Figure 27. Historic demand and predictions of demand to 2020

The anticipated shortage in supply has already had an impact on the spot price of Artemisinin which has increased from US$200/kg (June 2009) to US4380/kg (February 2010). All signs are the spot price will continue to increase and a return to prices in excess of US$1000 /kg cannot be ruled out. Whereas in 2006 where China was able to respond to changes in the market, the situation is not the same today as much of the required infrastructure to process the crop has disappeared over the past four years.

The new hybrids developed by NIAB over the past four years give a real opportunity for the UK to satisfy the shortfall in supply.

Table 13. Artemisinin yields per hectare of different cultivars

Leaf Yield Per Hectare	**Percentage Artemisinin in Leaf**				
	0.8% (1)	**1%**	**1.5% (2)**	**2%**	**2.5% (3)**
1000Kg	8	10	15	20	25
1500Kg	**12**	15	**22.5**	30	**37.5**
2000Kg	16	20	30	40	50
2500Kg	18	25	37.5	50	62.5
3000Kg	24	30	45	60	75

Note 1) Industry Average (source Artepal / FSC)
Note 2) NIAB line 1062
Note 3)NIAB new hybrids tested in 2009

4 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.

4.1 Artemisinin analysis and plant breeding approaches

Although absolute baseline separation was not achieved, it was demonstrated clearly that a high yield of Deoxyartemisinin-free Artemisinin can be produced using simple, inexpensive methods. Thus, using simple laboratory equipment, it has been demonstrated that it is possible to separate Artemisinin from the inactive contaminant Deoxyartemisinin. A two step column chromatography set-up was achieved: step 1, the two compounds were isolated from the other constituents of a crude extract, and step 2, the two compounds were pulled away from each other. For this last step, two modifications to the standard column chromatography set-up were required: (a) The use of silica with a particle size of 35 – 70 µm (as distinguished from the more usual 70 - 200 µm size) and (b) The use of flash chromatography.

The exploration of flavonoid profiles in *A. annua* began in 2007. Previous research had demonstrated the anti-cancer potential of methoxylated flavonoids (Kobayakawa *et al*, 2004), and it was thought that the presence of chrysosplenetin could add value to *A. annua* as a commercial crop – hence the interest in quantifying this compound in Consortium-grown material. It was during this quantification that the correlation with Artemisinin was noticed.

It was found that chrysosplenetin: Artemisinin ratios are constant only within batches (e.g., in material grown in one location during one summer, where material is harvested and dried in the same manner). In this case, the grower would have to determine the ratio for that batch early in the season – which could then be reliably presumed to remain constant. But the usefulness of such a system is fairly limited, and it is reasonable to suppose that the TLC densitometry system of Artemisinin quantification (described in HDC Year 3 Report) is a better means of quality assessment for those without access to LCMS.

4.2 Seed Production and Parentage

Since the project started, a consistent increase of the Artemisinin content of parent material was achieved each year. From the experiments so far, it was also shown that the % Artemisinin observed in plants can be affected by conditions after harvest. Additionally, since the success of this breeding programme has resulted in the need to maintain large numbers of parent lines, a system of micro-prop it was developed to maintain our best material. This has the advantage of providing security for our material and significant cost savings as it only requires propagation once a year.

Moreover, in 2009 new parents were selected from individually tested plants from within the best crosses as this had proven to be very successful in 2007. All 240 plants within the 3 best crosses tested in 2009 were sampled. After an initial analysis in early August the best plants were sampled a further 2 times to confirm the results. Within the crosses there was a range of genetic distance between the parents (estimated relative to pedigree) which will inform the project further about the effects of inbreeding.

4.3 Nutrition assay

In years 2 and 3, the consortium concluded that work should focus mainly on the optimisation of biomass production and Artemisinin yield in response to variation in nutritional supply for pot grown plants. The conclusions were as follows:

Boron concentration in the leaves of plants was increased as boron supplied increased from 0.1 mg/L to 0.6 mg/L, increasing the concentration supplied to the plants above this did not increase boron concentration in the leaves any further. This suggested that the uptake/demand for boron at the higher rates of applications (0.6 and 0.9 mg/L) was saturated.

