IN VITRO AND *IN VIVO* PRODUCTION OF ARTEMISININ BY *ARTEMISIA* SPECIES

By

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DECLARATION

I declare that this dissertation, for the degree of MASTERS IN SCIENCE (Medicinal Plant Science), has never been submitted for any degree at any university. The research work reported is the results of my own original investigation, except where acknowledged.

FRANCOIS JACOBUS LIEBENBERG KRUGER **DATE**

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Abstract

Artemisinin is produced in the leaves of *Artemisia annua* and is currently one of the most valuable antimalarial treatments. *A. annua* is of Asian origin but many other family members have been identified worldwide. *A. annua* however, is the only one that produces artemisinin. Synthetic production of artemisinin is not yet feasible, not to mention very expensive and the product yields are relatively low. The aims of this study were threefold: 1) To regenerate callus, cell cultures and plants from genetically modified root cultures of *A. afra* into which an artemisinin biosynthetic gene was inserted from *A. annua* 2) To investigate the probability that fungal endophytes are responsible for the production of artemisinin and 3) To establish two fields of high yielding varieties of *A. annua* plants and evaluate whether artemisinin production of these two locations will remain high.

Callus and cell cultures of the genetically modified *A. afra* root cultures were established, but no shoots have been produced as of yet and this is an on-going investigation. Fungal endophytes were sampled and none of the endophytes produced artemisinin. Five different lines of *A. annua* were cultivated, successfully grown and harvested. Measurements were taken at different stages of processing, these were compared and analysed using various methods such as height and mass comparisons. Comparisons revealed that the production of artemisinin is correlated to local sets of conditions rather than the variety of individual lines. The genetic potential to produce high quantities of artemisinin appears to have been lost, instead of being maintained. We confirmed that secondary compound production and specifically, artemisinin, is enhanced by certain stress factors on the plants.

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 *Artemisia annua* background

Artemisia annua L. is commonly known as sweet wormwood, sagewort, sweet Annie or Qinghaosu (Figure1.1). Due to the importance of *A. annua* it has been distributed from its Asian origin across the world and much cultivation has been attempted (Ferreira *et al.,* 1997). There are about 200 described species in the genus, *Artemisia*. The African family member known as *A. afra,* is found in South Africa and other regions of Africa up to Ethiopia (Van Wyk *et al.,* 1997). *A. afra* is also known in isiZulu as Umhlonyane. Most species of *Artemisia* have medicinal value in certain cultures and share the same bitter taste of which many stories and expressions have been told. *A. absynthia* is infamous for its powerful hallucinogenic properties in a drink known as, Absynth. This drink contains the detrimental compound thujone and has been banned in many countries outside of Europe preceding the 20th century (Silbernagel *et al.,* 1990). The levels of this compound are however neglectable in aqueous extracts, but treatment using this or any of the other related species for longer than three weeks, is not advised. In addition there is a naturalized American relative called *A. vulgaris* that is often confused with another medicinal plant, *Saint John's wort,* because of its common name, Saint John's plant (Wright, 2004).

A. annua is an aromatic annual herb which grows vigorously and can reach heights of up to three metres. The African relative is a perennial shrub which seldom reaches heights of over two metres and is usually found in groups. Both species of plants produce one main stem growing upwards but these stems however, can be replaced if the main growth points have been damaged. Thereafter other stems will develop from branching and continue with upward growth until the maximum allowed height is reached (Ferreira *et al.,* 1997; Van Wyk *et al.,* 1997).

A new market has developed around the *Artemisia* genus pertaining to the aromatic/ volatile compounds being produced and there are current investigations into the perennials for the production of essential oils for various products and consumables, including perfumes and scents (Graven*et al.,*1990).

Figure 1.1: *Artemisia annua*

The seeds are extremely small and oval shaped (Figure 1.2). They are carried on long inflorescent axes and dropped when ripe. The seeds have been found to stay viable for up to three years if kept in a cool, dry environment (Ferreira *et al.,* 1997). A high degree of similarity exists between *A. afra* and *A. annua* species with regard to the morphology of the stems, seeds, flowers and leaves.

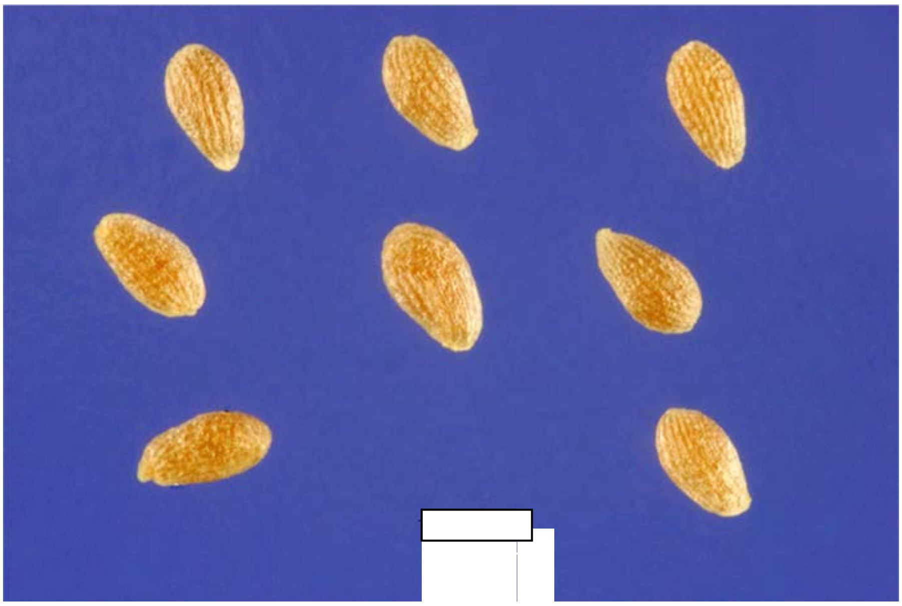

Figure 1.2: Seeds of *Artemisia annua* (Hurst, unknown)

Seeds are produced from the flowers (Figure 1.3) which are small, yellow and carried in green panicles. The florets are bisexual and contain little nectar. Glandular trichomes are found in the corolla and receptacle florets (Ferreira *et al.,* 1997).

Figure 1.3: Flowers of *Artemisia annua* (Peters, 2007)

The leaves contain the trichomes that are associated with the production of artemisinin (Figure 1.4). The African *A. afra* and other related species do not contain artemisinin in their trichomes. Artemisinin has also been found to be produced in the flowers but these concentrations are relatively low. The leaves are fernlike and alternate spirally (Figure 1.5). The leaves are also responsible for the strong odour associated with the plant. This is because of the aromatic compounds contained in the leaves. The green, finely pinnately dissected leaves can reach sizes of up to five centimetres in length (Ferreira *et al.,* 1997). A clear vein is found down the centre of the leaf with slightly smaller veins branching from it.

Figure 1.4: Trichome found on *Artemisia annua* leaves

Figure 1.5: The leaves of *Artemisia annua*

The uses of *A. annua* and its African relative are quite diverse. *A. annua* has been used for more than 2000 years in Asia as a Chinese herbal medicine. The dried leaf material was cooked and then the solid particles were filtered leaving a tea-like drink. The substance was used to treat symptoms of malaria and different types of fevers, tuberculosis, jaundice, anxiety, constipation and as an antiseptic, anti-periodic and for digestive problems.

African wormwood has been used in ethnobiology to treat conditions such as colic, headaches, intestinal parasites, moth repellent and is used as an organic insecticidal spray (Watt *et al.,* 1964). The raw leaves are often put into the nose of a patient to treat congestion of the nasal cavities and similarly to relieve ear pain, hence the Afrikaans common name of 'oorpynhoudjie' directly translated as ear pain wood. Later, it was found that these plants may yet hold more potential in the treatment of cancers (Peng *et al.*, 2006).

1.2 Artemisinin

No other members of the Asteraceae family, even the closely related species like *A. afra* and *A. vulgaris* show any production of artemisinin. Artemisinin is produced from artemisinic acid via the mevalonate pathway. It then undergoes various forms of processing to yield the different derivatives that are used in various treatments such as Artemisinin-based Combination Therapy (ACT therapies) (Figure 1.6) (Meshnick, 2002).

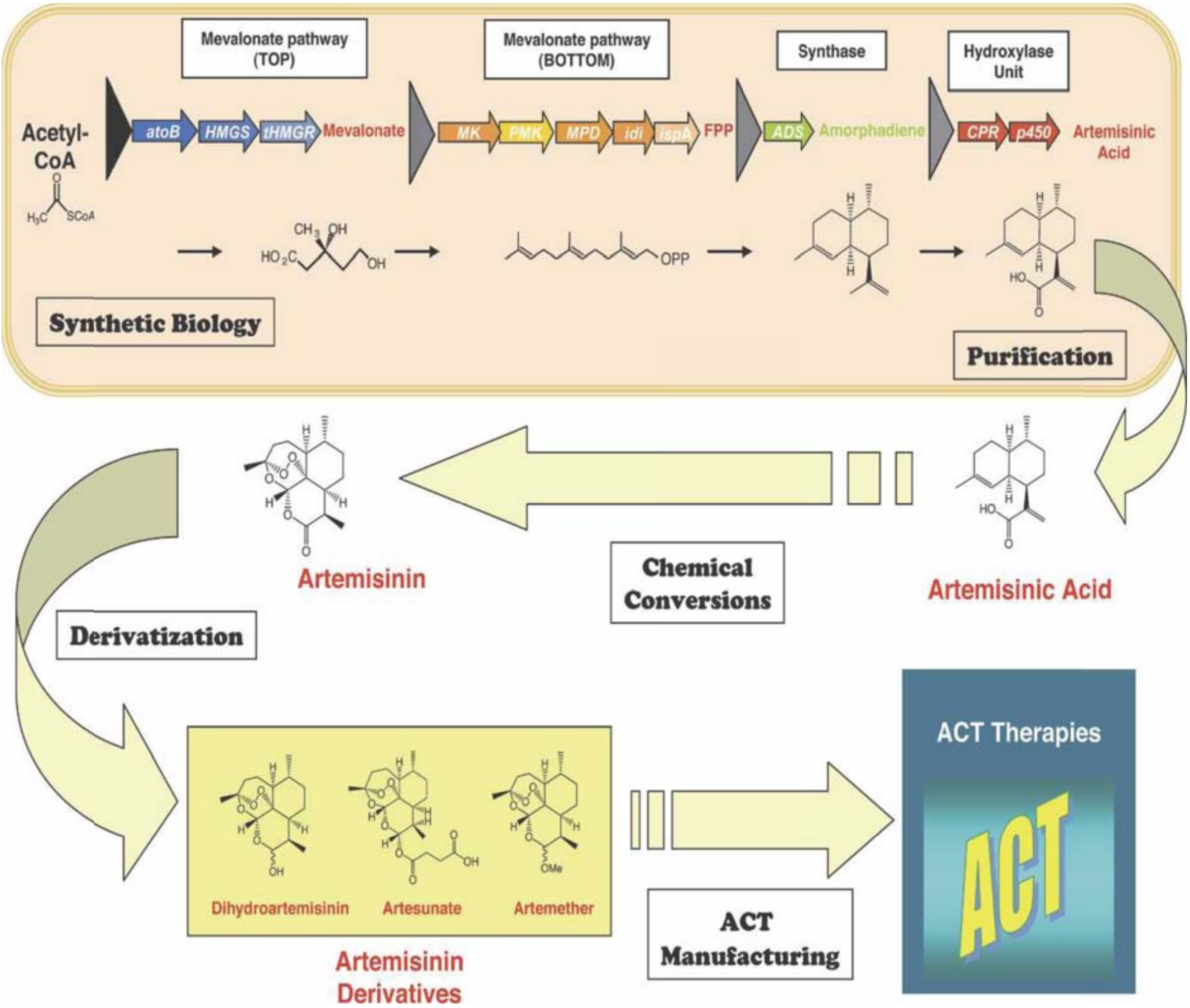

Figure 1.6: Production of ACT drugs (Hale *et al.,* 2007)

The best treatment to date comes from combinations of artemisinin and its derivatives with compounds derived from quinine. Monotherapy with just artemisinin showed recrudescence (treatment failures), and showed that combinations are more effective especially with parasites acquiring resistance to quinine. The combination of artenusate and chloroquine is a good example of this (Figures1.6 and 1.7) (Meshnick, 2002).

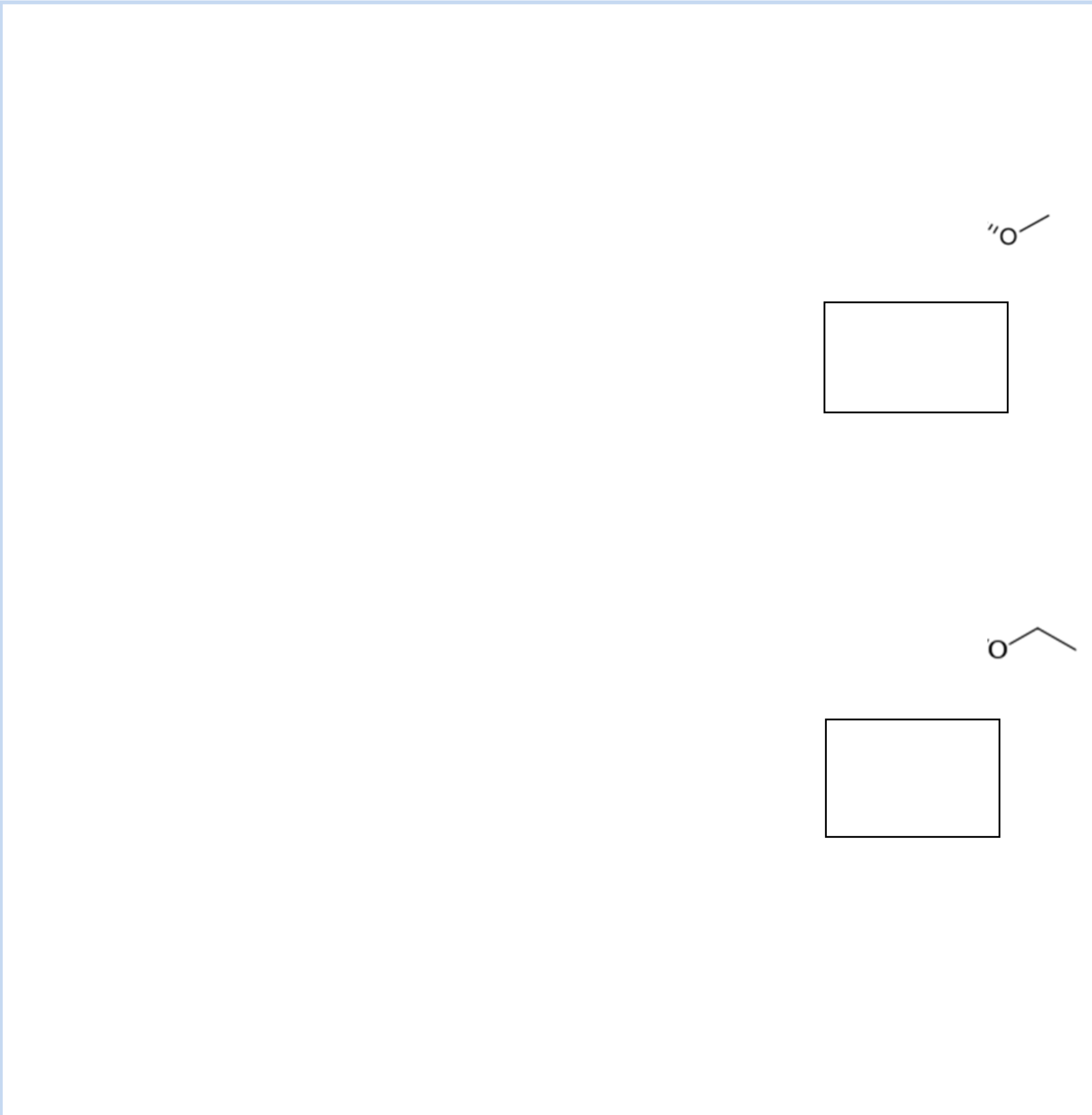

Figure 1.7: The chemical structures of artemisinin, its derivatives and chloroquine (Bengue and Bonnet-Delport, 2005)

Arterolane, also known as OZ-277 (Figure 1.8), is a synthetic compound derived from artemisinin which is being investigated and has passed a few of its preceding trial tests as well. This compound mimics the action of artemisinin and seems to be more efficient than pure artemisinin and can be used in combination with its derivatives. Further tests have however produced some contradicting results which lead to a reduction in research funding (Vennerstrom *et al.*, 2004).

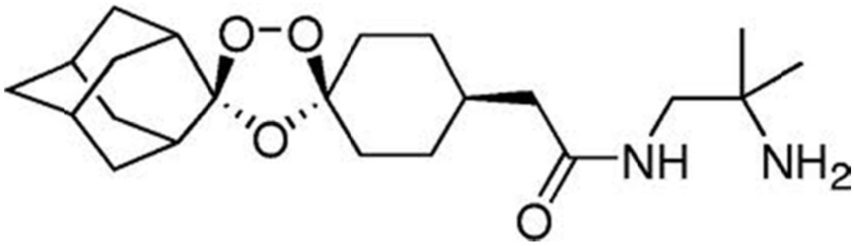

Figure 1.8: The chemical structure of OZ-277 (Kreidenweiss *et al.,* 2006)

1.3 Malaria

Malaria is one of the largest killers in the world, however the worst effects are substantially evident in Africa. The spread of AIDS in sub-Saharan Africa might worsen the casualties caused by malaria. This disease kills between one million and three million people per year. Malaria is a parasitic disease that originates from four different species, *Plasmodium vivax, P. ovale, P. malariae* of which *P. falciparum* is the most severe and linked to the most deaths usually associated with cerebral malaria (Snow *et al*., 2005).

This protozoan uses a mosquito (Figure 1.9) as its vector and thus many treatments have been developed to attempt to eradicate this mosquito. Chemical treatments and sprays have been used in areas where nets and preventative medication is difficult to obtain. South Africa underwent large scale projects in which dichlorodiphenyltrichloroethane(DDT) was used to treat sensitive areas in the past but this met opposition from environmental groups. Due to the remoteness of some of the areas where these mosquitoes are prevalent it is almost impossible to eradicate them (Snow *et al.,* 2005).

Figure 1.9: *Anopheles albimanus* mosquito feeding on a human arm (Vickers, 2006)

Malaria is transferred to humans through the blood when the mosquitoes feed. The parasite then distributes itself and targets the liver of humans. Sporozoites migrate to the liver where they then multiply to merozoites. These cells in turn rupture the liver cells and re-enter the bloodstream. Further development leads to trophozoites and schizonts which in turn produce further merozoites (Figure 1.10)(Strum *et al.,* 2006).

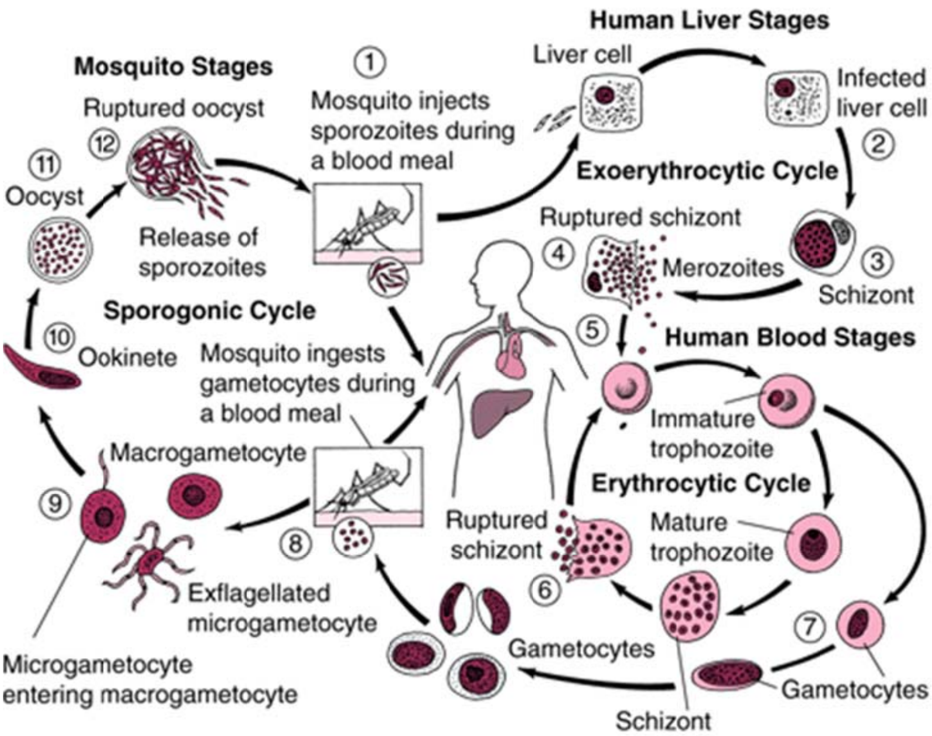

Figure 1.10: Lifecycle of malaria (Pearson, 2009)

Treatment against malaria gained more attention as an inoculation has not yet been successfully developed. Many treatments have originated from Asia. The two compounds that have been tried and tested are quinine and artemisinin. Some strains of the protozoa have shown increased resistance to quinine (Figure 1.11). Due to the rapid action and metabolism of the artemisinin derivatives in humans, the resistance to artemisinin and its derivatives are less likely to occur. Another confounding factor is the cost of production of these compounds that place them out of the financial reach of most sufferers (Wellems, 2002).

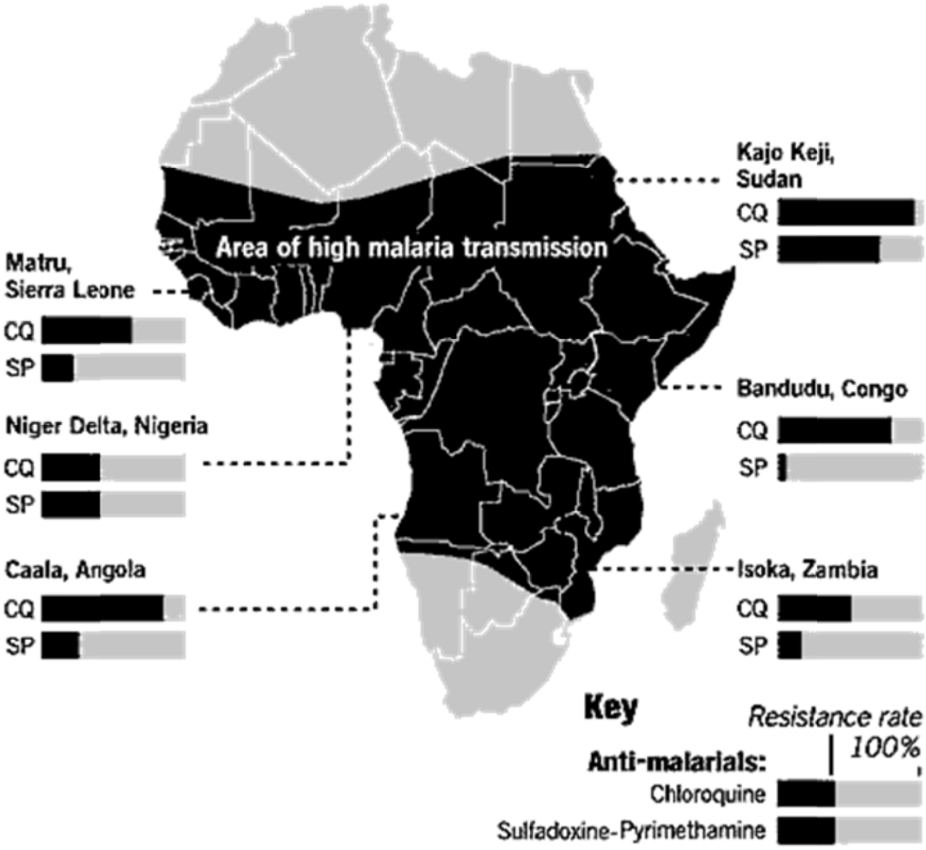

Figure 1.11: Areas in Africa affected by malaria and showing resistance to drug treatments (McNeij Jnr, 2004)

1.4 Artemisinin mode of action

The action of artemisinin against malaria appears to be related to the heme-mediated decomposition of the endoperoxide bridge. This produces free radicals with carbon centres. Heme is the iron and pigment containing part of haemoglobin while haemoglobin is the protein part of erythrocytes (red blood cells), where the malaria parasite is found, thus this is the target point for the action of artemisinin and its derivatives (Meshnick, 2002).

In comparison with other medication used for the treatment of malaria, artemisinin has a few additional advantages. The parasites responsible for malaria have acquired certain levels of resistance to most of the treatments of quinine-related drugs which used to be the leader in treatment against this epidemic. That is why research has switched to alternative medicines and alternative treatments (Cocquyt *et al*., 2011).

Artemisinin is readily taken up by the human system. It quickly spreads through the body binding to the parasite and its remnants, disabling them and leading to cell death. The dead cells are then removed from the system. This process happens with sufficient speed to prevent the parasite from building up resistance to the treatment in the body (Cocquyt *et al*., 2011).

There are however debates as to which mechanism is used and what the reason is for the rapid action (Figure 1.12). One theory is the potent protein alkylation ability of artemisinin. This alkylation of a protein molecule then leads to plasmodium death via another debated pathway (O'Neil and Paul, 2010). Another hypothesis is that there is interference with the endoplasmic/sarcoplasmic proteins and a third is damage to the normal mitochondrial functions of the plasmodium cells (Li and Zhou, 2010).

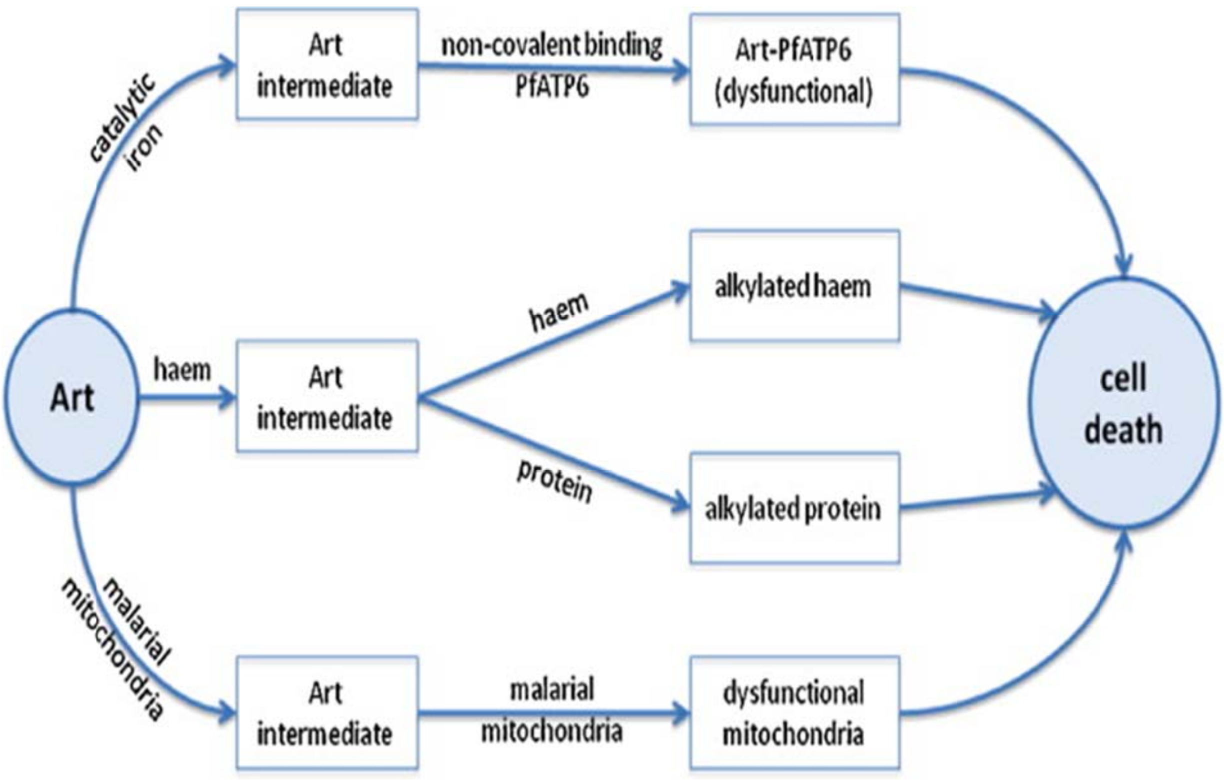

Figure 1.12: Proposed mechanisms of action of artemisinin leading to parasite death (Li and Zhou, 2010)

1.5 Extraction of Artemisinin

With the discovery of artemisinin and its antimalarial effects the next step was the synthetic production of this compound, but as of yet artemisinin has not been successfully synthesised to completion, although some of its precursors have been created chemically. Many treatments for malaria are simple and natural, making use of the combinational therapy concept. They usually use one to two heaped dining spoons of dried, finely ground, leaf material in combination with a litre of water as a day's treatment in the form of a drink or tea. This treatment then has to be taken for at least ten consecutive days to try and eradicate the parasite from the system (Wright *et al.,* 2002).

Extraction of artemisinin has mostly been done by hexane, but many other methods have since been investigated and developed. Each method has a number of advantages as well as drawbacks. While hexane is the cheapest, it is the least effective and is harmful to the environment (Lapkin*et al.,* 2006). Ethanol has almost been completely removed from the list of solvents because it is also dangerous and less effective. The tendency is to move away from flammable solvents and remove the chances of explosions during processing. Other solvents also being used are water, ethyl acetate and carbon dioxide (Lapkin*et al.,* 2006).

The market is tending towards an increase in the use of derivatives of artemisinin in combinational therapies and this too is putting pressure on the production of artemisinin as a whole. Artimether is created by reducing artemisinin with sodium borohydride to generate dihydroartemisinin and then treating it with methanol and an acid catalyst (Haynes and Vonwiller, 1994). In Table 1.1 the three main methods used to extract artemisinin with their relative efficacy, costs and environmental impact are discussed.

Table 1.1: Extraction methods and their characteristics (TechnoServe, 2004)

Extraction method	**Process efficiency (inc. solubility and selectivity)**	**Total capital and running costs**	**Environmental impact assessment**
Mixed liquid extraction ethyl acetate / n-hexane	Ethyl acetate has the best *solubility* properties, while carbon dioxide and n-hexane have the best *selectivity* characteristics.	Significantly higher for carbon dioxide than for either ethanol or mixed solvents	Impact greater with mixed solvent than with a carbon dioxide extraction plant
Hypercritical carbon dioxide extraction	Only carbon dioxide can significantly alter its properties through changes in temperature and pressure and may have wider alternative uses than ethanol or mixed solvent.	Carbon dioxide plant of approximately the same capacity as a mixed solvent plant requires almost 100 % greater capital cost (estimated).	However, newer equipment can minimize solvent losses in conventional mixed solvent extraction plant.
Ethanol extraction	Ethanol was determined not to be a recommended option because mixed solvents are more selective solvents than ethanol, and the latter is more expensive (due to special tax).	In addition, carbon dioxide plant requires additional maintenance and repair of high pressure equipment (up to 50bar).	Major competitors in developing countries are utilizing mixed solvent extraction plants.

1.6 NMR methods and uses

Analyses of plant material for artemisinin can be done using various methods. LC-MS (liquid chromatography mass spectrometry) is one of the most popular methods. It combines two methods i.e. physical separation by chromatography through a column and then analysis by spectrometry. Mass spectrometry measures the mass to charge ratio of the charged particles contained in a sample while chromatography is basically a filtration system that filters out compounds in to different categories dependant on size and charge.

In this study the focus will be on nuclear magnetic resonance spectrometry (NMR) to quantify artemisinin (Liu *et al.,* 2010). NMR gives data on the molecular conformation of a compound, which can be "translated'' into chemical structures. It does this by reading the spin and charges of the components of a compound. Many methods have been developed from basic NMR principles of which the best known is in the health sector, MRI (magnetic resonance imaging) (Edwards, 2006).

NMR was developed by a group of dedicated scientists at Massachusetts Institute of Technology and University of Stanford in the U.S.A. during the 1950's. NMR makes use of a very large magnet and the fact that the nuclei of atoms have magnetic properties contained in their centres. Each part has a spin but they usually cancel out in most atoms because they are paired, except the ones with uneven proton and neutron numbers e.g. ^{1}H, ^{13}C, ^{31}P, ^{15}N, ^{19}Fetc. These atoms have spin in their nuclei. It is due to these properties that NMR can give detailed images of chemical structures or suggestions for chemical structures dependant on which software is used (Edwards, 2006).

Artemisinin has a unique structure and molecular composition that shows characteristic peaks at specific places on an NMR spectrum. To quantify it comparisons with an internal standard of known concentration using the integrals, are done. The integrals are then compared and the concentrations calculated via a formula containing the molecular mass of the two different compounds (Liu *et al.*, 2010).

1.7 Endophytes

Schulz and Boyle, (2005) define the term "endophyte" by those bacteria and fungi that can be detected at a particular moment within the tissues of apparently healthy plant hosts. The definitions and descriptions of endophytes are often quite diverse due to new discoveries being made. In this project the best fitting definition from a plant science perspective aligns with the Schulz and Boyle description. Figure 1.13 shows the relationships where the endophyte aids the plant in defence against disease and even the transformation of endophyte to pathogen by environmental factors

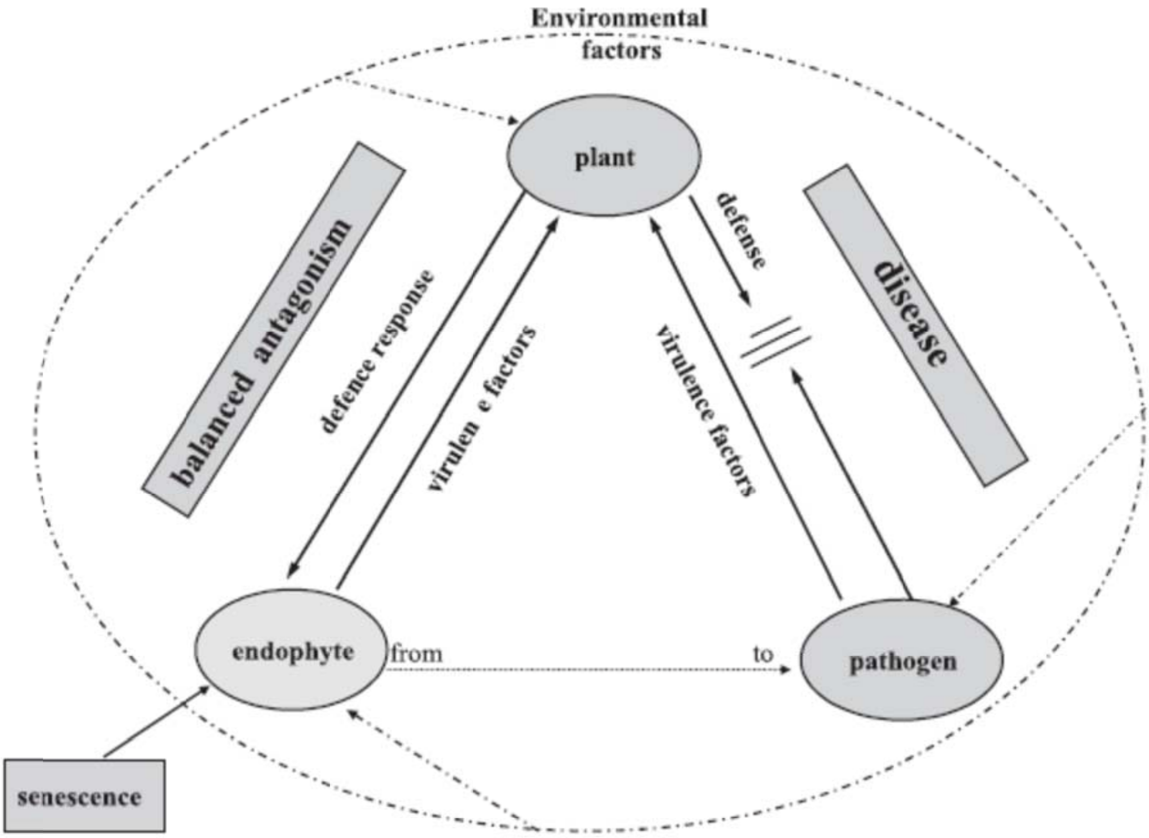

Figure 1.13: Illustration of relationships between endophytes and plants (Schulz and Boyle, 2005)

Endophytes are divided into specific categories, those that are fungal by nature and those that are bacterial by nature. In most cases there are mutualistic or symbiotic relationships between the plant host and the 'in-living' endophytes. The relationships sometimes entail protection by production of a certain chemical compound being produced by the endophyte and the plant host provides nutrients for the endophyte.

Many interesting and novel compounds have been found to be produced by endophytes, several of these are antifungal agents, but host specifity plays a crucial role. One compound that is of particular interest in the medical world is the production of taxol which is a highly rated compound to treat cancer (Strobel, 2003).

A small number of the thousands of plant species in the world have had their full spectrum of endophytes identified. These are mostly grass species which leaves a substantial lack of information because most plants species have not all been fully examined. A vast number of endophyte species are contained in a single plant which could provide potential medical advances in the treatment of many disorders and diseases (Strobel, 2003).

Some of the endophytes that have already been identified from *A. annua* show novel compounds being produced such as 3β,5α-dihydroxy-6β-acetoxyergosta-7,22-diene and 3β,5α-dihydroxy-6β-phenylacetoxyergosta-7,22-diene, which are steroids produced by a fungal endophyte, *Colletotrichum* sp. These steroids and others collected from *A. annua* showed antifungal properties against certain crop pathogens. This endophyte has also been found to have the capability of promoting the growth of the host callus (Lu*et al.,* 2000).

C. gloeosporioides an endophytic fungus from another species in the *Artemisia* genus (*A. mongolica*) was found to produce a novel antimicrobial tridepsidec olletotric acid (Zhou*et al.,* 2000).

1.8 Genetically modified organisms and tissue cultures

The concept of genetically modified organisms (GMO) is currently a hot topic of debate worldwide. This is often because of the misconception that the foreign genes that have been inserted would be harmful. Further, religious groups have accused scientists of breaking ethical laws (Winter and Gallegos, 2006; Key *et al.*, 2008). GMO's are also referred to as transformed plant's i.e. plants which have a foreign gene/ sets of genes inserted.

World hunger has been a driving force in the development of GMO crops. With the population increasing at its current rate and the amount of arable land decreasing, GMO crops became a possible solution. This is especially the case in rural and famine stricken parts of the world where poverty and skilled farm practises as well as pest control are severely lacking. None of the claims that GMO foods are detrimental have been confirmed to date. But the problems that might occur with GMO crops in specific areas are that they might become resistant to herbicides. The herbicide resistance may lead to weeds attaining these properties through crossbreeding, leading to super weeds. In the case of insects, it is possible that they might develop an affinity for the developed "insect resistant" crops by adaption (Uzogara, 2000; Konig *et al.,* 2004).

Tissue culture (also known as micropropagation) refers to the growing of a cell or specific tissue on a growth medium (liquid or solid), outside of the donor organism. The growth is usually in a new sterile, artificial environment that has been supplemented with nutrients. Tissue culture is most often used for the growth of a newly transformed species into which foreign DNA has been inserted (Hildebrandt, 1972).The pieces of the plant that are used are referred to as the explants. Selection is usually made for explants that are in a young and fast growing phase to aid in the uptake and growth after transformation. The development and growth of the transformed plant *in vitro* can then be controlled by the addition of compounds and hormones in different concentrations. The initiation is usually followed by the production of callus at the open ends of the wounds and areas in contact with the growth medium. Calli or calluses (these terms are used ambiguously) are tissue cells that are undefined in function and are omnipotent. This means that they have the potential to differentiate into any plant organ, determined by the stimulation of hormones (Sathyanarayana and Varghese, 2001).

1.9 Plant hormones

Plant growth hormones or phytohormones as they are also known, are chemical compounds that influence growth and the plant organ development linked to it. They can act as chemical triggers for various plant growth functions at very low concentrations. The influence of plant hormones can be found at various levels of transcription. Each cell has the potential to produce plant hormones. 'Plant growth regulators' is a term coined for the production of synthetic i.e. man-made plant hormones. Many plant processes are usually controlled by hormones and their onset. Examples include flowering, fruit production and senescence etc. (Kende and Zeevaart, 1997).

Plant hormones can be divided into five main classes. There are however other classes of hormones but their roles are smaller and more specifically linked than the larger classes. The first two discovered and most widely studied are: Auxins and cytokinines. Auxins are the hormones that are closely linked to the initiation of root production while cytokinines are linked to shoot propagation. Under these two groups, synthetic and naturally occurring hormones can be found. Combinations of auxins and cytokinines are often added to growth media for the development of different plant organs at different stages of tissue cultures (Liu *et al.,* 2003).

Auxins generally stimulate cell enlargement and elongation and the most common example of an auxin is indole-3-acetic acid (IAA). Cytokinines are linked to cell division and the onset of senescence. It is also believed to be involved with the transport of auxins through the plant systems. Zeatin is the most commonly found cytokinine in plants (Kende and Zeevaart, 1997).

Abscisic acid (ABA) is associated with inhibitory roles, for example the closing of stomata with water stress and inhibiting shoot growth but may even sometimes aid it. Ethylene is a hormone that is a gas. It is formed from the disassembly of methionine which is present in most plant cells. It is also known to have a tripple response in stimulating shoot and root growth and differentiation but is most commonly associated with ripening of fruit. Gibberillins (GA) have many compounds in its class. Most gibberillins share the gibberellane skeleton and gibberillic acid (GA_3) was the first discovered in this class. They are mostly associated with elongation of stems (internode elongation) and growth by counteracting the effect of ABA. In addition, they stimulate bolting and flowering because of day length differences (Kende and Zeevaart, 1997).

Plant hormones can be added directly to an area where the plant has been wounded and some experiments even include injecting or superficial addition of plant hormones to attempt to stimulate the development of a different plant organ at a particular stage of development.

1.10 Methods used to increase artemisinin yield

Various methods have been attempted to increase the amount of artemisinin produced by plants. Dissimilar soil compositions have been tested where different elements have been removed from the soil and their effects tested on the yield of artemisinin (Figure 1.14). Other methods involve stress. Stress can be divided into categories such as water stress, light intensities and nutrient availability as well as spacing competition. It would also seem that the time of harvest and different developmental stages have an effect on the amount of artemisinin produced (Delabays *et al.,* 2001).

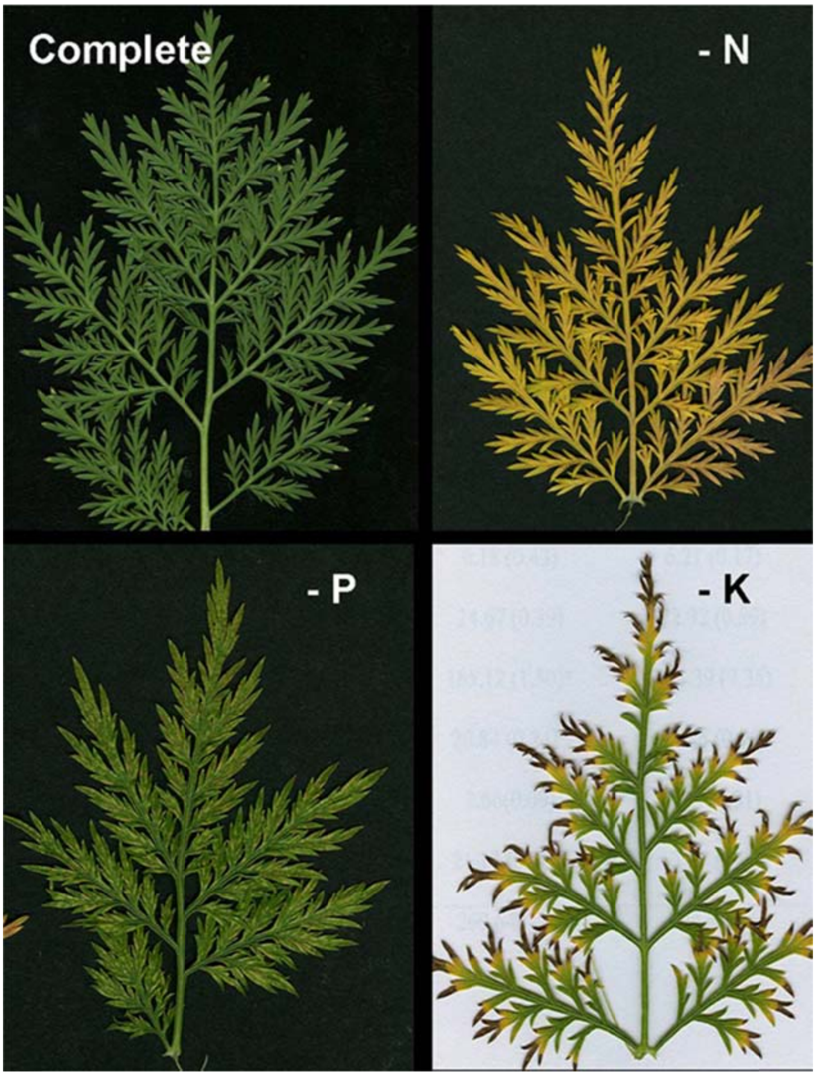

Figure 1.14: Effects on morphology of removal of different elements from the soil on *Artemisia annua* (Ferreira, 2007)

A salinity stress was done by Qian *et al*. (2007) on *A. annua* and it was found to increase the artemisinin content by a percentage of dry weight. Wang *et al.* (2001) tested different types of light showing that white and red light had the most promising results of increasing the amount of artemisinin. Water stress results are contradictory; Charles *et al.* (1990) showed that water stress had little effect on the artemisinin content except causing a decrease at extreme stress levels before harvest. They also suggest that different drying methods might increase the level of artemisinin. Sun *et al.'s* (2009) paper contradicts these results and showed that plants at 50% soil moisture had the highest production of artemisinin.

1.11 Aims and objectives of study

The aims of this study were to firstly establish calli from hairy root cultures of a GMO and determine whether they produce artemisinin and also to attempt to induce the formation of plants from the calli.

Secondly, establish endophyte cultures from *A. annua* and subject the endophyte cultures to NMR examination to detect whether it might be the endophytes producing the artemisinin, rather than the plant.