- Application of boron across the range evaluated had no significant effect on *Artemisia* growth rate or total plant biomass production.
- Artemisinin concentration in the leaves of *Artemisia* did not increase when boron application increased above 0.6 mg/L (although there was an increase in Artemisinin concentration between plants receiving 0.1 and 0.6 mg/L, this was just outside the 5% significance level).

The total yield of Artemisinin was not increased when boron application was raised above 0.6 mg/L

Increasing the concentration of boron in irrigation water above 0.6 mg/L did not have any effect on Artemisinin concentration or Artemisinin yield.

4.4 Commercial cropping of *A. annua* in the UK

Significant effort was made to establish trials at higher seed rates, at NIAB in Cambridge, using irrigation. However all of the trials failed to establish. Soil moisture and sowing depth were

confirmed to be critical which means that establishment of this crop in the spring will always be a risk. However it was found that the crop can be established over winter if the seed is put on the surface of a stale seed bed. Establishment was possible in May and June. Additionally, reliable establishment was possible during the winter period which had the added benefit of allowing an earlier harvest.

4.5 Commercial context

As the extraction percentage was achieved to approximately 50%, the consortium could deliver 20.75Kg/ha of commercial product. Currently the price of atremisinin is between $200(2009) & $380(2010) which would give a potential value of $4150/ha to $7885/ha. However, even at 1t/ha of dried leaf the value would be $1660 and due to the projected world shortages the price per Kg has been steadily increasing.

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 very well; however there is always space for further improvements. Moreover, considerable effort has been being made, particularly from Frontier Agriculture, to tackle this aspect of the business. Invaluable input from farmers and vegetable growers through Frontier and seed production with Humber VHB has been achieved.

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

8.1 Appendix 1. Table of predicted means for aprent lines tested in 2009

Parent Number	Parent Breeding Codes		Mean % Artemisinin yield	Corrected for block effect Mean % Artemisinin yield
P1	1015		1.51	1.509
P2	1001-1		1.40	1.425
P3	1001-3		1.32	1.303
P4	1001-9		1.41	1.432
P5	1038-1		1.38	1.377
P6	1045-3		1.57	1.556
P7	1046-5		1.55	1.568
P8	1046-7		1.58	1.542
P9	1062-1		2.13	2.118
P10	1062-2		2.14	2.131
P11	1062-3		2.12	2.145
P12	1062-4		1.94	1.971
P13	1053-1		1.80	1.766
P14	1053-3		1.78	1.767
P15	1053-4		1.55	1.534
P16	535		1.66	1.693
P17	679		1.79	1.79
P18	697		1.89	1.873
P19	708		2.16	2.136
P20	182		2.10	2.085
P21	268		1.68	1.683
P22	585		2.00	1.97
P23	589		2.19	2.224
P24	636		1.91	1.974

REML analysis corrects the % yield of Artemisinin by taking into account the Block effects.

REML analysis for differences between varieties for Art_%_w_w

Response variate: Art_%_w_w
Fixed model: Constant + VTY
Random model: REP + BLK
Number of units: 96

Residual term has been added to model

Sparse algorithm with AI optimisation

Estimated variance components

Random term	component	s.e.
REP	0.00071	0.00169
BLK	0.00515	0.00564

Residual variance model

Term	Factor	Model(order)	Parameter	Estimate	s.e.
Residual		Identity	Sigma2	0.0319	0.00554

Tests for fixed effects

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
VTY	229.02	23	9.96	66.7	<0.001 *** (0.1%)

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
VTY	229.02	23	9.96	66.7	<0.001 *** (0.1%)

Message: denominator degrees of freedom for approximate F-tests are calculated using algebraic derivatives ignoring fixed/boundary/singular variance parameters.

Table of predicted means for Constant

1.774 Standard error: 0.0424

Standard errors of differences

Average:	0.1283
Maximum:	0.1347
Minimum:	0.1263

180 VLSD [PRINT=lsd; FACTORIAL=32; LSDLEVEL=5; SAVE=_remlsave]

Approximate least significant differences (5% level) of REML means

Average:	0.2535
Maximum:	0.2625
Minimum:	0.2521