Thirdly, compare different varieties of *A. annua* that have been grown at two locations with two different sets of conditions. Analysis was done on the plant growth and production of artemisinin, with the latter being analysed using NMR analysis.

1.12 Scope of dissertation

Chapter 1 gives an introduction to *A. annua* and some plant background followed by an overview of techniques and terms associated with the practices around artemisinin. Chapter 2 is the first experimental chapter focussing on the production of GMO calli of *A. afra* and the attempts to produce plantlets of these calli by the addition of hormones. Chapter 3 investigates the possibility that artemisinin production might be linked to endophytes and cultures were accordingly tested. The final experimental chapter is Chapter 4 which shows that location conditions play an important role in the production of artemisinin from different varieties produced in the field. Chapter 5 gives a general discussion and concluding remarks on the experiments, while Chapters 6 and 7 are comprised of the references and statistical data used for the production of this dissertation.

Chapter 2: Tissue culturing of GMO *Artemisia afra*

2.1Introduction

Not much research has been done on the genes required for the production of artemisinin. Investigation into the South African species *A. afra* showed that it contained most of the genes required to produce the precursors required in the metabolic path to the end product, artemisinin. However one of the last genes required for the final conversion to artemisinin, amorpha-4,11-diene synthase (ADS) is lacking (Figure 1.6). This enzyme is required for the conversion from artemisinic acid to artemisinin. If the successful insertion of the gene and production of genetically modified *A. afra* plants could be established, it could change the way in which artemisinin is produced and harvested. *A. afra* is a perennial plant compared to the annual *A. annua*. This could mean that if a GMO is produced successful, consecutive seasons of planting would no longer be necessary and different harvesting practices could be developed and costs could be saved. In addition, insertion of multiple copies of the genes in combination with improved agricultural practices could lead to higher yields of artemisinin being obtained. All these factors combined could lead to cheaper and more efficient ways of treating malaria.

Whipkey *et al.* (1992) did tissue culturing of *A. annua* on Murashige and Skoog (MS) medium with supplementation of different plant hormones and found 6-benzylamino purine to be the best for producing shoots from the leaf material. Wang *et al.* (2001) used *Agrobacterium rhizobium* co-culture with leaf discs of *A. annua* to produce hairy roots on hormone free MS medium. Nair *et al*. (1986) also used MS medium for the culturing of different plant parts of *A. annua*. He supplemented the MS with naphthalene acetic acid and 6-benzyladenine. All the literature used sucrose concentrations of approximately 3% and constant temperatures of 25°C. The concentrations of the hormones al ranged from 0.5mg/l to 2.5mg/l.

2.2 Materials & Methods

Genetically modified (GM) hairy root cultures of *A. afra* were obtained from a previous study with Professor Toshiya Muranaka, of the University of Osaka. The hairy roots were cut in 3 mm pieces using a sterilized blade in a laminar flow cabinet. The cuttings were then placed on solidified medium (25ml) under sterile conditions. The medium consisted of a solution of half strength Murashige and Skoog shoot multiplication media, 3% sucrose, 0.8% agarose, at a pH of 5.8 and various concentrations of plant hormones were added in a number of combinations (Table 2.1). The hormones used were naphthalene acetic acid (NAA) (2mg/l, 1mg/l and 0.5mg/l) and zeatin (1mg/l, 0.5mg/l and 0.1mg/l). These applications were adapted from the methods used by Nair *et al*. (1986) and other supplementary literature.

Table 2.1: The first sets of combinations of plant hormones

Flask numbers	**Plant hormone concentrations**
1-3	2.0mg/l NAA
4-6	1.0mg/l NAA
7-9	0.5mg/l NAA
10-12	1.0mg/l Zeatin
13-15	0.5mg/l Zeatin
16-18	0.1mg/l Zeatin
19-21	2.0mg/l NAA + 1.0mg/l Zeatin
22-24	1.00mg/l NAA + 0.5mg/l Zeatin
25-27	0.5mg/l NAA + 0.1mg/l Zeatin
28-30	2.0mg/l NAA + 0.1mg/l Zeatin
31-33	0.5mg/l NAA + 1.0mg/l Zeatin
34-36	No plant hormones were added

The samples were placed in an incubator that provided a 16 hour light period and 8 hour dark period at a temperature of 25˚C (Figure 2.1).

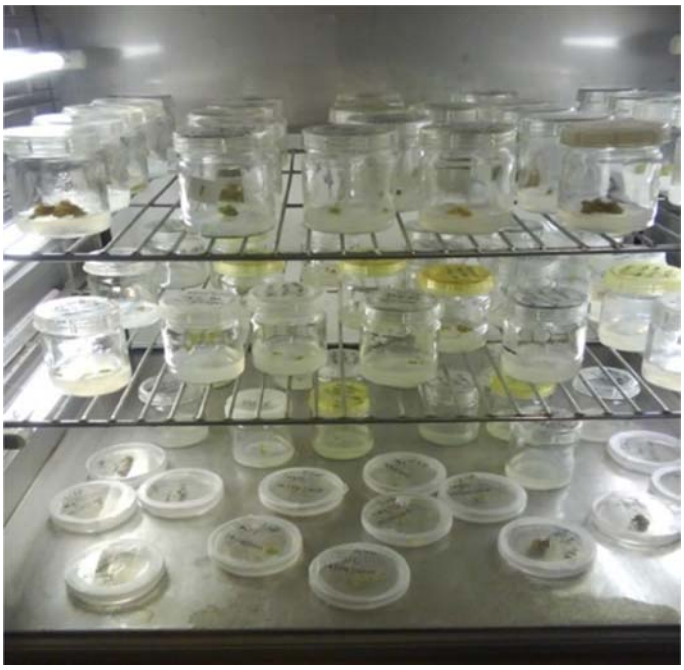

Figure 2.1: The incubator and tissue culture flasks

Developed callus and roots were sub-cultured a second time. The hormone, 2,4-dichlorophenoxyacetic acid (2,4-D) was added to the standard medium. Concentrations of 2mg/l and 1mg/l NAA was again used and combined with 2mg/l 2,4-D and 1mg/ 2,4-D. Half of these samples were covered in foil to submit them to permanent darkness. These samples were placed in the incubator under the same conditions as previously described.

A third sub-culturing of the callus and roots were subjected to treatment with gibberellic acid (GA_3) (0.5mg/l). Again NAA was added in concentrations of 1mg/l. These samples were also placed in the incubator under the same conditions.

A forth sub-culturing of the callus and roots received 1mg/l 6-benzyladenine (BA) and 1mg/l NAA. Half of the cultures were added to a solid medium and half were added to a liquid medium which lacked agarose. The samples on solid medium were placed in the incubator while the samples in liquid medium were subjected to shaking at 100 revolutions per minute in an incubator, set at 25˚C and constant darkness.

A fifth sub-culturing was done to keep a constant stock and supply. Stocks were maintained and treated with combination of other hormones in an attempt to regenerate shoots. These hormones were TDZ (thidiazuron) and kinetin and were added in concentrations of 1mg/l and 2mg/l.

When contamination was encountered the samples were either discarded or sterilized by submerging plant material in 70% alcohol solution for 3 seconds, rinsing in distilled water and then sub-culturing.

Cultures were regularly harvested, ground in liquid nitrogen, mixed with distilled chloroform, concentrated, dried and subjected to NMR analysis. These were done on a 200MHz Varian NMR in deuterated chloroform.

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2.3 Results & Discussions

Many calli were produced on the media with the concentration of 1mg/l NAA having the highest yield (Figure 2.2). Calli were also produced on the 2,4-D hormone but a reduced amount in comparison to those treated with NAA. Only three samples sprouted roots (Figure 2.2) but no shoots or leaves were formed.

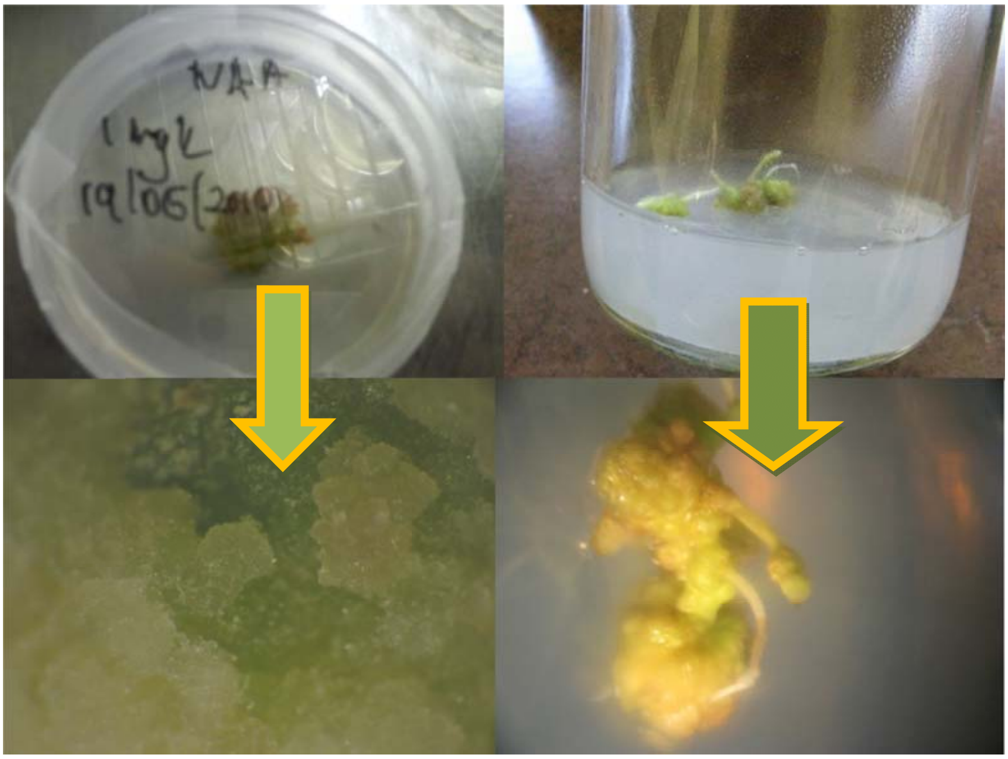
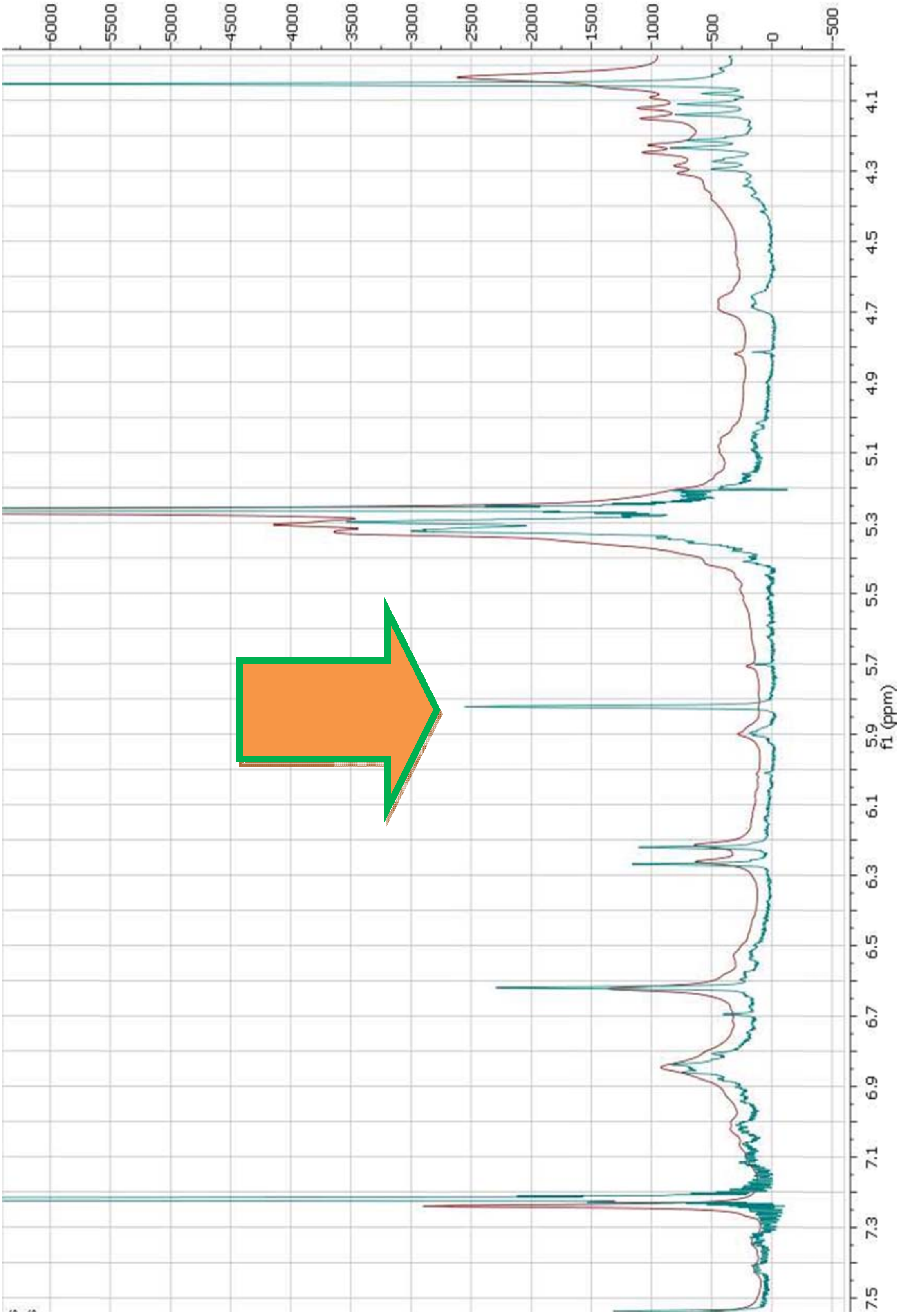


Figure 2.2: Some of the calli and roots produced

The NMR spectra showed two interesting peaks around the area where the H15 peak for artemisinin is usually found, 5.8ppm. Pure artemisinin was added to the ground calli mixture for comparison however it produced a signal that occurred between the two suspected peaks when the two spectra were overlayed using MestReNova. This proved that artemisinin was not produced in the GMO calli (Figure 2.3).


f1 (ppm)

The production of an *A. afra* GMO plant that synthesis artemisinin needs more investigation. The successful production of such a plant can be utilized in the battle against malaria in countries where medication is very expensive and out of the reach of most of the infected. A large scale Artemisia crop producing more of the active compound with less intense farming practices and better adapted to African conditions, would be the best choice.

It has been reported in literature that artemisinin can be produced by calli. Nair *et al.* (1986) stated that artemisinin was produced in tissue culture calli, but only in calli originating from stems and leaves and not those originating from roots. This could be because the production of artemisinin is usually associated with occurrence of trichomes, which are found on leaves and flowers and to a lesser extent on stems. Most of the studies used leaf and stem cuttings to produce calli. However Wang *et al.* (2001) showed that hairy root cultures of *A. annua* did produce artemisinin with the introduction of external stimuli but again leaf discs were used to create the hairy root cultures.

Hairy root cultures are usually used for the insertion of foreign genes, but the material used to create the hairy roots may pass certain elements on to the hairy roots. These elements might be carried on to calli. This could be the reason for the occurrence of artemisinin in tissue cultures and calli suggested by literature earlier.

The explants used in our experiment were from hairy root cultures which did not contain trichomes. This might be the reason why there is no artemisinin production as artemisinin is usually associated with trichomes.

Chapter 3: Production of artemisinin by endophytes

3.1 Introduction

Endophytes are known to live commonly in plants and usually share in symbiotic relationships with them. In the case of most secondary metabolites, like artemisinin, their original reason for production might have been lost through evolutionary changes. For example a compound being produced by a plant to ward off a herbivore, but the herbivore is now extinct, yet the plant still produces the metabolite.

In symbiotic relationships between endophytes and plants one would many times find that the endophyte produces a compound to be used either for warding off of an attacker or parasite in return for protection or nutrients shared by the plant. Wang *et al*. (2001) induced increased production of artemisinin in sterile tissue culture with the addition of a fungal endophyte elicitor. This specific endophyte is usually found on the stems of *A. annua.*

Artemisinin might be produced by endophytes in *A. annua* and culturing of the endophytes might lead to different ways of producing artemisinin and further investigation. *Eurotium amstelodani* and *Aspergillus niger* are two microbes that have been used to produce novel derivatives from artemisinin (Parshikov et al., 2006). No production of artemisinin by microbes or endophytes other than transformed *Escherichia coli* and *Agrobacterium tumefaciens* could be found in literature.

3.2 Materials & Methods

Various samples were taken from an *A. annua* plant that was kindly donated by Riana Kleynhans of the Agricultural Research Council (ARC) Roodeplaat. This plant had been growing under shade nets and in a pot of about 1m^2.

Samples were taken from the leaves, the stem and the roots, cut into approximately five mm by five mm pieces and then cleansed to clear away bacterial contaminants inside a laminar flow cabinet. This was done by submerging the cut plant parts in a 3% solution of bleach. The samples were submerged for approximately 5 seconds, removed and rinsed in sterilized distilled water. The stem and root samples were then cut open and the open parts as well as the uncut leaves were then placed on different media.

The media were soy flower media (SFM) with 1% PDA (potato dextrose agar) and pure PDA. The samples were placed in Petri dishes inside a Labotec IncoCool incubator with no light at 25° C. The plates were left for a few days before observations were made.

Extensive microbial growth resulted and pure, single fungal colonies were selected. These selected colonies were grown up in four one litre containers on a shaker inside a temperature controlled incubator and then extracted.

The fungal broth were thoroughly mixed and poured in separating funnels and extracted using distilled chloroform (artemisinin dissolves well in chloroform). The mixtures were then collected leaving the more polar compounds behind in the separating funnel, dried and concentrated using a Buchi rotary evaporator. The dried and concentrated samples were dissolved in 1ml deuterated DCM (dichloromethane) and subjected to NMR analysis.

A 200 MHz nuclear magnetic resonance (NMR) machine was then used to determine if artemisinin was present within each sample by comparing it to the spectrum of pure artemisinin. Pure artemisinin was added to the samples after their first round of analysis and they were again subjected to NMR analysis. This was done because pH and contaminants may cause shifting of the spectra. The two sets of spectra were then compared and superimposed.

3.3 Results & Discussion

Selection on Petri dishes was made for fungal endophytes trying to avoid bacterial endophytes by comparing cultures to those of known bacterial nature (Figure 3.1). The Petri dishes with the largest amount of single fungal colonies for each plant organ was selected for extraction. The NMR spectra for root endophytes showed small peaks close to the characteristic H15 peak of artemisinin as seen in Figures 3.2 and 3.3. It is unknown whether some of the endophytes might have been mycorrhizae as the investigation was for artemisinin production.

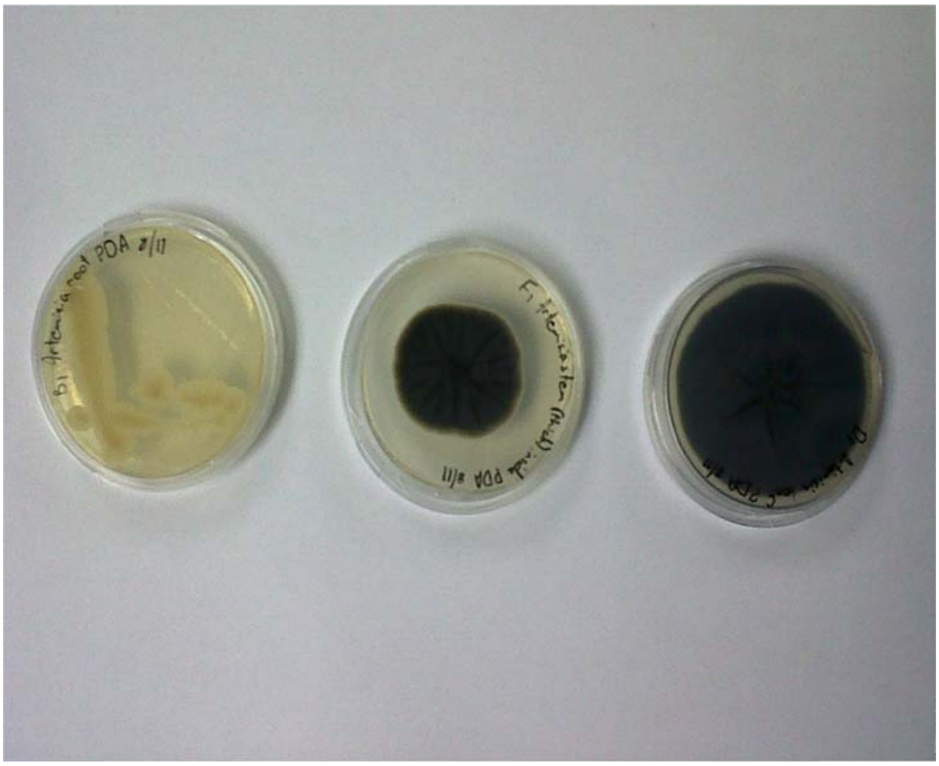
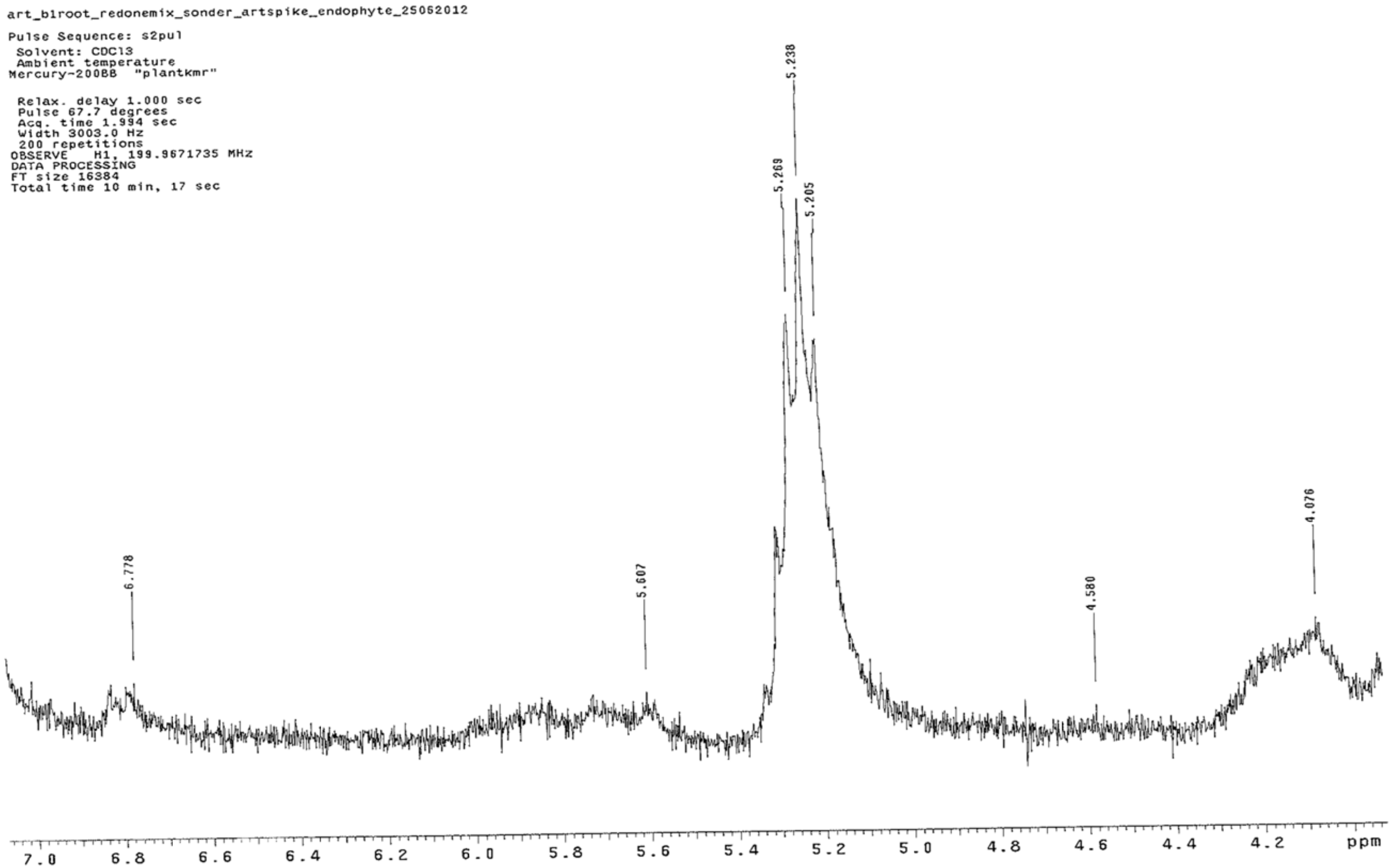
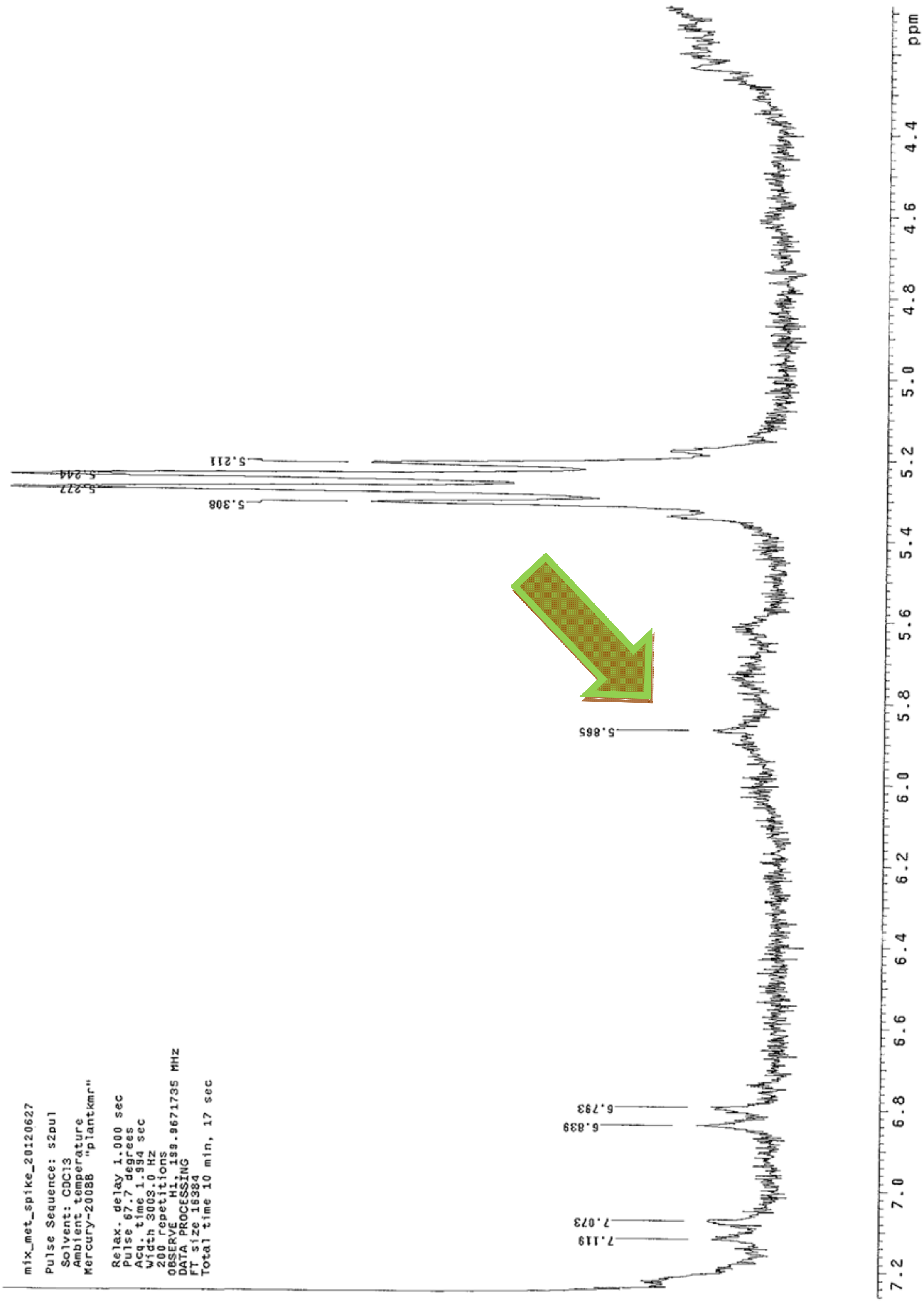


Figure 3.1: Examples of the endophytes


art_b1root_redonemix_sonder_artspike_endophyte_25062012
Pulse Sequence: s2pul
Solvent: CDCl3
Ambient temperature
Mercury-200BB "plantkmr"
Relax. delay 1.000 sec
Pulse 67.7 degrees
Acq. time 1.994 sec
Width 3003.0 Hz
200 repetitions
OBSERVE H1, 199.9671735 MHz
DATA PROCESSING
FT size 16384
Total time 10 min, 17 sec
6.778
5.607
5.269
5.238
5.205
4.580
4.076
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
ppm


mix_met_spike_20120627
Pulse Sequence: s2pul
Solvent: CDCl3
Ambient temperature
Mercury-200BB "plantkmr"
Relax. delay 1.000 sec
Pulse 67.7 degrees
Acq. time 1.994 sec
Width 3003.0 Hz
200 repetitions
OBSERVE H1, 199.9671735 MHz
DATA PROCESSING
FT size 16384
Total time 10 min, 17 sec
5.211
5.244
5.277
5.308
5.865
6.793
6.839
7.073
7.119
ppm
4.4
4.6
4.8
5.0
5.2
5.4
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2

Figures 3.2 and 3.3 showed small peaks in the area where the characteristic H12 peak for artemisinin usually occurs. With artemisinin added (Figure 3.3) there was a small peak in at 5.8 ppm. The resolution on the 200 MHz NMR might have been too low to detect artemisinin in the samples with only 200 scan cycles. The experiment was redone running the samples for 3000 scan cycles and adding a substantially higher amount of purified artemisinin. The NMR spectra were then obtained, combined and superimposed using MestReNova (Figure 3.4). From the figure it can be seen that there was in fact no artemisinin being produced by the fungal colony. None of the other plant organs' endophytes showed any signs of artemisinin production.

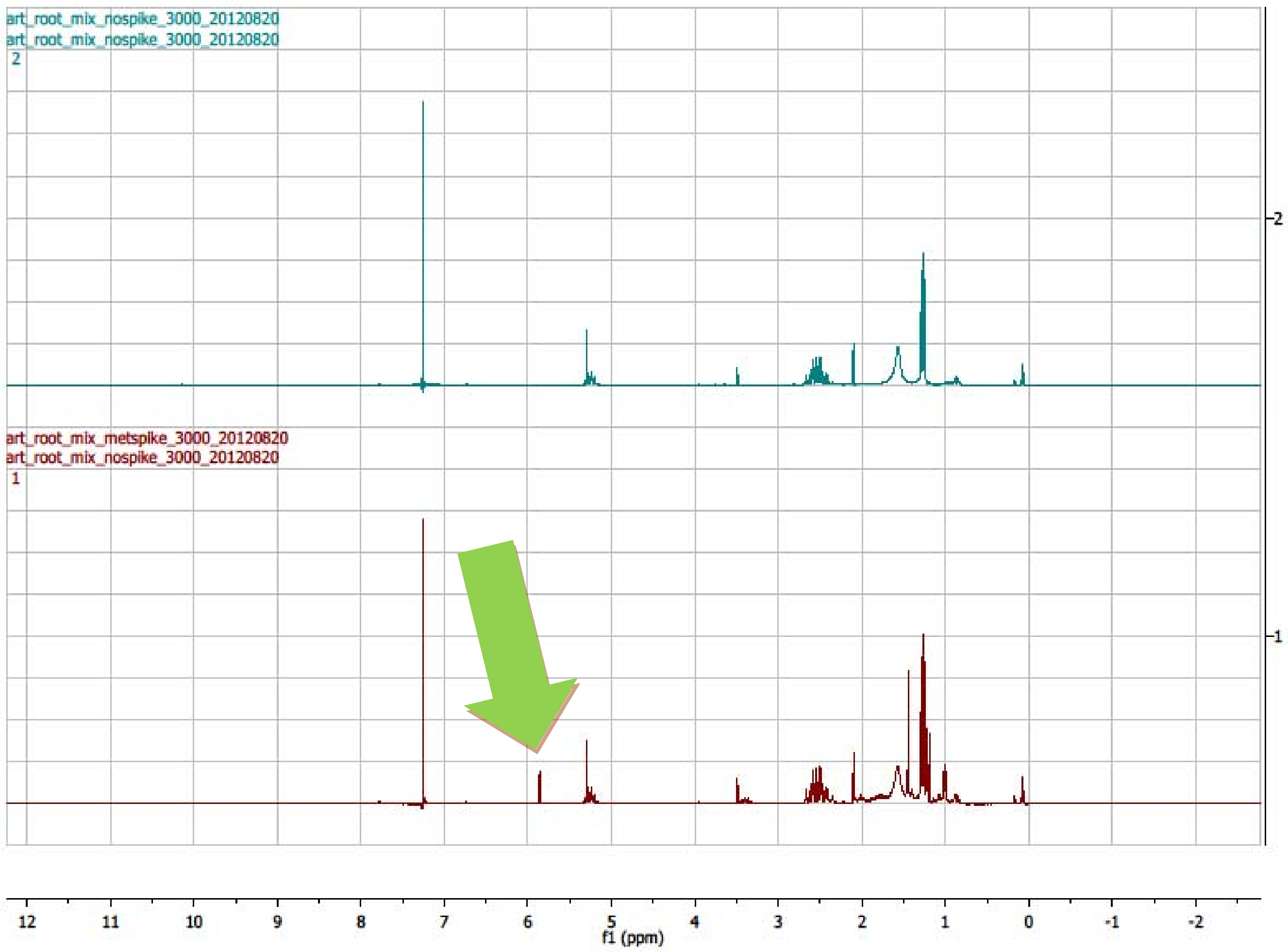


Figure 3.4: NMR spectra for fungal root growth of *Artemisia annua* with the bottom spectrum containing a purified addition of artemisinin, indicated by the arrow.

Wang *et al.* (2001) has discovered that endophytes play an important role in host plant secondary metabolism. An endophyte (*Colletotrichum* sp.) identified in *A. annua* was added to hairy root cultures, originating from leaf discs, and induced higher production levels of artemisinin. However no literature could be found where endophytes themselves produce artemisinin.

Chapter 4: Influence of location on artemisinin varieties

4.1 Introduction

Artemisinin yields are very low per plant and effort has been made to try and increase the yields. It would appear as if location might play a role as varied yields have been attained all over the world. Wallaart *et al.* (2000) stated that there might be chemotypes associated with geographical location. This means that different yields of artemisinin might be found at different locations and that the plants at one location might differ from plants at other locations. Delabays *et al*. (2001) stated that large variations in artemisinin have been observed in leaves originating from different sources.

The malarial drug market needs higher yielding varieties as extraction is quite expensive and of less use if the percentages extracted are low. Importers usually only purchase for production if the percentages are adequate. In South Africa we lack artemisinin processing facilities however there are some institutes that might be able to produce at a large enough scale to market products.

A great deal of focus has also been put into breeding higher yield varieties. Arsenault *et al*. (2010) discussed the over-expression of certain genes in GMO *A. annua* varieties but the results vary. He also mentioned the selection for hybrids with high yields of artemisinin for a location. Some literature claim very high artemisinin yields but whether these high yields will be produced in consecutive seasons under different sets of circumstances remains to be seen (Damtew *et al*., 2011).

This chapter deals with the differences between varieties of *A. annua* and the effect that locations might have on the yield of artemisinin. Criteria like soil composition and rainfall for each location is noted and compared and effect on artemisinin yield discussed between the varieties. Proton NMR and multivariate data analysis software were used for the analysis between the varieties. This software included MestReNova 8.1.1 (Mestrelab Research), Excell (Microsoft Excel 2010) and SIMCA-P 13.0.0 (Umetrics, Umeå, Sweden).

MestReNova converts the NMR spectra to a more user-friendly interface and allows for simpler editing of data. Fields that can be edited include baseline correction, normalization, scaling and binning (Heyman, Unpublished).

SIMCA is designed to show patterns and similarities based on the statistical analyses of data. SIMCA can group samples and show their similarities and can be used to differentiate between two slightly different samples at compound and concentration level. This can be used to show for example differences in the metabolic pathways of samples as they will also be separated at compound level (Hedenström *et al.,* 2008).

Principal component analysis (PCA) can be performed with SIMCA which is a pattern recognition technique that does not ''discriminate'' between the data being analysed. Another pattern recognition technique used by SIMCA is orthogonal projection to latent structure-discriminate analysis (OPLS-DA). This discriminating method contains a filter more suited to noisy variables commonly associated with biological data (Bylesjö *et al.,* 2006).

4.2 Materials & Methods

4.2.1 Seed germination and seedling establishment

Five different varieties of *A. annua* seeds were obtained for field trials. Two high yielding varieties were obtained from Dr. Frank van der Kooy of the University of Leiden. One variety was produced during earlier stress-induced studies at the University of Pretoria and the other two varieties were received from the Agricultural Research Council of South Africa (ARC) at Roodeplaat, courtesy of Riana Kleynhans (Table 4.1).

Table 4.1: Key for the respective lines of *Artemisia annua*

Variety	Supplier	Area of origin	Colour
f0	Prof Meyer	Univ of Pta	
f1	Dr. Frank van der Kooy	Eastern Europe	
f2	Dr. Frank van der Kooy	Eastern Europe	
r1	Riana Kleynhans	ARC- Roodeplaat	
r2	Riana Kleynhans	ARC- Roodeplaat	

The seeds of the varieties were planted in Hygromix (a mixture of peat and polystyrene) containing vermiculite (a clay compound that has the potential to expand and contract and absorb water, it is usually used in combinations with soil to add air and water content and absorption to soil for germination mixes). This was done by mixing the growth mixture with water until a wet mass was formed, this was then used to fill small sterilized polystyrene planting trays, which were either 20 or 12 welled with a surface area of about 60cm^2. The volume of each well was approximately 30cm^3 (Figure 4.1).

Figure 4.1: Trays in which the seeds were planted

The seeds were then sown on top of the mixture and the trays were marked according to the varieties planted. All the trays were transferred to a glass house at the ARC Roodeplaat's facilities with a controlled temperature at 25°C, receiving sun most of the day. The seeds and seedlings were watered daily or as appropriately needed and a second set of seeds were added where germination was too low. After a month the seedlings were transplanted to other planting trays in order to have a single plant in each well and thereby removing competition. These seedlings were allowed to grow for a month after which they were transplanted to the field at the specific locations. One ARC variety (r2=purple) had really low germination rates and these seedlings only sprouted later and were transplanted later.

4.2.2 Location of field trials

The Agricultural Research Council Vegetable and Ornamental Plant Institute (ARC-VOPI) situated at Roodeplaat (coordinates: 25°35'59.81"S 28°21'45.49"E, elevation 1164m) and the University of Pretoria's (UP) LC de Villiers experimental farm (coordinates: 25°45'02.15''S 28°14'46.48''E, elevation 1305m) were the two localities for the experiment. The two areas are about 30km apart with two different soil compositions and altitudes (Figure 4.2).

Soil sampling was done (Figure 4.3) and sent to the ARC Institute for Soil, Climate and Water (ARC-ISCW) for analysis and comparison. Samples were taken of the top 30cm of soil (0cm to 30cm) followed by the second layer of soil (30cm to 60cm) in a grid layout over the plot areas.

Figurer 4.2: A satellite view showing the topographical view of the two sites. (Google Earth)

Figure 4.3: Soil sampling being done by the author

4.2.3 Experimental layout

The field transplanting commenced in February 2012 in Latin squares with five repeats of the five varieties in a scattered pattern, per location (Figure 4.4). The blocks consisted of 35 plants in five rows with seven plants per row with 60cm spacing between the 5 rows and 70cm between the seven plants. The samples for analysis were taken from the inner block of 15 plants per block (Figure 4.5). This was done in attempt to eradicate variations as the plants on the outside of the blocks that may have received altered weather conditions because they are not protected by other plants. Plants received water with planting and onwards every second day for three hours by piped sprinkler systems. The sprinklers delivered about 8-10mm per hour.

ıre

E	J	O	T	Y
D	I	N	S	X
C	H	M	R	W
B	G	L	Q	V
A	F	K	P	U

E	J	O	T	Y
D	I	N	S	X
C	H	M	R	W
B	G	L	Q	V
A	F	K	P	U

Figure 4.4 Showing the Latin square patterns per location each colour representing a different variety shown by Table 4.1's key.

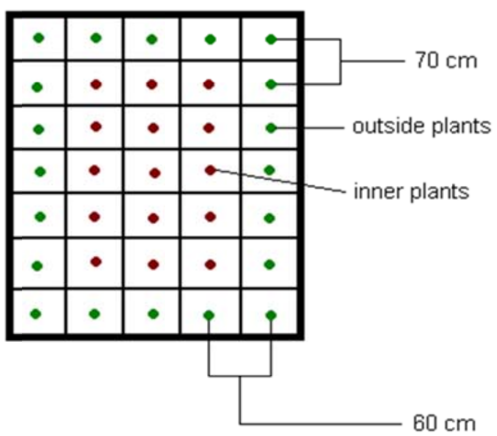


Figure 4.5: Representation of a single block of plants

Plants that died were replaced to establish uniform trial blocks for experimentation. Careful attention was paid to the onset of flowering. The plants at the ARC had to be treated with Termic (termite eradicator) as it was found that the soil contained termites that could lead to some plant loss.

4.2.4 Harvesting practices

Plants that had reached a height of between 1.1 and 1.3 metres (Figure 4.6) were severed above ground level during the week of the 14th of May 2012, the plants were in the field for nearly four months at this stage (Figure 4.7).

Figure 4.6: Four month old plants just before harvest

Figure 4.7: Harvesting on the 14th of May 2012

The 15 plants representing the sample plants from each block were grouped together and weighed to determine the wet mass. The wet mass was determined to two decimal places (Figure 4.8).

Figure 4.8: Determining the wet mass with a levelled scale (inserted picture shows that the scale was levelled).

The harvested plants were placed in storerooms on diamond mesh metal sheets shelves (Figures 4.9). The shelves were about 1.2m in diameter and 10m in length.

Figure 4.9: The metal shelves with mesh that were used to dry the plant material.

The plant material was left for a week to dry after which they were again weighed to give the dry mass. The dried plants were stripped of all leaf material and dry leaf mass was determined. The stripping was done by dragging a clinched fisted hand from the thicker part of stems down to the thinner parts. The powdered leaf material was mixed and subsequently weighed. The sample bags containing the representative samples of each sample block were then labelled and stored (Figure 4.10).

Figure 4.10: Dried leaf material in brown paper bags for weighing.

4.2.5 Sample preparation

A representative sample of 1.0g of each bag of plant material was then taken and set for extraction as seen in Figure 4.11.

Figure 4.11: A representative sample of a bag of plant material.

Extraction was done on a Buchi speed extractor (E-916). It involved taking 1.0 g of dried leaf material and inserting it into the metal tubes of the speed extractor. Labotec filters (1cm diameter) were placed at the small end of the tube and covered with the metal stopper and screwed closed with special sealing cap. Tubes were filled with sand and 1.0g of plant material and covered with a large Labotec filter (2.5cm diameter).

The speed extractor was set to 50°C, 100bar pressure, 100% dichloromethane (DCM), three cycles, double flushing with solvent and then with nitrogen gas. 50 samples of nearly identical volumes were obtained. A Genvac (EZ-2) was used to concentrate the samples to dryness in specialized politops. The dried samples were then dissolved in 8ml DCM and 2ml maleic acid with a concentration of 2mg/ml in methanol. This was done to obtain a maleic internal standard for later comparison to artemisinin. The samples were again dried in the Genvac dryer and dissolved in 1ml deuterated methanol.

4.2.6 NMR and Multivariate data analysis

These samples were subjected to 600 MHz NMR analysis courtesy of UNISA and the CSIR and spectra were obtained and reduced to ACSII files using the analytical software, MestReNova 8.1.1 (Mestrelab Research). Normalisation was done by scaling the spectral intensities to 0.1% TMS. The region of 0.00 to 10.00 ppm was reduced to bins of 0.04 ppm in width. A second set of ASCII files were generated and then imported to Microsoft Excel 2010 for secondary variable labelling and transposing. The transposed and labelled Excel files were then imported to statistical software SIMCA-P 13.0.0 (Umetrics, Umeå, Sweden). Data was Pareto scaled before being subjected to PCA and OPLS analysis (Heyman *et al.,* Unpublished 2013).

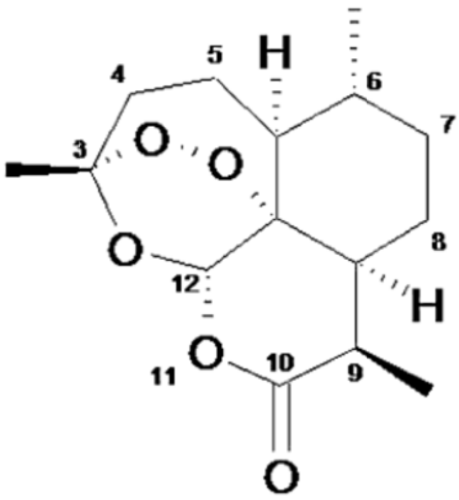
The integrals of the maleic acid peak (6.1 ppm) and the H-12 artemisinin peak (5.9 ppm) were inserted into a formula (Equation 4.1) to calculate the concentration of artemisinin (Liu *et al*., 2010).

$$\left(\frac{\int \; Art}{\int \; Mal}\right) \times 2 \times \left(\frac{282.332}{116.1}\right) \times 0.2$$

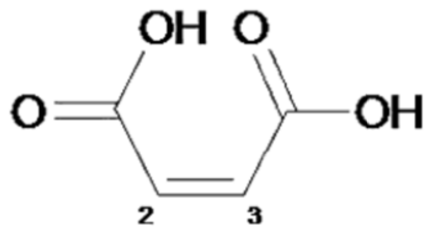
Equation 4.1: The equation to calculate artemisinin concentration from a NMR spectrum

Maleic acid contains two hydrogen atoms that are bonded to carbons two and three in its chemical structure. NMR uses the spin of protons to fulfil its diagnostic functions. Artemisinin has a characterising proton at C-12 which forms a singlet peak at 5.9 ppm on the NMR spectrum (Figure 4.13).

The equation takes the integral value of artemisinin divided by the integral value of maleic acid multiplied by 2 (for the 2 protons of maleic acid). This value was then multiplied by the molecular mass of artemisinin, divided by the molecular mass of maleic acid and then finally multiplied by 0.2 representing the concentration of maleic acid that was added.



1



2

Figure 4.12: Chemical structures of artemisinin and maleic acid (internal standard for NMR analysis): 1= Artemisinin, the proton at C-12 was used for quantification; 2=maleic acid the protons at C-2 and C-3 were used for the artemisinin quantification (Liu *et al.,* 2010).

These methods were previously validated by comparisons of results with HPLC results and other methods by Casthilho *et al.* (2007) and Liu *et al.* (2010).

4.3 Results &Discussion

4.3.1. Rainfall and soil analysis

Appendixes B and C contain weather station information that can be used to compare the different climates and what role they played in our trial. The ARC received 50mm more rainfall than UP during the time that the experiment was conducted. Higher solar radiation was received at the ARC and this would lead to higher evaporative values. The difference in moisture content between the two locations could perhaps be nullified by the ARC having both higher rainfall and solar radiation values. Some readings are missing on the rainfall chart for UP because of power outages, but it would be safe to mention that no rain fell during those periods as no rain fell during the same time at the ARC and this was also in the dry winter months.

Charles *et al.* (1993) stated that water stress can be related to retardation in growth of *A. annua*. They also stated that artemisinin content is negatively influenced by water stress and this statement is contradicting to the work of Fluck, 1955 and Gershenzon, 1984 that share the opinion that secondary metabolites are positively influenced by plant stresses. In this study the locations only differed slightly in rainfall. Considering this statement, the location that received the most rain and least stress should produce the highest plants and the highest yields of artemisinin according to Charles *et al.* (1993), but the inverse is observed. Investigation in to other factors could hold the reasons.

Factors like mean temperatures and wind are relatively similar for the two locations and no influences on the performance can be observed or great differences between the two locations (Appendix B and Appendix C).

The influence of soil composition is shown in Table 4.2 and this indicates that there are some differences in composition between the two locations. There is no element lacking at either site but the concentrations per location differ with the ARC having higher concentrations per block and on average in Table 4.3 of everything except nitrogen.

Table 4.2: The soil analysis of the two locations, ('Dd' in the last two rows represents ARC E)

			1	2	3	4	5	6	7	8	9
T	LabNo	SENDER_NR	Bulk Density	Total N	K	Ca	Mg	Na	pH(H2O)	P	WHC
			t/cub. M	%	mg/kg	mg/kg	mg/kg	mg/kg	Water	mg/kg	%
M	1569	LC A top 1		0.09	80.60	611.5	202.3	45.24	6.27	20.95	2.06
M	1570	LC A bottom 2	3.097	0.06							1.96
M	1571	LC B top 3	2.837	0.08	84.91	536.5	168.1	52.26	5.82	13.76	1.97
M	1572	LC B bottom 4	2.851	0.05							1.33
M	1573	LC C top 5	2.879	0.08	133.9	664.9	197.6	50.27	6.08	13.25	2.16
M	1574	LC C bottom 6	2.866	0.05							2.0
M	1575	LC D top 7	2.931	0.07	66.87	632.7	181.7	49.58	6.38	39.65	6.01
M	1576	LC D bottom 8	2.992	0.06							5.53
M	1577	LC E top 9	2.984	0.09	89.83	640.0	203.1	67.56	6.12	30.44	9.99
M	1578	LC E bottom 10	3.084	0.07							9.85
M	1579	ARC A top 11	3.26	0.07	302.5	873.9	323.2	85.65	6.59	74.29	6.99
M	1580	ARC A bottom 12	3.12	0.06							9.85
M	1581	ARC B top 13	3.239	0.07	241.8	1037	297.7	67.60	7.04	86.22	9.4
M	1582	ARC B bottom 14	3.337	0.07							10.84
M	1583	ARC C top 15	3.329	0.06	217.1	854.7	259.7	84.68	6.94	88.60	9.75
M	1584	ARC C bottom 16	3.36	0.06							15.33
M	1585	ARC D top 17	3.348	0.07	255.7	823.4	289.4	79.62	6.80	37.55	2.28
M	1586	ARC D bottom 18	3.221	0.05							2.03
M	1587	ARC Dd top 19	3.218	0.09	307.4	885.7	309.4	89.40	6.73	9.57	3.56
M	1588	ARC Dd bottom 20	3.175	0.06							7.85

Table 4.3: The soil composition averages between the ARC and UP (WHC-water holding capcity)

	Bulk Density	Total N	K	Ca	Mg	Na	pH (H2O)	P	WHC
	t/cub. m	%	mg/kg	mg/kg	mg/kg	mg/kg	water	mg/kg	%
ARC	3.26	0.07	264.90	894.94	295.88	81.39	6.82	59.25	7.79
UP	2.95	0.07	211.62	617.12	190.56	52.98	6.13	23.61	4.29

Soil composition could be one of the causes of the plant varieties at UP containing higher artemisinin than the plants at the ARC. Selmar and Kleinwachter (2013) suggested that medicinal plants produce higher amounts of medicinal compounds of interest under certain sets of stress conditions. This is also supported by Fluck (1955) and Gershenzon (1984). They both stated that stress factors reduce primary metabolism and that the chemical structures are assigned to secondary metabolism to attempt to overcome the stress problem.

The reduced amount of compounds like phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na) could lead to a type of stress condition which could lead to greater artemisinin production. Omer *et al.* (2013) stated that artemisinin percentages were increased in the cultivation on sandy loam soil compared to clay soil. In this experiment however the soils were of the same type only differing in concentration of elemental composition. Our results differ slightly, this is because even though the soils are similar, the soil with the highest amount of clay (23.2% compared to 20.6%) produced the highest artemisinin percentage. Omer *et al.* (2013) also stated that clay soils yield better growing plants which our data supports. Soils higher in clay usually contain more macro elements (Omer *et al*., 2013) but at UP previous trials may have depleted some sources.

Fertilizer trials mostly focus on N, P and K levels. Singh (2000) showed that an introduction of N could increase the yield of artemisinin but only to a certain extent. When the optimal concentration is reached adding more N will not really increase the production of artemisinin. The effects of P and K are extremely small and not noteworthy. In our experiment N was the same for both locations while all the other elements were found in a lower concentration at UP.

Salinity stress does not have an effect on artemisinin according to Prasad *et al.* (1998) but the ratio of potassium to sodium does (K:Na) does have a negative effect on dry mass production. This supports our data where the ration at the ARC is 1:3 and UP is 1:4 and UP plants produced lower wet and dry masses for all the varieties.

4.3.2 Growth rate and plant height

Figures 4.13 and 4.14 contain the relative height measurements for the locations in their Latin squares. Fall out plants (plants that died) are represented by zeros. The inner plants were all measured and their heights were averaged per location and per variety (Table 4.4).

73	166	48	5	0	65	80	0	0	60	90	78	104	85	100
80	73	70	70	55	15	78	90	63	72	85	0	70	90	107
70	30	80	70	90	60	95	65	70	89	94	0	90	100	90
64	60	70	100	101	15	86	0	50	96	100	64	90	90	100
57	75	42	70	80	80	84	51	85	95	100	70	90	112	112
45	75	52	80	80	95	55	98	107	100	73	88	75	80	68
80	42	77	37	70	90	73	100	94	98	80	86	85	70	60
90	75	60	62	10	50	0	115	97	95	87	77	50	68	70
80	82	60	62	30	0	107	100	89	97	58	84	80	72	88
78	63	70	60	0	76	110	87	87	74	90	75	60	35	50
60	100	75	70	0	75	90	0	42	78	74	37	80	68	75
106	100	85	68	80	70	72	34	0	89	12	45	75	70	70
0	90	80	20	65	68	62	82	44	47	80	33	70	40	90
90	75	90	84	65	88	78	0	22	78	10	98	70	82	70
78	90	100	10	80	0	64	95	90	76	89	80	30	75	30
64	60	80	78	90	90	70	54	75	85	93	78	90	112	90
50	70	72	73	85	65	90	48	80	52	76	72	100	90	80
68	30	27	80	90	80	68	102	85	77	8	79	90	80	88
50	40	60	101	85	0	82	80	35	77	85	70	80	80	90
68	60	0	90	92	80	52	32	82	82	75	59	50	95	85
76	65	60	15	150	80	97	120	85	85	107	114	80	70	80
70	35	35	76	80	58	87	89	85	86	96	92	80	85	70
40	67	50	80	120	50	95	78	75	107	91	100	90	60	65
70	42	80	90	23	100	110	110	72	100	100	92	70	75	80
40	30	0	100	100	80	116	112	80	110	100	110	80	60	72

f0=orange	f1=green	f2=blue	r1=red	r2=purple

Figure 4.13: The plant heights measured in cm at the ARC on the 17th of April 2012

100	100	107	75	120	105	23	13	12	80	40	60	80	85	87
110	113	116	107	100	110	10	20	12	70	76	90	40	100	110
90	105	99	93	110	110	13	13	20	70	80	70	0	70	90
102	110	100	90	110	110	10	13	20	80	90	60	100	0	80
110	116	100	104	100	100	20	16	15	83	85	75	100	75	102
83	70	100	10	15	6	90	75	55	40	90	99	85	110	40
55	80	100	23	15	15	105	70	92	70	73	92	100	85	75
100	90	69	12	16	12	100	110	93	80	80	95	82	100	87
77	70	101	25	25	16	80	10	110	80	75	80	75	82	100
70	85	80	12	21	12	93	95	0	62	85	100	80	85	90
75	0	98	100	100	110	92	0	97	25	15	11	90	80	60
83	85	95	100	60	60	85	55	100	11	20	15	65	70	70
70	90	90	110	122	80	30	90	82	20	15	20	0	60	60
75	94	120	110	122	110	0	100	100	15	22	10	50	90	65
82	102	103	118	90	110	90	100	70	15	15	20	50	35	62
20	17	17	90	110	90	75	92	80	80	86	95	82	80	67
10	13	120	90	90	80	112	100	85	90	90	90	90	80	80
15	15	10	102	115	90	110	110	80	85	70	65	70	90	82
10	30	16	100	114	117	110	110	110	92	75	82	80	95	110
19	16	9	90	115	110	100	90	70	79	65	92	80	80	110
110	90	93	100	85	110	105	85	80	95	80	80	60	25	20
110	111	109	115	100	90	100	115	100	80	70	70	65	22	15
93	90	102	110	54	120	80	104	86	87	78	70	25	13	15
93	102	0	10	84	110	108	100	75	80	87	90	20	10	19
93	70	89	90	102	90	100	90	70	80	0	73	12	25	15

f0=orange | f1=green | f2=blue | r1=red | r2=purple

Figure 4.14: UP experimental farm heights of plants measured in cm on the 19th of April 2012

Table 4.4: The average height of varieties of plants for the two locations

ARC	f0=orange	f1=green	f2=blue	r1=red	r2=purple
averages	75.70	60.30	69.80	64.40	88.50
UP	f0=orange	f1=green	f2=blue	r1=red	r2=purple
averages	85.20	87.60	80.70	80.70	16.60

Statistical analysis and comparison was done to identify the best performing variety in the various categories. The software program used, GenStat (version 15.1), uses algorithms that are specifically designed for use with the Latin square designed experiments. The data produced showed a significant difference between the varieties. The second variety received from the ARC (r2=purple) were the shortest since they were planted at the UP experimental farm at a later stage, as there was a lack of viable plants in trays ready for transplantation. Therefore these datasets (r2=purple) were excluded for the plant height aspects and the statistical analysis was redone. The Latin square software's parameters could therefore not be used for this analysis and instead the data was entered into the same programme but as four different sets of experiments. This result showed no specific variety had an increase in rate of growth (Appendix A). However, all varieties planted at UP's experimental farm, are taller than the varieties at the ARC. The average height at UP (83.53 cm) is higher than the average height at ARC (67.53 cm), these values are statistically significantly different between the locations.

The differences between the two locations can be because the plants at the ARC were planted on top of ridges (30cm from level with 45° inclination) whereas the plants at UP's experimental farm were planted on a more level surface. Planting on top of the ridges could have given rise to more side branch development and less to height growth as is also observed by Simon *et al*. (1990)'s spacing trials.

4.3.3. Wet plant mass

The wet mass of aerial plant parts per variety per block per location are shown in Figure 4.15 and 4.16.

11.80	13.90	17.50	16.50	22.90
13.40	15.60	22.30	17.30	17.15
17.95	12.75	16.00	14.30	14.10
12.05	19.65	13.80	17.85	18.75
5.60	12.15	16.00	19.35	14.35

Figure 4.15: ARC wet mass of aerial plant parts in kg per block

16.95	16.00	0.75	10.00	9.75
15.75	2.40	13.45	11.15	11.95
18.65	16.75	13.20	1.10	5.80
2.40	18.00	17.09	11.66	10.05
19.95	21.16	17.5	12.55	2.70

Figure 4.16: UP experimental farm wet mass in kg per block

The values from Figures 4.15 and 4.16 were averaged in Table 4.5 as per variety of plants per area.

Table 4.5: Averages of the variety per area for wet mass (kg)

ARC:	f0=orange	f1=green	f2=blue	r1=red	r2=purple
averages	15.48	14.13	13.84	14.72	20.43
UP:	f0=orange	f1=green	f2=blue	r1=red	r2=purple
averages	14.31	14.53	13.96	14.67	1.87

The Genstat data with the four different sets of combined experiments excluding the second variety received from the ARC (r2=purple), revealed no statistically significant differences between the wet mass of the different varieties of the plants (Appendix A pages 100 and 116). These results seem to be supporting the height data that showed no specific variety doing better. The wet mass averages for the two locations were also similar (ARC: 14.54kg to LC: 14.38kg).

4.3.4. Dry plant mass

The dry mass of the aerial plant parts (Figures 4.17 and 4.18) are between 25% and 40% of what the wet mass was. In Table 4.6 the variety averages between the different locations are compared.

4.60	4.95	5.70	4.30	6.90
4.50	5.35	7.45	6.20	5.90
5.85	4.40	4.95	4.70	5.80
4.65	7.20	4.90	5.70	7.40
2.15	5.05	5.40	7.10	5.55

Figure 4.17: The average dry plant mass per block for the ARC in kg.

5.70	5.80	0.20	3.70	2.35
5.25	0.60	3.50	2.50	3.70
6.30	2.40	5.30	0.30	2.15
0.60	5.90	4.65	3.35	3.35
5.10	6.80	5.85	2.50	0.65

Figure 4.18: The average dry mass per block for UP in kg

Table 4.6: The average dry mass of plant material per variety per location in kg

ARC:	f0=orange	f1=green	f2=blue	r1=red	r2=purple	Total average
averages	5.20	4.94	5.03	5.26	6.90	5.11
UP:	f0=orange	f1=green	f2=blue	r1=red	r2=purple	Total average
averages	3.34	4.62	4.51	4.76	0.47	4.31

The dry mass yield trend was similar to the wet mass yield as the group with the highest wet mass produces the highest dry mass. This differs slightly with the plant heights seeing as the plants from UP's experimental farm were on average 13cm higher than the plants at the ARC. The differences between the averages per location (UP 4.31kg and ARC 5.10kg) are not statistically significant.

4.3.5. Dry leaf mass

The results of the dry leaf mass can be seen in Table 4.7 and Figures 4.19 and 4.20.

1.89	0.93	1.05	0.86	1.59
0.66	0.92	1.28	1.14	1.13
1.41	1.00	1.09	0.90	1.25
0.99	1.43	1.11	1.09	1.37
0.49	1.105	1.28	1.42	0.90

Figure 4.19: Dry leaf mass of different blocks at the ARC in kg

1.21	1.15	0.10	0.59	0.70
0.98	0.15	0.89	0.68	0.78
1.28	0.96	0.55	0.09	0.49
0.13	1.08	1.01	0.92	0.75
1.07	0.96	0.84	0.57	0.15

Figure 4.20: Dry leaf mass of different blocks at the UP in kg

Table 4.7: Comparing of the average dry leaf mass per variety in kg

ARC:	f0=orange	f1=green	f2=blue	r1=red	r2=purple	Total average
Averages	1.03	0.96	1.23	1.01	1.42	1.13
UP:	f0=orange	f1=green	f2=blue	r1=red	r2=purple	Total average
Averages	0.90	0.83	0.80	0.94	0.12	0.72

In the above data one can see that no variety is outperforming another. It would however seem that the plants at the ARC might have performed a little better than the plants of UP in all cases, except for height, but the numbers are not statistically significant at $p<0.001$ (appendix A). One probable reason for the ARC plants having a higher mass production with lower height is the production of more side branches. Side branches would lead to higher amounts of leaf production and so an increase its biomass. The plants planted on top of the rows might have had more space for side branch development.

4.3.6. Artemisinin yields

The software program MestReNova allows one to standardise the NMR results by setting the integral value of maleic acid to 1. The artemisinin integrals subsequently adjust by the same ratio (Table 4.8). The differences in artemisinin percentages are averaged and shown between the two different locations and the different varieties (Tables 4.15, 4.16 and 4.17).

Table 4.8: The integrals and calculation of percentage artemisinin per gram of dry leaf mass

	UP		ARC	% Art / g dry leaf mass		
Block	Art integral		Art Integral	UP		ARC
A	0.38		0.34	0.37		0.33
B	0.27		0.28	0.26		0.27
C	0.45		0.29	0.44		0.28
D	0.31		0.52	0.30		0.51
E	0.5		0.25	0.49		0.24
F	0.43		0.24	0.42		0.23
G	0.55		0.31	0.53		0.30
H	0.24		0.40	0.23		0.39
I	0.24		0.27	0.23		0.26
J	0.61		0.37	0.59		0.36
K	0.42		0.33	0.41		0.32
L	0.48		0.23	0.47		0.22
M	0.57		0.31	0.55		0.30
N	0.37		0.3	0.36		0.29
O	0.23		0.32	0.22		0.31
P	0.55		0.27	0.53		0.26
Q	0.35		0.34	0.34		0.33
R	0.3		0.29	0.29		0.28
S	0.53		0.32	0.52		0.31
T	0.48		0.28	0.47		0.27
U	0.31		0.34	0.30		0.33
V	0.53		0.36	0.52		0.35
W	0.40		0.30	0.39		0.29
X	0.60		0.24	0.58		0.23
Y	0.38		0.34	0.37		0.33

Table 4.9: The percentage yield of artemisinin per variety and per location

Location ARC	f0=orange	f1=green	f2=blue	r1=red	r2=purple
	0.35	0.29	0.23	0.33	0.33
	0.27	0.33	0.28	0.31	0.26
	0.32	0.31	0.22	0.30	0.29
	0.39	0.26	0.23	0.36	0.30
	0.51	0.33	0.24	0.27	0.28
Average at ARC per variety	0.37	0.30	0.24	0.31	0.29
Total average at ARC combined varieties					0.30
Location UP	f0=orange	f1=green	f2=blue	r2=red	r2=purple
	0.37	0.58	0.39	0.52	0.30
	0.34	0.53	0.47	0.52	0.29
	0.36	0.55	0.47	0.41	0.22
	0.23	0.53	0.42	0.59	0.23
	0.37	0.49	0.30	0.44	0.26
Average UP per variety	0.33	0.54	0.41	0.50	0.30
Total average per variety	0.35	0.42	0.33	0.41	0.28
Total average at UP combined varieties					0.41

The yields within a variety are similar per location but between the locations performances of varieties differed (Table 4.7). This could indicate differences between the two locations.

It appears that the variety supplied by Prof Meyer (yellow variety, f0), was the highest artemisinin yielding variety at the ARC and that the first variety received from Dr Van der Kooy (green variety, f1), is the highest producing variety of artemisinin at the UP location. If one averages the percentage of yield over both locations for the best variety for the production of artemisinin, Dr Van der Kooy's first variety (green, f1) is the best. However, the Genstat program can identify only slight significant differences (p<0.001) between the varieties. This is because the varieties that are first and second in production of artemisinin differ only slightly from each other but both differ greatly from the lowest producing variety. The roles are however interchanged with a change of location.

If one compares the averages of the artemisinin production per location, the plants at UP outperformed the plants at the ARC location ($p<0.001$). Both of these sets of results are statistically significant. In other words, UP produced the highest plants with the highest artemisinin concentrations, while the ARC produced the plants with the greatest wet mass, dry mass and dry leaf mass.

It has already been suggested that the plants at the ARC had increased development of side branches leading to more plant material but it would seem as if the production of artemisinin could be linked to height of the plants or a competitive stress increasing the yield of artemisinin. Simon *et al.* (1990) and Damtew *et al.* (2011)'s data agrees with other studies showing a positive correlation between plant height and planting density in *A. annua* and other plants. There is however a threshold of plant density, when this is exceeded (plants planted too close to each other) height growth is also negatively influenced. Damtew *et al.* (2011)'s work also states that there is a negative correlation between the production of side branches and height in *A. annua.*

4.3.7. Metabolomics evaluation

Metabolomic evaluation was done by importing the NMR spectra into MestReNova for editing and then for statistical evaluation into SIMCA. Results are shown in Figures 4.21 to 4.26. The PCA spectrum in Figure 4.21 shows a comparison between al the samples. It does not ''discriminate'' on the varieties but slightly on the localities.

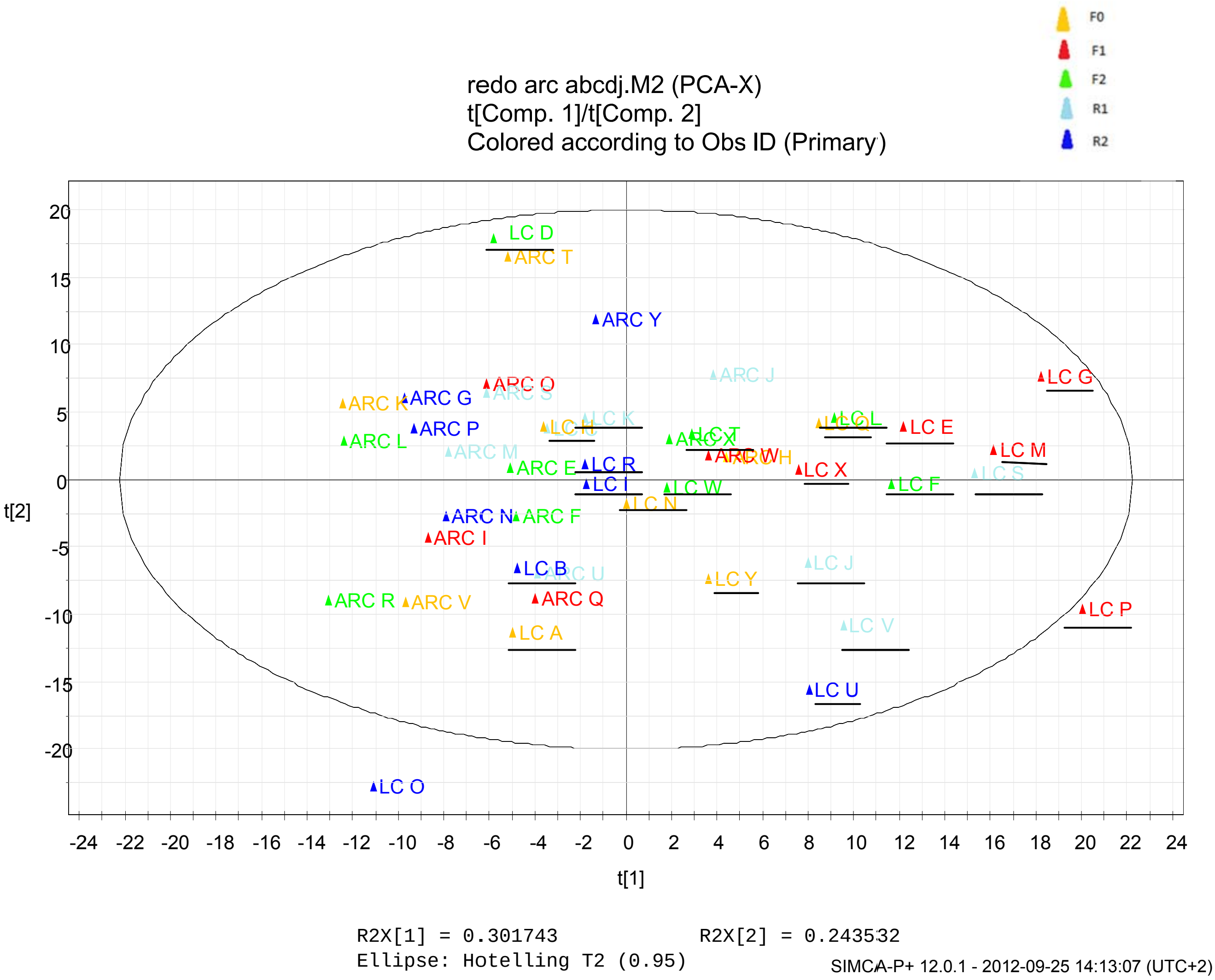


Figure 4.21: PCA spectrum generated by SIMCA, comparing the five different varieties, each coloured differently (underlined samples are the UP samples)

The varieties do not group well and are scattered (Figure 4.21). However one can already see from this PCA figure that there seems to be a grouping on the two localities. ARC plants group more to the left and the UP plants more to the right, irrespective of varieties. The different localities were then individually put through the same process (Figures 4.22 and 4.23) giving only the grouping for a single location.

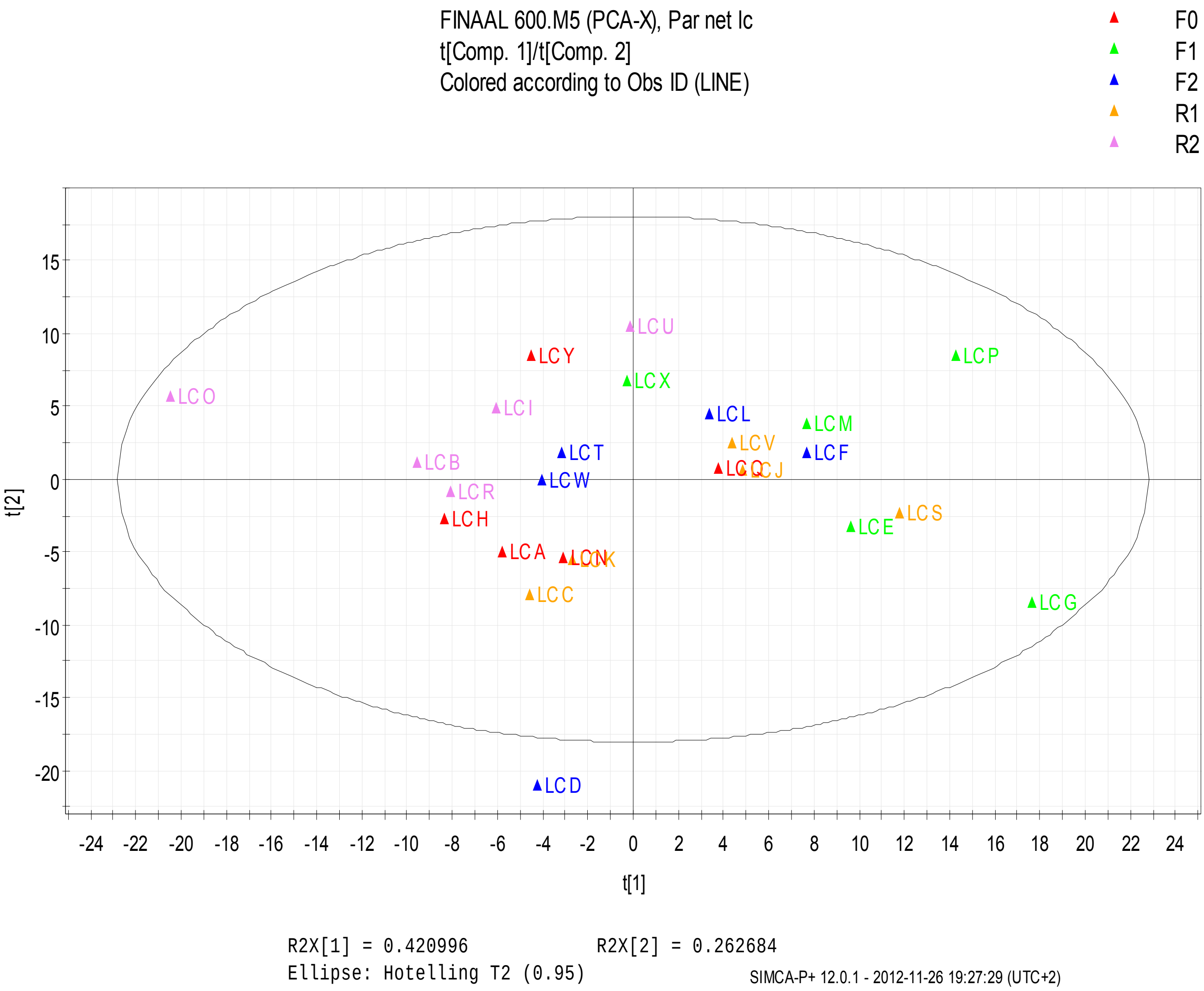


Figure 4.22: PCA plot for the different varieties just at UP

There is slight grouping visible for the different varieties. Strong grouping might indicate a variety that contains something unique not present in the others. It could possibly provide a reason for increase in yields between varieties and indicate differences that might lead to Wallaart *et al.* (2000)'s proposed existence of chemotypes. Wallaart *et al.* (2000) proposes the idea that chemotypes exist between locations of origin of *A. annua*. In our experiment the varieties were obtained from two different continents. Three varieties have been propagated in South Africa for a few trial years and the other varieties were produced in Eastern Europe. This was the first trial year for the European varieties in South Africa and a slight grouping might indicate the chemotype or another influence.

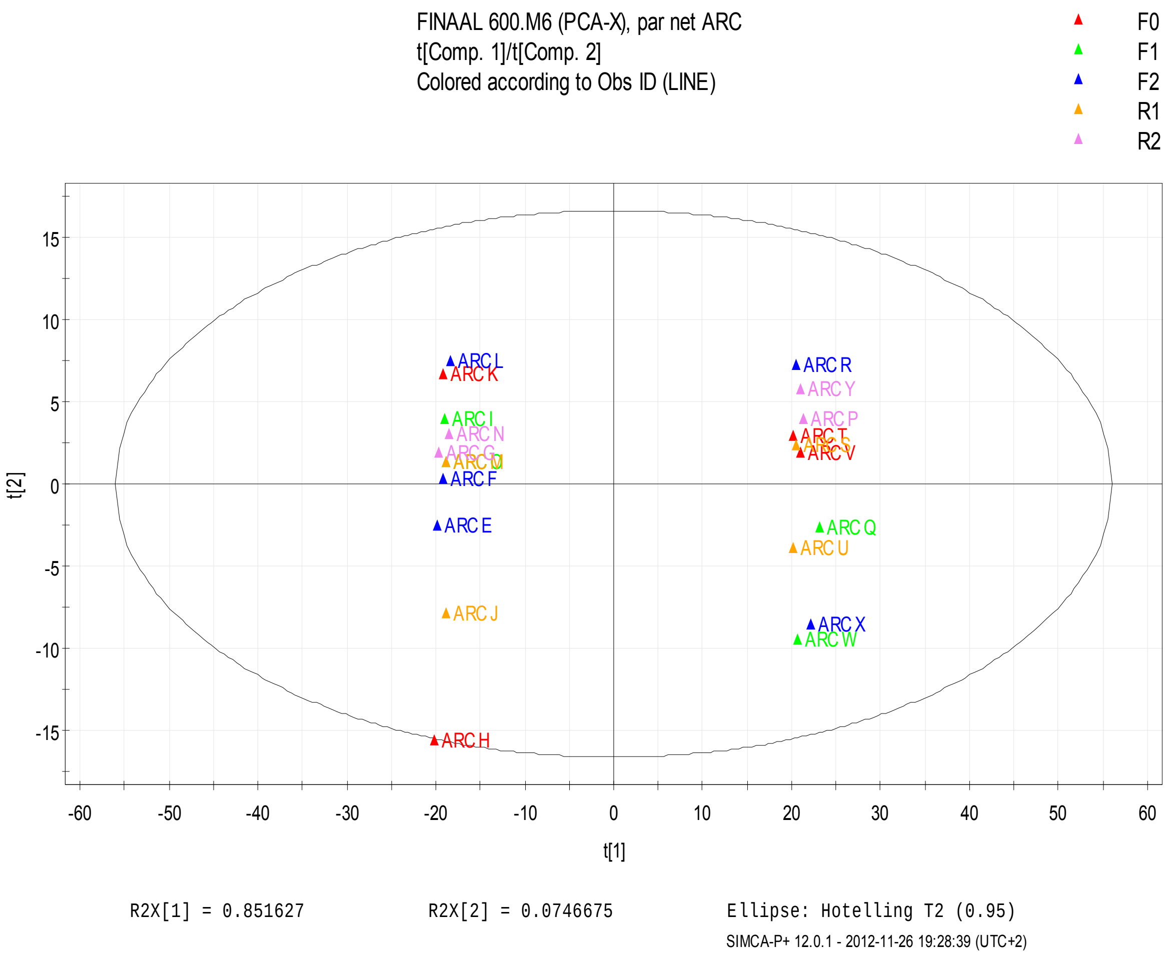


Figure 4.23: PCA spectrum for the varieties just at ARC

Figure 4.22 and 4.23 differ completely. Figure 4.23 show the comparison of the varieties at the ARC only. There is a great amount of grouping but the grouping is not because of the varieties. At the ARC the varieties are still not grouping, but there appears to be clear difference between some blocks. This was further investigated to determine the cause of the split. The spectrum was redone excluding chemical shifts of water and methanol (Figure 4.24). This was done because it was recalled that two separate methanol bottles were used during the NMR extraction.

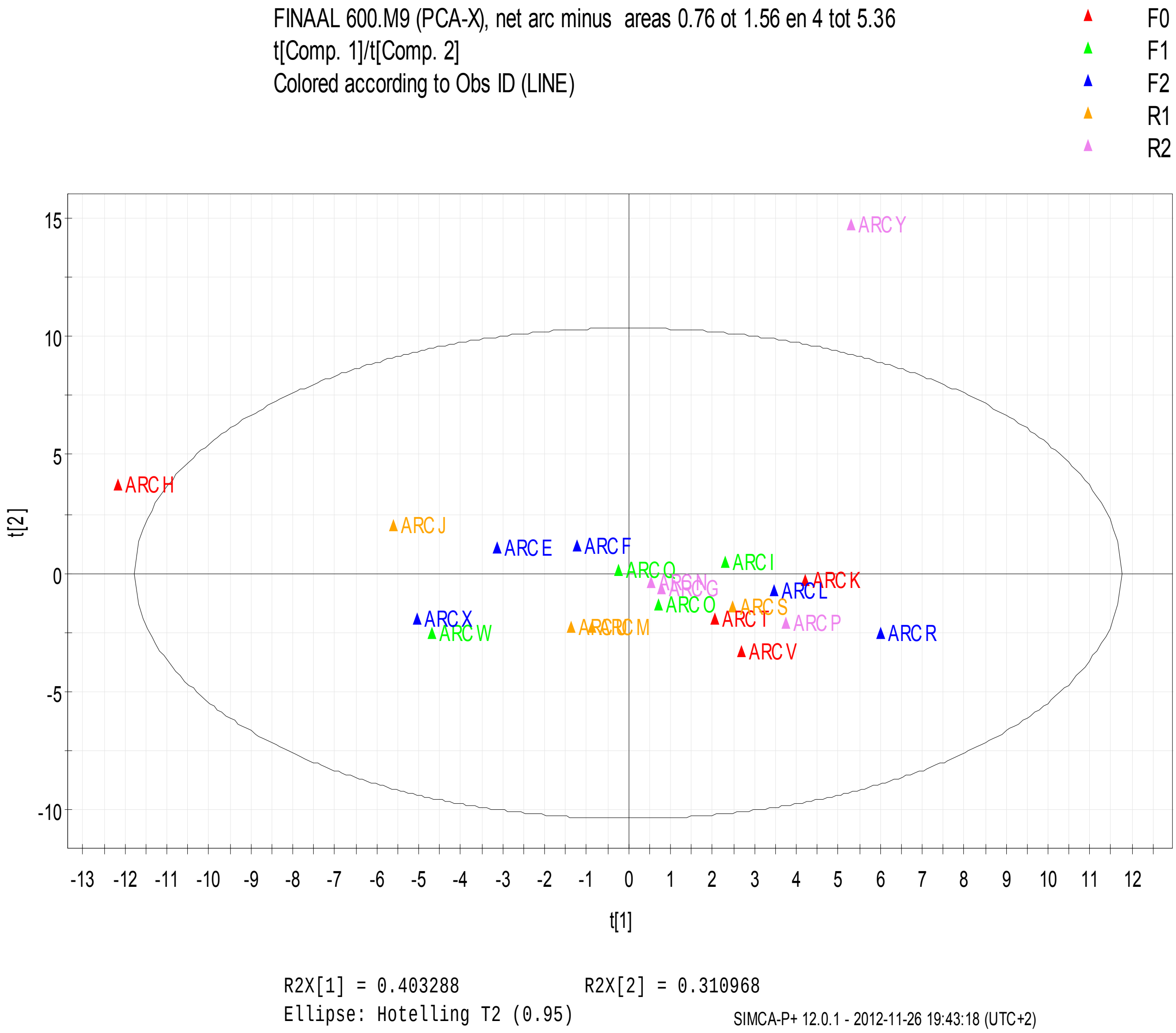


Figure 4.24: PCA spectrum of plants grown at the ARC with H_20 and MeOH chemical shifts excluded that probably lead to the severe split in Figure 4.23.

With the removal of values of methanol and water from the data, no grouping is seen between varieties. It is interesting that the metabolic analysis could discriminate between two bottles of methanol from different suppliers. In summary, grouping could not be clearly seen from the PCA plots on variety but slight grouping was seen per location.

OPLS spectra (Figure 4.26) in general are more sensitive and ''discriminate'' more efficiently than standard PCA spectra. This is because discrimination factors can be entered. In Figure 4.26 the discriminating factor entered was the locations of the samples. Clear grouping occurs between the samples from the ARC and UP. The varieties again only showed slight grouping.

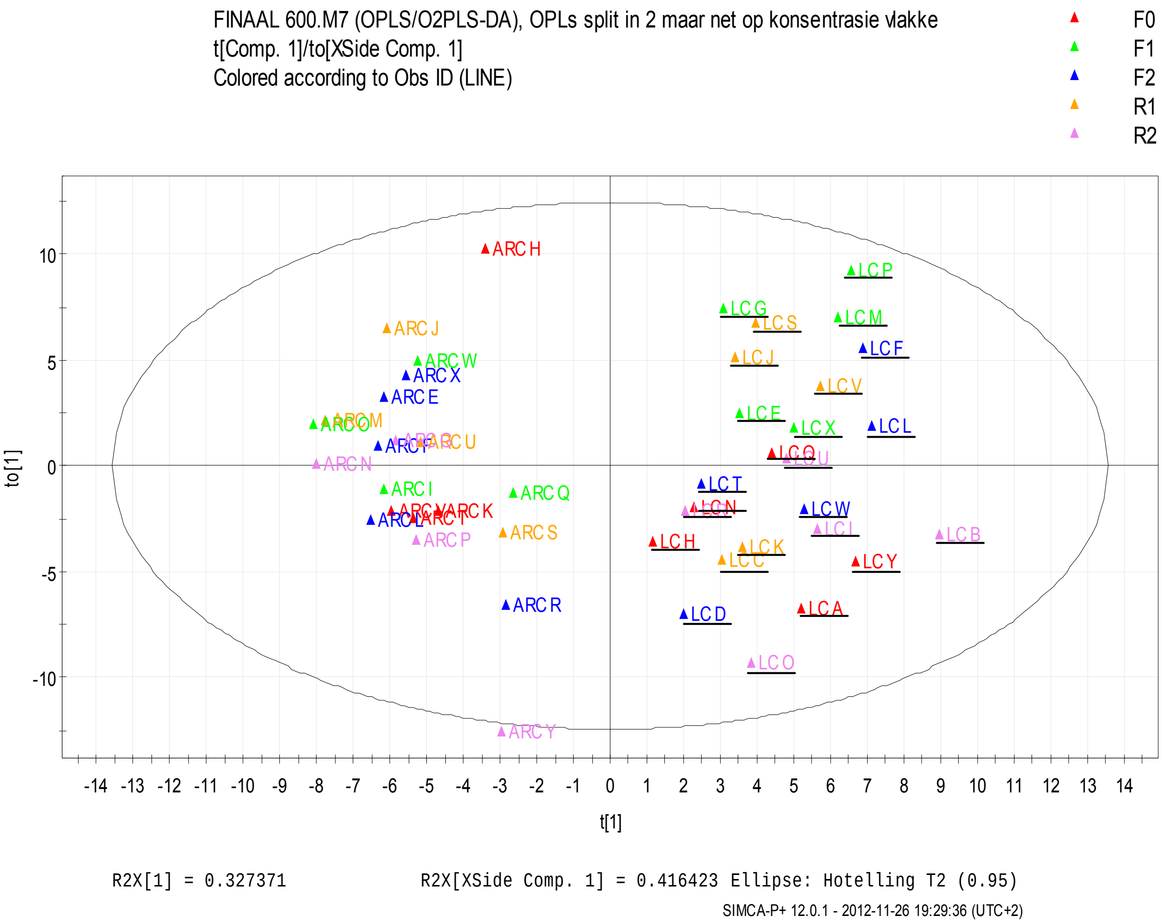


Figure 4.25: OPLS plot of the different varieties with the two locations grouping well (UP varieties underlined)

It is apparent that the grouping occurs between the two locations and this suggests that there are differences between the locations as seen in the data produced by the growth rates/heights, relative masses and artemisinin yields. A contribution plot was drawn up (Figure 4.26) and the differences between the two locations seem to be only on one factor, the concentrations of compounds, which could correlate with the findings pertaining to the soil compositions and concentrations of artemisinin produced. Other chemicals, precursors and metabolic paths pertaining to higher secondary metabolite productions due to stress would also be higher.

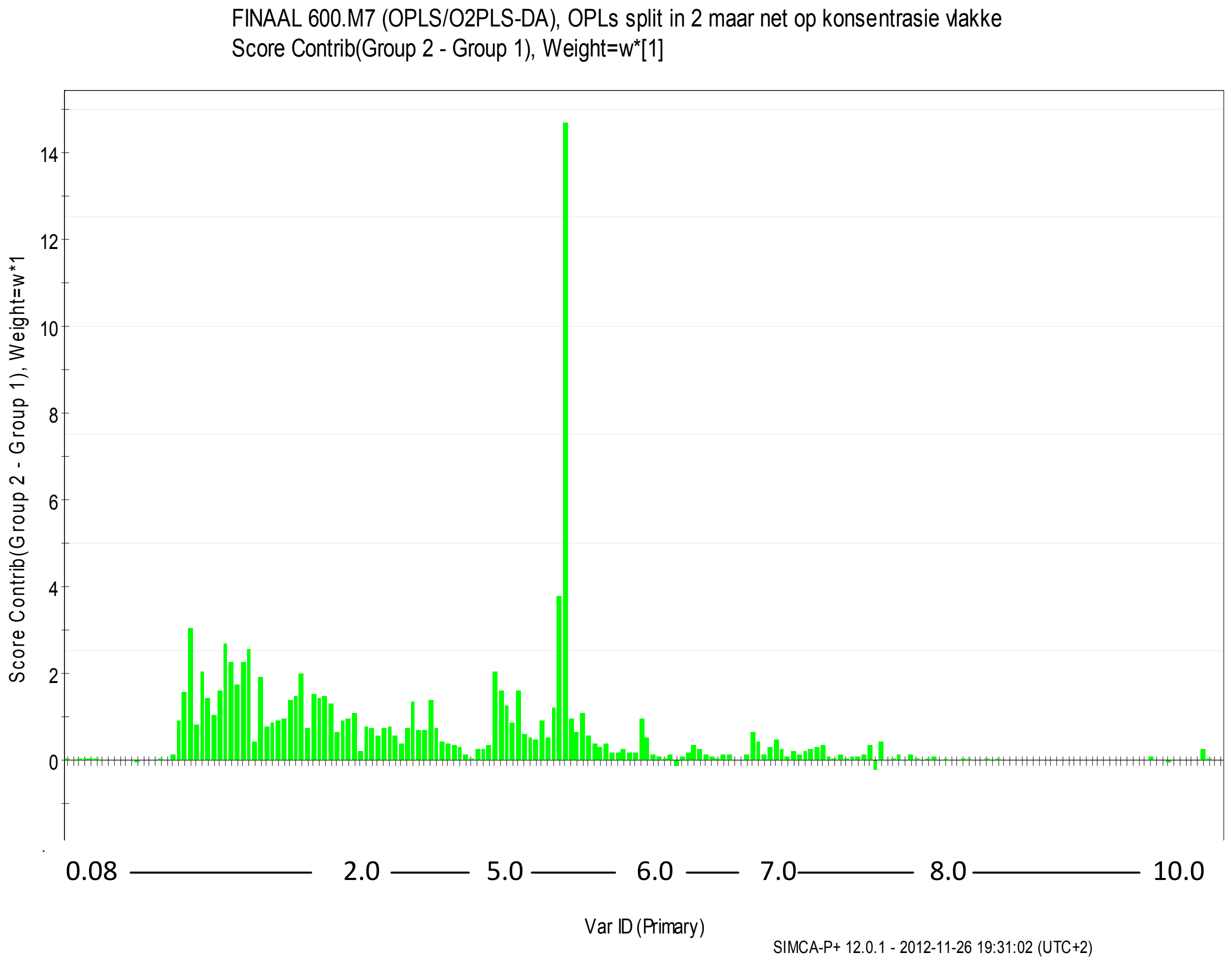


Figure 4.26: Contribution plot of all varieties on both locations. ARC plants (top) showing higher concentrations in general (buckets 3-4 removed).

The soil of the ARC contained higher concentrations of elements and also produced higher plant mass. The ARC samples could contain higher concentrations of most compounds except for artemisinin and other secondary compounds which could be higher at UP. The positive bars represent higher concentrations of compounds at the ARC and the negative bars represent compounds that occur at higher concentrations at UP. The few negative bars could possibly represent compounds involved in the metabolomic pathway of artemisinin.

A summary of all the parameters analysed can be seen in Table 4.10 which summarizes the data in all the different categories for the different varieties and two locations. The values are also shown for the yields that can be obtained per hectare.

The yields of artemisinin are low at an average of 0.3% as yields of more than 1.0% are required to meet the current market trend.

In Table 4.10 the column, % yield of dry mass on wet mass, gives an estimation of the water content of the plants. On average the plant's wet mass contained 65% water mass. The percentages can be useful when conducting experiments to compare water loss between varieties. The average dry mass of just the leaves is 20% of the total dry mass of the plant including stems. The average percentage of the dry leaf mass that will be harvested from the initial wet mass is about 6.50%. That suggests for the sale of a kg of dry leaf material for about 15kg wet mass plant material will be required.

The variety that produced highest artemisinin percentage at the ARC was the variety produced by Professor Meyer (f0=yellow), this variety might have a better epigenetic background as the seeds were collected from a water stressed (speculated to increase artemisinin yield) trial group, and at UP it was Dr Van der Kooy's first variety (f1=green). The variety that best performed in this category considering an average between both locations was again Dr Van der Kooy's first variety.

The plants that produced the highest wet mass and thus the highest dry leaf mass at the ARC was Professor Meyer's variety and at the UP was ARC's first variety (r1=red). The best average between the two locations was however Dr Van der Kooy's second variety (f2=blue).

Table 4.10: A summary of all the growth data and artemisinin concentrations.

	Varieties Averages for ARC						Varieties Averages for UP						Total varieties average				
Locality	ARC	ARC	ARC	ARC	ARC	ARC Total Average	UP	UP	UP	UP	UP	UP Total Average	Averages of two areas combined				
Varieties	f0=orange	f1=green	f2=blue	r1=red	r2=purple	All varieties at ARC	f0=orange	f1=green	f2=blue	r1=red	r2=purple	All varieties at UP	f0=orange	f1=green	f2=blue	r1=red	r2=purple
Plant height @1 month (cm)	28.49	19.61	23.19	15.81	18.01	21.02	40.81	48.17	41.69	35.11	4.69	34.10	34.65	33.89	32.44	25.46	11.35
Plant height @ 3 months (cm)	75.68	60.29	69.76	64.39	88.49	71.72	85.20	87.57	80.67	80.67	16.61	70.14	80.44	73.93	75.21	72.53	52.55
Wet mass (kg/ha)	24571.40	22428.55	21968.23	23365.06	32428.54	24952.36	22717.44	23063.47	22158.71	23285.69	2968.25	18838.71	23644.42	22746.01	22063.47	23325.37	17698.40
Dry mass (kg/ha)	8253.96	7841.26	7984.12	8349.20	10952.37	8676.18	5301.58	7333.33	7158.72	7555.55	746.03	5619.04	6777.77	7587.29	7571.42	7952.37	5849.20
Dry leaf mass (kg/ha)	1636.51	1520.63	1946.03	1601.59	2260.32	1793.01	1434.92	1323.81	1274.60	1485.71	192.06	1142.22	1535.71	1422.22	1610.32	1543.65	1226.19
% yield of dry mass on wet mass	33.59	34.96	36.34	35.73	33.77	34.88	23.34	31.80	32.31	32.45	25.13	29.00	28.46	33.38	34.33	34.09	29.45
% yield of dry leaf mass on wet mass	6.66	6.78	8.86	6.85	6.97	7.22	6.32	5.74	5.75	6.38	6.47	6.13	6.49	6.26	7.31	6.62	6.72
% yield of dry leaf mass on dry mass	19.83	19.39	24.37	19.18	20.64	20.68	27.07	18.05	17.80	19.66	25.74	21.67	23.45	18.72	21.09	19.42	23.19
Artemisinin %	0.37	0.31	0.24	0.32	0.29	0.31	0.33	0.54	0.41	0.49	0.26	0.41	0.35	0.42	0.33	0.40	0.28
Artemisinin (kg/ha)	6.02	4.64	4.73	5.05	6.64	5.42	4.80	7.13	5.21	7.34	0.50	5.00	5.41	5.89	4.97	6.19	3.57

The best overall variety is difficult to select. This is because of the criteria for selection. If selection is to be made according to an economical scale for the best combination (percentage yield and wet mass) for large scale production the best performing line is Kleynhans' second variety (r2=red). This is because of the interactions involved in the two processes. If one produces optimal factors for primary metabolite production secondary metabolite production will not be as high and the inverse is true too. Omer *et al.* 2013 showed this by comparing the gram artemisinin per plant Figure 4.27. His results had two different yields of artemisinin per soil type (sandy soil had higher production of artemisinin, clay soil better wet mass production). Our results also support this concept when examining the last variety in Table 4.10 showing the kg artemisinin produced per hectare and that the amounts are more evenly distributed.

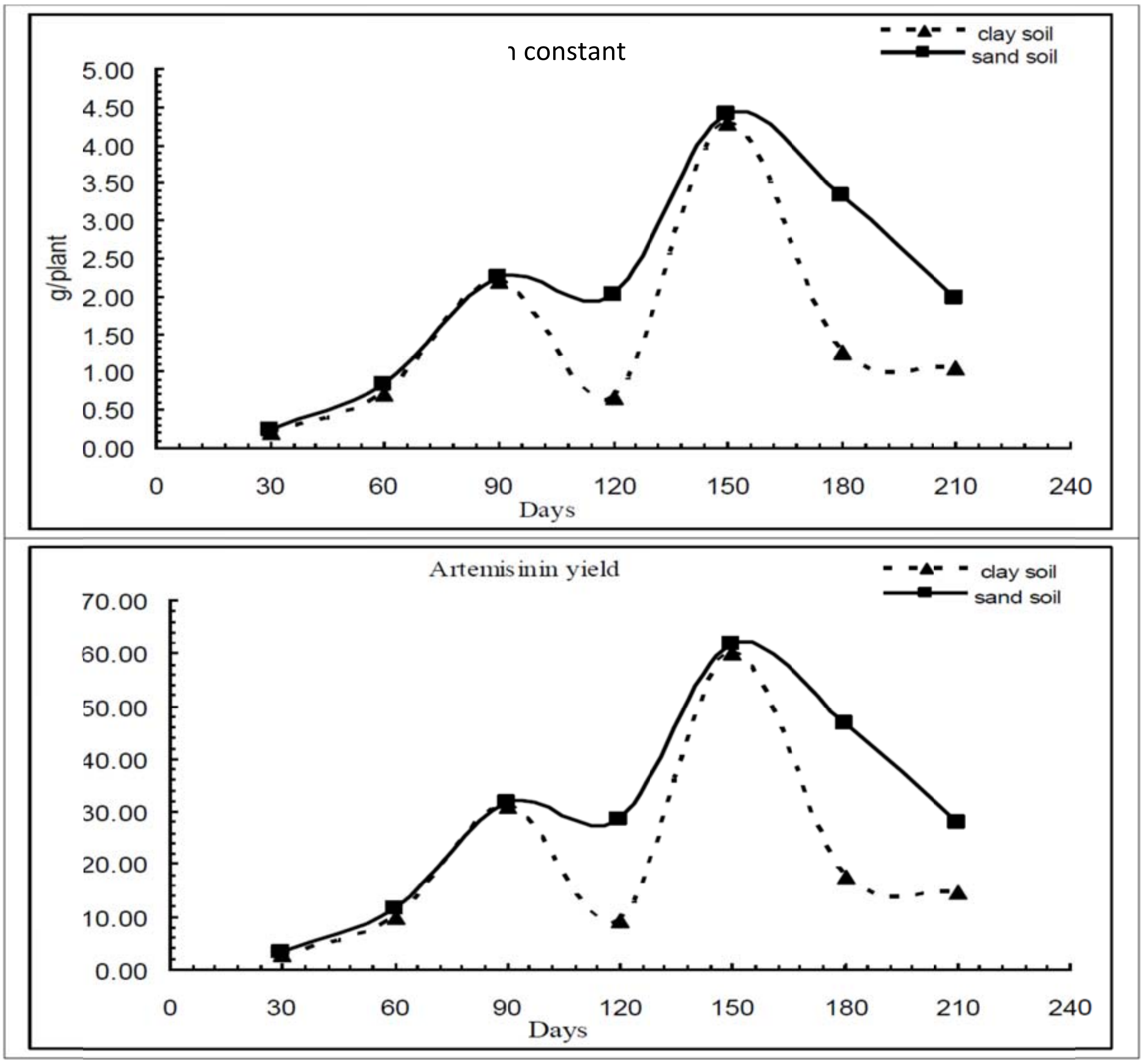


Figure 4.27: The amount of artemisinin per plant and per field (Omer *et al.*, 2013).

Chapter 5: General discussion and conclusion

In conclusion it would seem as if there are a number of factors which can influence the production of artemisinin. Stresses like competition, soil nutrients and even water could lead to the production of greater yields. Varieties bred for high production can be used for follow-up years of high production if conditions are kept appropriately constant, however the effects of location and the stresses paired with the large scale production might be sufficient to lower the yields.

Much can be learnt from this study and conditions can be adapted in order to produce even higher concentrations of artemisinin in *Artemisia annua* which could lead to even greater gains on a larger scale.

The correct manipulation of circumstances could lead to great increases in an array of yield possibilities. This study showed that plant spacing can be used to manipulate the length or mass and the production of side branches in *A. annua*. However, if the increase in the yield of artemisinin is the goal, these factors' roles could be reversed. Careful notification must be made of the stresses received by the plants as this study showed that a new stress on the plant increased its artemisinin yield, this has to be followed up in consecutive seasons as this trial was only to screen for the effects of more natural South African conditions on *A. annua*.

This study also showed that there are many avenues for the production of an artemisinin GMO to be explored. Even with the limited success of the tissue culturing of the GMO *A. afra* it showed promise for further attempts at this process. An alteration in the protocol to make use of the leaf material of a host plant for calli production is advised, even if shooting might not occur the calli could produce artemisinin in that case.

The idea of endophytes producing artemisinin might not be too far from accurate as it has already been shown that the addition of endophytic elicitors increased the production of artemisinin in the host *A. annua* plants. The application of endophytes could even be applied in tissue culture scenarios as another avenue to be explored.

5.1. TISSUE CULTURING OF GMO *ARTEMISIA AFRA*

Artemisinin can be produced in tissue cultures of *A. annua* as proven by Wang *et al.* (2001) but the mother material should be from leaves as our study found and this agrees with Nair *et al.'s* (1986) finding. This could be because trichomes are found lacking on roots and they do not naturally produce artemisinin. The leaf trichomes and material originating from leaf cuttings contain trichomes and more readily produce artemisinin. It was also shown by Liu *et al.* (2010) that the host plant *A. afra* does not produce artemisinin. Thus if gene transfer was successful and the callus originated from leaf not root material, leaves would still have to be produced for the expression of artemisinin to be realised.

5.2. PRODUCTION OF ARTEMISININ BY ENDOPHYTES

Many endophytes have been identified in Artemisia species as mentioned by Wang *et al.* (2001), but none have been identified in *A. annua* that might be responsible for the production of artemisinin as our study also showed. However the influence of endophytes cannot be underestimated as shown by Kampoor *et al.* (2007). Their studies show that the addition of certain mycorrhiza and their interactions with the host plants could increase the density of trichomes and overall artemisinin production. This information is of great importance in the studies to increase the yields of artemisinin without detrimental effects on the general production of the plant.

5.3. INFLUENCE OF LOCATION ON HIGH YIELDING VARIETIES OF ARTEMISININ

Stress factors have an enormous role to play in the synthesis of secondary compounds in most plants as many studies have showed. Most studies focused on the general production of plants i.e. height, amount of foliage, etc. Limiting stress factors are the main goals of most of these studies. They attempt to increase traits like height, biomass, yield, etc., usually linked to primary metabolism and production of the plants, by stimulating them with additional positive factors like fertilization and nutrient supplementation. In *A. annua* the production of these traits can be increased by a manipulation of the environment (i.e. plant density) or addition of a nutrient source (N-fertilizer). These points were confirmed by Damtew *et al.* (2011) and Singh (2000), but they also showed that there are threshold values related to these factors and that production will decrease once these thresholds have been passed.

Stress factors are believed to be responsible for the increase in production of secondary metabolites (Fluck, 1955; Gershenzon, 1984; Selmar and Kleinwachter, 2013). Artemisinin is a secondary metabolite thus it is expected that stress factors will increase its production. From our study and confirmed by Omer *et al.* (2012), there seems to be a negative relationship between primary and secondary metabolism. With the increase of stress factors the primary production declines and secondary production increases. But these effects are nullified on the ‘economic production scale’ because a stressed plant might produce more secondary metabolites but at the cost of plant mass (a primary production point).

From this study and suggestions by Omer *et al.* (2012) and Kampoor *et al*. (2007) it is suggested that focus for the increased production of artemisinin should rather take into consideration the effect on primary plant production. Methods should be investigated that can increase the yield of artemisinin without limiting primary production. Omer *et al.* (2012) showed that micro-elements like Zn and Mn might play a role without limiting production and Kampoor *et al*. (2007) found that yields increased by introduction of mycorrhiza.

The locations will have an influence on general production and the synthesis of artemisinin, as our study showed each location has its own set of stress factors to be accounted for and taken into consideration.

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Appendix A: Comprehensive Statistics:

file Artemisia annua 2012.gen
================== Artemisia annua data

Message: You have input sufficient data, READ terminated.

Identifier	Minimum	Mean	Maximum	Values	Missing	
Plantht1	3.470	28.62	55.53	50	0	
Plantht2	15.33	74.71	105.2	50	0	
Flowering	0.0000	0.5000	3.000	50	0	Skew
Freshmass	0.7500	13.79	22.90	50	0	
Drylfmass	0.09000	0.9276	1.890	50	0	
Artemisinin	0.2300	0.3664	0.6100	50	0	

Identifier	Values	Missing	Levels
LOC	50	0	2
ROW	50	0	5
COL	50	0	5
LINE	50	0	5
REP	50	0	5

======================== All five lines ===================

Analysis of variance

Variate: Plantht1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	2120.31	2120.31	35.13	<.001
Residual	8	482.90	60.36	2.48	
Loc.REP.*Units* stratum					
LINE	4	4407.31	1101.83	45.33	<.001
LOC.LINE	4	2414.12	603.53	24.83	<.001
Residual	32	777.82	24.31		
Total	49	10202.46			

Tables of means

Variate: Plantht1

Grand mean 28.62

LOC	Roodeplaat	LC
	22.11	35.13

LINE	1	2	3	4	5
	35.59	36.66	33.65	25.71	11.48

LOC	LINE	1	2	3	4	5
Roodeplaat		28.13	23.22	25.09	15.81	18.27
LC		43.04	50.11	42.21	35.60	4.69

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	25	10	5
e.s.e.	1.554	1.559	2.511
d.f.	8	32	33.07
Except when comparing means with the same level(s) of			
LOC			2.205
d.f.			32

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	25	10	5
l.s.d.	5.067	4.491	7.223
d.f.	8	32	33.07
Except when comparing means with the same level(s) of			
LOC			6.351
d.f.			32

Stratum standard errors and coefficients of variation

Variate: Plantht1

Stratum	d.f.	s.e.	cv%
Loc.REP	8	3.475	12.1
Loc.REP.*Units*	32	4.930	17.2

Fisher's protected least significant difference test

LINE

	Mean	
2	36.66	a
1	35.59	a
3	33.65	a
4	25.71	b
5	11.48	c

Fisher's protected least significant difference test

LOC.LINE

	Mean	
LC 2	50.11	a
LC 1	43.04	b
LC 3	42.21	b
LC 4	35.60	c
Roodeplaat 1	28.13	d
Roodeplaat 3	25.09	d
Roodeplaat 2	23.22	de
Roodeplaat 5	18.27	ef
Roodeplaat 4	15.81	f
LC 5	4.69	g

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	28.13	80.14	43.04	28.61
2	23.22	65.13	50.11	18.01
3	25.09	59.75	42.21	17.07
4	15.81	19.31	35.60	17.95
5	18.27	8.54	4.69	0.68
Margin	22.11	59.81	35.13	276.95

LOC	Margin	
	Mean	Variance
LINE		
1	35.59	110.07
2	36.66	237.77
3	33.65	115.48
4	25.71	125.31
5	11.48	55.28
Margin	28.62	208.21

LOC	REP	COL	LINE	Plantht1	FITTED	RESIDUAL
Roodeplaat	1	1	3	22.86	23.63	-0.769
Roodeplaat	1	2	4	18.40	14.35	4.051
Roodeplaat	1	3	2	16.23	21.75	-5.523
Roodeplaat	1	4	1	29.38	26.67	2.713
Roodeplaat	1	5	5	16.33	16.80	-0.473
Roodeplaat	2	1	1	22.23	25.23	-2.997
Roodeplaat	2	2	2	18.50	20.31	-1.813
Roodeplaat	2	3	5	17.67	15.36	2.307
Roodeplaat	2	4	4	18.87	12.91	5.961
Roodeplaat	2	5	3	18.73	22.19	-3.459
Roodeplaat	3	1	5	19.07	13.62	5.449
Roodeplaat	3	2	1	16.15	23.48	-7.335
Roodeplaat	3	3	4	16.00	11.17	4.833
Roodeplaat	3	4	3	18.21	20.45	-2.237
Roodeplaat	3	5	2	17.86	18.57	-0.711
Roodeplaat	4	1	4	8.20	17.15	-8.951
Roodeplaat	4	2	5	15.40	19.60	-4.205
Roodeplaat	4	3	3	29.21	26.43	2.779
Roodeplaat	4	4	2	29.00	24.55	4.445
Roodeplaat	4	5	1	35.40	29.47	5.931
Roodeplaat	5	1	2	34.50	30.90	3.601
Roodeplaat	5	2	3	36.46	32.77	3.685
Roodeplaat	5	3	1	37.50	35.81	1.687
Roodeplaat	5	4	5	22.87	25.95	-3.079
Roodeplaat	5	5	4	17.60	23.49	-5.895
LC	1	1	2	55.53	50.96	4.571
LC	1	2	4	42.40	36.45	5.947
LC	1	3	5	4.73	5.55	-0.817
LC	1	4	3	39.33	43.06	-3.729
LC	1	5	1	37.92	43.89	-5.973
LC	2	1	3	40.07	40.79	-0.717
LC	2	2	5	4.40	3.27	1.125
LC	2	3	1	44.21	41.62	2.589
LC	2	4	4	31.67	34.18	-2.511
LC	2	5	2	48.20	48.69	-0.487
LC	3	1	4	36.86	36.47	0.385
LC	3	2	1	49.00	43.91	5.085
LC	3	3	2	50.46	50.98	-0.521
LC	3	4	5	5.27	5.57	-0.299
LC	3	5	3	38.43	43.08	-4.651
LC	4	1	5	3.47	4.36	-0.895
LC	4	2	2	52.13	49.78	2.353
LC	4	3	3	47.73	41.88	5.853
LC	4	4	1	37.07	42.71	-5.641
LC	4	5	4	33.60	35.27	-1.671
LC	5	1	1	47.00	43.06	3.939
LC	5	2	3	45.47	42.23	3.243
LC	5	3	4	33.47	35.62	-2.151
LC	5	4	2	44.21	50.13	-5.917
LC	5	5	5	5.60	4.71	0.885

Analysis of variance

Variate: Plantht2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	8.95	8.95	0.11	0.749
Residual	8	652.52	81.56	0.94	
Loc.REP.*Units* stratum					
LINE	4	5089.05	1272.26	14.71	<.001
LOC.LINE	4	16766.74	4191.69	48.47	<.001
Residual	32	2767.54	86.49		
Total	49	25284.80			

Tables of means

Variate: Plantht2

Grand mean 74.7

LOC	Roodeplaat	LC
	75.1	74.3

LINE	1	2	3	4	5
	84.8	78.3	75.6	79.4	55.4

LOC	LINE	1	2	3	4	5
Roodeplaat		79.7	65.7	69.8	68.6	92.0
LC		89.9	90.9	81.5	90.3	18.8

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	25	10	5
e.s.e.	1.81	2.94	4.14
d.f.	8	32	39.98
Except when comparing means with the same level(s) of			
LOC			4.16
d.f.			32

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	25	10	5
l.s.d.	5.89	8.47	11.82
d.f.	8	32	39.98
Except when comparing means with the same level(s) of			
LOC			11.98
d.f.			32

Stratum standard errors and coefficients of variation

Variate: Plantht2

Stratum	d.f.	s.e.	cv%
Loc.REP	8	4.04	5.4
Loc.REP.*Units*	32	9.30	12.4

Fisher's protected least significant difference test

LINE

	Mean	
1	84.78	a
4	79.42	ab
2	78.27	ab
3	75.64	b
5	55.43	c

Fisher's protected least significant difference test

LOC.LINE

		Mean	
Roodeplaat	5	92.03	a
LC	2	90.88	ab
LC	4	90.28	ab
LC	1	89.91	ab
LC	3	81.53	abc
Roodeplaat	1	79.65	bcd
Roodeplaat	3	69.76	cde
Roodeplaat	4	68.56	de
Roodeplaat	2	65.65	e
LC	5	18.83	f

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	79.65	154.5	89.91	64.0
2	65.65	64.7	90.88	123.1
3	69.76	44.7	81.53	158.1
4	68.56	115.3	90.28	75.0
5	92.03	38.7	18.83	17.0
Margin	75.13	167.1	74.29	886.0

LOC	Margin	
	Mean	Variance
LINE		
1	84.78	126.3
2	78.27	260.2
3	75.64	128.6
4	79.42	215.6
5	55.43	1513.2
Margin	74.71	516.0

LOC	REP	COL	LINE	Plantht2	FITTED	RESIDUAL
Roodeplaat	1	1	3	70.53	72.08	-1.548
Roodeplaat	1	2	4	62.57	70.88	-8.314
Roodeplaat	1	3	2	74.75	67.97	6.778
Roodeplaat	1	4	1	84.08	81.97	2.106
Roodeplaat	1	5	5	95.33	94.35	0.980
Roodeplaat	2	1	1	68.60	79.73	-11.128
Roodeplaat	2	2	2	61.69	65.73	-4.036
Roodeplaat	2	3	5	94.21	92.10	2.106
Roodeplaat	2	4	4	84.13	68.64	15.492
Roodeplaat	2	5	3	67.40	69.83	-2.432
Roodeplaat	3	1	5	87.07	85.81	1.260
Roodeplaat	3	2	1	64.85	73.43	-8.584
Roodeplaat	3	3	4	64.58	62.34	2.236
Roodeplaat	3	4	3	61.73	63.54	-1.808
Roodeplaat	3	5	2	66.33	59.43	6.898
Roodeplaat	4	1	4	57.07	67.06	-9.992
Roodeplaat	4	2	5	84.21	90.53	-6.318
Roodeplaat	4	3	3	69.00	68.26	0.744
Roodeplaat	4	4	2	71.20	64.15	7.050
Roodeplaat	4	5	1	86.67	78.15	8.518
Roodeplaat	5	1	2	54.29	70.98	-16.688
Roodeplaat	5	2	3	80.13	75.08	5.046
Roodeplaat	5	3	1	94.07	84.98	9.090
Roodeplaat	5	4	5	99.33	97.36	1.974
Roodeplaat	5	5	4	74.47	73.89	0.580
LC	1	1	2	105.20	93.29	11.913
LC	1	2	4	102.93	92.69	10.239
LC	1	3	5	15.33	21.24	-5.907
LC	1	4	3	73.93	83.94	-10.009
LC	1	5	1	86.08	92.32	-6.237
LC	2	1	3	82.00	76.63	5.365
LC	2	2	5	15.67	13.93	1.737
LC	2	3	1	84.14	85.01	-0.873

LC	2	4	4	80.07	85.39	-5.317
LC	2	5	2	85.07	85.98	-0.913
LC	3	1	4	90.14	87.11	3.027
LC	3	2	1	100.13	86.74	13.391
LC	3	3	2	83.92	87.71	-3.789
LC	3	4	5	16.60	15.66	0.941
LC	3	5	3	64.79	78.36	-13.571
LC	4	1	5	22.47	21.69	0.779
LC	4	2	2	100.20	93.74	6.459
LC	4	3	3	95.60	84.39	11.207
LC	4	4	1	82.40	92.77	-10.371
LC	4	5	4	85.07	93.14	-8.075
LC	5	1	1	96.79	92.70	4.089
LC	5	2	3	91.33	84.32	7.007
LC	5	3	4	93.20	93.07	0.125
LC	5	4	2	80.00	93.67	-13.671
LC	5	5	5	24.07	21.62	2.449

Analysis of variance

Variate: Freshmass

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	185.44	185.44	14.90	0.005
Residual	8	99.55	12.44	1.04	
Loc.REP.*Units* stratum					
LINE	4	93.15	23.29	1.95	0.126
LOC.LINE	4	679.60	169.90	14.22	<.001
Residual	32	382.41	11.95		
Total	49	1440.16			

Tables of means

Variate: Freshmass

Grand mean 13.79

LOC	Roodeplaat	LC
	15.72	11.87

LINE	1	2	3	4	5
	14.90	14.33	13.90	14.70	11.15

LOC	LINE	1	2	3	4	5
Roodeplaat		15.48	14.13	13.84	14.72	20.43
LC		14.31	14.53	13.96	14.67	1.87

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	25	10	5
e.s.e.	0.706	1.093	1.552

d.f.	8	32	39.99
Except when comparing means with the same level(s) of			
LOC			1.546
d.f.			32

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	25	10	5
l.s.d.	2.301	3.149	4.437
d.f.	8	32	39.99
Except when comparing means with the same level(s) of			
LOC			4.453
d.f.			32

Stratum standard errors and coefficients of variation

Variate: Freshmass

Stratum	d.f.	s.e.	cv%
Loc.REP	8	1.578	11.4
Loc.REP.*Units*	32	3.457	25.1

Fisher's protected least significant difference test

LINE

Warning 2, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LINE is not significant.

Fisher's protected least significant difference test

LOC.LINE

	Mean	
Roodeplaat 5	20.43	a
Roodeplaat 1	15.48	b
Roodeplaat 4	14.72	b
LC 4	14.67	b
LC 2	14.53	b
LC 1	14.31	b
Roodeplaat 2	14.13	b
LC 3	13.96	b
Roodeplaat 3	13.84	b
LC 5	1.87	c

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	15.48	5.95	14.31	16.58
2	14.13	25.03	14.53	7.56
3	13.84	4.55	13.96	36.78
4	14.72	4.06	14.67	14.84
5	20.43	4.38	1.87	0.77
Margin	15.72	13.43	11.87	38.85

LOC	Margin	
	Mean	Variance
LINE		
1	14.90	10.39
2	14.33	14.53
3	13.90	18.37
4	14.70	8.40
5	11.15	97.98
Margin	13.79	29.39

LOC	REP	COL	LINE	Freshmass	FITTED	RESIDUAL
Roodeplaat	1	1	3	11.80	14.64	-2.840
Roodeplaat	1	2	4	13.90	15.52	-1.620
Roodeplaat	1	3	2	17.50	14.93	2.570
Roodeplaat	1	4	1	16.50	16.28	0.220
Roodeplaat	1	5	5	22.90	21.23	1.670
Roodeplaat	2	1	1	13.40	16.91	-3.510
Roodeplaat	2	2	2	15.60	15.56	0.040
Roodeplaat	2	3	5	22.30	21.86	0.440
Roodeplaat	2	4	4	17.30	16.15	1.150
Roodeplaat	2	5	3	17.15	15.27	1.880
Roodeplaat	3	1	5	17.95	19.73	-1.780
Roodeplaat	3	2	1	12.75	14.78	-2.030
Roodeplaat	3	3	4	16.00	14.02	1.980
Roodeplaat	3	4	3	14.30	13.14	1.160
Roodeplaat	3	5	2	14.10	13.43	0.670
Roodeplaat	4	1	4	12.05	15.42	-3.370
Roodeplaat	4	2	5	19.65	21.13	-1.480
Roodeplaat	4	3	3	13.80	14.54	-0.740
Roodeplaat	4	4	2	17.85	14.83	3.020
Roodeplaat	4	5	1	18.75	16.18	2.570
Roodeplaat	5	1	2	5.60	11.90	-6.300
Roodeplaat	5	2	3	12.15	11.61	0.540
Roodeplaat	5	3	1	16.00	13.25	2.750
Roodeplaat	5	4	5	19.35	18.20	1.150
Roodeplaat	5	5	4	14.35	12.49	1.860
LC	1	1	2	16.95	13.35	3.598
LC	1	2	4	16.00	13.49	2.508
LC	1	3	5	0.75	0.69	0.058
LC	1	4	3	10.00	12.78	-2.782
LC	1	5	1	9.75	13.13	-3.384
LC	2	1	3	15.75	13.03	2.718

LC	2	2	5	2.40	0.94	1.458
LC	2	3	1	13.45	13.38	0.066
LC	2	4	4	11.15	13.74	-2.592
LC	2	5	2	11.95	13.60	-1.652
LC	3	1	4	18.65	13.90	4.748
LC	3	2	1	16.75	13.54	3.206
LC	3	3	2	13.20	13.76	-0.562
LC	3	4	5	1.10	1.10	-0.002
LC	3	5	3	5.80	13.19	-7.392
LC	4	1	5	2.40	1.84	0.558
LC	4	2	2	18.00	14.50	3.498
LC	4	3	3	17.09	13.93	3.158
LC	4	4	1	11.66	14.28	-2.624
LC	4	5	4	10.05	14.64	-4.592
LC	5	1	1	19.95	17.22	2.734
LC	5	2	3	21.16	16.86	4.296
LC	5	3	4	17.50	17.57	-0.074
LC	5	4	2	12.55	17.43	-4.884
LC	5	5	5	2.70	4.77	-2.074

Analysis of variance

Variate: Drylfmass

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	2.08897	2.08897	69.67	<.001
Residual	8	0.23986	0.02998	0.47	
Loc.REP.*Units* stratum					
LINE	4	0.35923	0.08981	1.40	0.257
LOC.LINE	4	2.69643	0.67411	10.50	<.001
Residual	32	2.05402	0.06419		
Total	49	7.43851			

Tables of means

Variate: Drylfmass

Grand mean 0.928

LOC	Roodeplaat	LC
	1.132	0.723

LINE	1	2	3	4	5
	0.971	0.899	1.017	0.975	0.776

LOC	LINE	1	2	3	4	5
Roodeplaat		1.034	0.960	1.228	1.010	1.428
LC		0.908	0.838	0.806	0.940	0.124

Standard errors of means

Table	LOC	LINE	LOC LINE

rep.	25	10	5
e.s.e.	0.0346	0.0801	0.1071
d.f.	8	32	37.85
Except when comparing means with the same level(s) of			
LOC			0.1133
d.f.			32

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	25	10	5
l.s.d.	0.1129	0.2308	0.3066
d.f.	8	32	37.85
Except when comparing means with the same level(s) of			
LOC			0.3264
d.f.			32

Stratum standard errors and coefficients of variation

Variate: Drylfmass

Stratum	d.f.	s.e.	cv%
Loc.REP	8	0.0774	8.3
Loc.REP.*Units*	32	0.2534	27.3

Fisher's protected least significant difference test

LINE

Warning 3, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LINE is not significant.

Fisher's protected least significant difference test

LOC.LINE

	Mean	
Roodeplaat 5	1.4280	a
Roodeplaat 3	1.2280	ab
Roodeplaat 1	1.0340	bc
Roodeplaat 4	1.0100	bc
Roodeplaat 2	0.9600	bc
LC 4	0.9400	bc
LC 1	0.9080	c
LC 2	0.8380	c
LC 3	0.8060	c

LC 5 0.1240 d

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	1.0340	0.08618	0.9080	0.01817
2	0.9600	0.08290	0.8380	0.08877
3	1.2280	0.14582	0.8060	0.06053
4	1.0100	0.01055	0.9400	0.06835
5	1.4280	0.01142	0.1240	0.00078
Margin	1.1320	0.08758	0.7232	0.13531

LOC	Margin	
	Mean	Variance
LINE		
1	0.9710	0.05079
2	0.8990	0.08043
3	1.0170	0.14118
4	0.9750	0.03643
5	0.7760	0.47776
Margin	0.9276	0.15181

LOC	REP	COL	LINE	Drylfmass	FITTED	RESIDUAL
Roodeplaat	1	1	3	1.8900	1.3600	0.5300
Roodeplaat	1	2	4	0.9300	1.1420	-0.2120
Roodeplaat	1	3	2	1.0500	1.0920	-0.0420
Roodeplaat	1	4	1	0.8600	1.1660	-0.3060
Roodeplaat	1	5	5	1.5900	1.5600	0.0300
Roodeplaat	2	1	1	0.6600	0.9300	-0.2700
Roodeplaat	2	2	2	0.9200	0.8560	0.0640
Roodeplaat	2	3	5	1.2900	1.3240	-0.0340
Roodeplaat	2	4	4	1.1400	0.9060	0.2340
Roodeplaat	2	5	3	1.1300	1.1240	0.0060
Roodeplaat	3	1	5	1.4100	1.4260	-0.0160
Roodeplaat	3	2	1	1.0000	1.0320	-0.0320
Roodeplaat	3	3	4	1.0900	1.0080	0.0820
Roodeplaat	3	4	3	0.9000	1.2260	-0.3260
Roodeplaat	3	5	2	1.2500	0.9580	0.2920
Roodeplaat	4	1	4	0.9900	1.0760	-0.0860
Roodeplaat	4	2	5	1.4300	1.4940	-0.0640
Roodeplaat	4	3	3	1.1100	1.2940	-0.1840
Roodeplaat	4	4	2	1.0900	1.0260	0.0640
Roodeplaat	4	5	1	1.3700	1.1000	0.2700
Roodeplaat	5	1	2	0.4900	0.8680	-0.3780
Roodeplaat	5	2	3	1.1100	1.1360	-0.0260
Roodeplaat	5	3	1	1.2800	0.9420	0.3380
Roodeplaat	5	4	5	1.4200	1.3360	0.0840
Roodeplaat	5	5	4	0.9000	0.9180	-0.0180
LC	1	1	2	1.2100	0.8648	0.3452
LC	1	2	4	1.1500	0.9668	0.1832
LC	1	3	5	0.1000	0.1508	-0.0508
LC	1	4	3	0.5900	0.8328	-0.2428

LC	1	5	1	0.7000	0.9348	-0.2348
LC	2	1	3	0.9800	0.7788	0.2012
LC	2	2	5	0.1500	0.0968	0.0532
LC	2	3	1	0.8900	0.8808	0.0092
LC	2	4	4	0.6800	0.9128	-0.2328
LC	2	5	2	0.7800	0.8108	-0.0308
LC	3	1	4	1.2800	0.8908	0.3892
LC	3	2	1	0.9600	0.8588	0.1012
LC	3	3	2	0.5500	0.7888	-0.2388
LC	3	4	5	0.0900	0.0748	0.0152
LC	3	5	3	0.4900	0.7568	-0.2668
LC	4	1	5	0.1300	0.1788	-0.0488
LC	4	2	2	1.0800	0.8928	0.1872
LC	4	3	3	1.0100	0.8608	0.1492
LC	4	4	1	0.9200	0.9628	-0.0428
LC	4	5	4	0.7500	0.9948	-0.2448
LC	5	1	1	1.0700	0.9028	0.1672
LC	5	2	3	0.9600	0.8008	0.1592
LC	5	3	4	0.8400	0.9348	-0.0948
LC	5	4	2	0.5700	0.8328	-0.2628
LC	5	5	5	0.1500	0.1188	0.0312

Analysis of variance

Variate: Artemisinin

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	0.139392	0.139392	110.63	<.001
Residual	8	0.010080	0.001260	0.39	
Loc.REP.*Units* stratum					
LINE	4	0.145092	0.036273	11.33	<.001
LOC.LINE	4	0.166948	0.041737	13.04	<.001
Residual	32	0.102440	0.003201		
Total	49	0.563952			

Tables of means

Variate: Artemisinin

Grand mean 0.3664

LOC	Roodeplaat	LC
	0.3136	0.4192

LINE	1	2	3	4	5
	0.3610	0.4340	0.3350	0.4160	0.2860

LOC	LINE	1	2	3	4	5
Roodeplaat		0.3780	0.3140	0.2500	0.3240	0.3020
LC		0.3440	0.5540	0.4200	0.5080	0.2700

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	25	10	5
e.s.e.	0.00710	0.01789	0.02372
d.f.	8	32	37.17
Except when comparing means with the same level(s) of			
LOC			0.02530
d.f.			32

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	25	10	5
l.s.d.	0.02315	0.05154	0.06796
d.f.	8	32	37.17
Except when comparing means with the same level(s) of			
LOC			0.07289
d.f.			32

Stratum standard errors and coefficients of variation

Variate: Artemisinin

Stratum	d.f.	s.e.	cv%
Loc.REP	8	0.01587	4.3
Loc.REP.*Units*	32	0.05658	15.4

Fisher's protected least significant difference test

LINE

	Mean	
2	0.4340	a
4	0.4160	a
1	0.3610	b
3	0.3350	bc
5	0.2860	c

Fisher's protected least significant difference test

LOC.LINE

	Mean	
LC 2	0.5540	a
LC 4	0.5080	a
LC 3	0.4200	b
Roodeplaat 1	0.3780	bc
LC 1	0.3440	cd
Roodeplaat 4	0.3240	cde
Roodeplaat 2	0.3140	cdef
Roodeplaat 5	0.3020	def
LC 5	0.2700	ef
Roodeplaat 3	0.2500	f

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	0.3780	0.008220	0.3440	0.003530
2	0.3140	0.000880	0.5540	0.001330
3	0.2500	0.000550	0.4200	0.004950
4	0.3240	0.001130	0.5080	0.005620
5	0.3020	0.000670	0.2700	0.001250
Margin	0.3136	0.003666	0.4192	0.014024

LOC	Margin	
	Mean	Variance
LINE		
1	0.3610	0.005543
2	0.4340	0.016982
3	0.3350	0.010472
4	0.4160	0.012404
5	0.2860	0.001138
Margin	0.3664	0.011509

LOC	REP	COL	LINE	Artemisinin	FITTED	RESIDUAL
Roodeplaat	1	1	3	0.2500	0.2484	0.00160
Roodeplaat	1	2	4	0.3700	0.3224	0.04760
Roodeplaat	1	3	2	0.3200	0.3124	0.00760
Roodeplaat	1	4	1	0.2800	0.3764	-0.09640
Roodeplaat	1	5	5	0.3400	0.3004	0.03960
Roodeplaat	2	1	1	0.5200	0.3944	0.12560
Roodeplaat	2	2	2	0.2700	0.3304	-0.06040
Roodeplaat	2	3	5	0.3000	0.3184	-0.01840
Roodeplaat	2	4	4	0.3200	0.3404	-0.02040
Roodeplaat	2	5	3	0.2400	0.2664	-0.02640
Roodeplaat	3	1	5	0.2900	0.3064	-0.01640
Roodeplaat	3	2	1	0.4000	0.3824	0.01760
Roodeplaat	3	3	4	0.3100	0.3284	-0.01840
Roodeplaat	3	4	3	0.2900	0.2544	0.03560
Roodeplaat	3	5	2	0.3000	0.3184	-0.01840
Roodeplaat	4	1	4	0.2800	0.3144	-0.03440
Roodeplaat	4	2	5	0.3100	0.2924	0.01760
Roodeplaat	4	3	3	0.2300	0.2404	-0.01040
Roodeplaat	4	4	2	0.3400	0.3044	0.03560

Roodeplaat	4	5	1	0.3600	0.3684	-0.00840
Roodeplaat	5	1	2	0.3400	0.3044	0.03560
Roodeplaat	5	2	3	0.2400	0.2404	-0.00040
Roodeplaat	5	3	1	0.3300	0.3684	-0.03840
Roodeplaat	5	4	5	0.2700	0.2924	-0.02240
Roodeplaat	5	5	4	0.3400	0.3144	0.02560
LC	1	1	2	0.5000	0.5748	-0.07480
LC	1	2	4	0.6100	0.5288	0.08120
LC	1	3	5	0.2300	0.2908	-0.06080
LC	1	4	3	0.4800	0.4408	0.03920
LC	1	5	1	0.3800	0.3648	0.01520
LC	2	1	3	0.3100	0.4108	-0.10080
LC	2	2	5	0.2400	0.2608	-0.02080
LC	2	3	1	0.3700	0.3348	0.03520
LC	2	4	4	0.5300	0.4988	0.03120
LC	2	5	2	0.6000	0.5448	0.05520
LC	3	1	4	0.4500	0.4808	-0.03080
LC	3	2	1	0.2400	0.3168	-0.07680
LC	3	3	2	0.5700	0.5268	0.04320
LC	3	4	5	0.3000	0.2428	0.05720
LC	3	5	3	0.4000	0.3928	0.00720
LC	4	1	5	0.2700	0.2868	-0.01680
LC	4	2	2	0.5500	0.5708	-0.02080
LC	4	3	3	0.4800	0.4368	0.04320
LC	4	4	1	0.3500	0.3608	-0.01080
LC	4	5	4	0.5300	0.5248	0.00520
LC	5	1	1	0.3800	0.3428	0.03720
LC	5	2	3	0.4300	0.4188	0.01120
LC	5	3	4	0.4200	0.5068	-0.08680
LC	5	4	2	0.5500	0.5528	-0.00280
LC	5	5	5	0.3100	0.2688	0.04120

========================= Without line 5 ====================

Analysis of variance

Variate: Plantht1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	3870.47	3870.47	58.59	<.001
Residual	8	528.51	66.06	2.28	
Loc.REP.*Units* stratum					
LINE	3	736.69	245.56	8.48	<.001
LOC.LINE	3	203.33	67.78	2.34	0.099
Residual	24	695.31	28.97		
Total	39	6034.31			

Tables of means

Variate: Plantht1

Grand mean 32.90

LOC	Roodeplaat	LC
	23.06	42.74

LINE	1	2	3	4	5
	35.59	36.66	33.65	25.71	

LOC	LINE	1	2	3	4	5
Roodeplaat		28.13	23.22	25.09	15.81	
LC		43.04	50.11	42.21	35.60	

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	20	10	5
e.s.e.	1.817	1.702	2.766
d.f.	8	24	27.20
Except when comparing means with the same level(s) of			
LOC			2.407
d.f.			24

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	20	10	5
l.s.d.	5.927	4.968	8.022
d.f.	8	24	27.20
Except when comparing means with the same level(s) of			
LOC			7.026
d.f.			24

Stratum standard errors and coefficients of variation

Variate: Plantht1

Stratum	d.f.	s.e.	cv%
Loc.REP	8	4.064	12.4
Loc.REP.*Units*	24	5.383	16.4

Fisher's protected least significant difference test

LINE

	Mean	
2	36.66	a
1	35.59	a
3	33.65	a
4	25.71	b

Fisher's protected least significant difference test

LOC.LINE

Warning 4, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LOC.LINE is not significant.

======= Summary of data =======

LOC	Roodeplaat Mean	Variance	LC Mean	Variance
LINE				
1	28.13	80.14	43.04	28.61
2	23.22	65.13	50.11	18.01
3	25.09	59.75	42.21	17.07
4	15.81	19.31	35.60	17.95
5	*	*	*	*
Margin	23.06	68.91	42.74	44.98

LOC	Margin Mean	Variance
LINE		
1	35.59	110.07
2	36.66	237.77
3	33.65	115.48
4	25.71	125.31

5	*	*
Margin	32.90	154.73

LOC	REP	COL	LINE	Plantht1	FITTED	RESIDUAL
Roodeplaat	1	1	3	22.86	23.75	-0.887
Roodeplaat	1	2	4	18.40	14.47	3.933
Roodeplaat	1	3	2	16.23	21.87	-5.641
Roodeplaat	1	4	1	29.38	26.79	2.595
Roodeplaat	2	1	1	22.23	24.65	-2.420
Roodeplaat	2	2	2	18.50	19.74	-1.236
Roodeplaat	2	4	4	18.87	12.33	6.538
Roodeplaat	2	5	3	18.73	21.61	-2.882
Roodeplaat	3	2	1	16.15	22.12	-5.973
Roodeplaat	3	3	4	16.00	9.80	6.196
Roodeplaat	3	4	3	18.21	19.08	-0.874
Roodeplaat	3	5	2	17.86	17.21	0.652
Roodeplaat	4	1	4	8.20	18.20	-10.002
Roodeplaat	4	3	3	29.21	27.48	1.728
Roodeplaat	4	4	2	29.00	25.61	3.394
Roodeplaat	4	5	1	35.40	30.52	4.880
Roodeplaat	5	1	2	34.50	31.67	2.832
Roodeplaat	5	2	3	36.46	33.54	2.916
Roodeplaat	5	3	1	37.50	36.58	0.917
Roodeplaat	5	5	4	17.60	24.26	-6.664
LC	1	1	2	55.53	51.16	4.367
LC	1	2	4	42.40	36.66	5.743
LC	1	4	3	39.33	43.26	-3.933
LC	1	5	1	37.92	44.10	-6.177
LC	2	1	3	40.07	40.51	-0.435
LC	2	3	1	44.21	41.34	2.870
LC	2	4	4	31.67	33.90	-2.229
LC	2	5	2	48.20	48.41	-0.206
LC	3	1	4	36.86	36.55	0.310
LC	3	2	1	49.00	43.99	5.010
LC	3	3	2	50.46	51.06	-0.596
LC	3	5	3	38.43	43.16	-4.725
LC	4	2	2	52.13	50.00	2.130
LC	4	3	3	47.73	42.10	5.629
LC	4	4	1	37.07	42.93	-5.865
LC	4	5	4	33.60	35.49	-1.894
LC	5	1	1	47.00	42.84	4.160
LC	5	2	3	45.47	42.01	3.465
LC	5	3	4	33.47	35.40	-1.930
LC	5	4	2	44.21	49.91	-5.696

Analysis of variance

Variate: Plantht2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	2973.0	2973.0	43.23	<.001
Residual	8	550.2	68.8	0.62	
Loc.REP.*Units* stratum					

LINE	3	442.9	147.6	1.34	0.285
LOC.LINE	3	406.3	135.4	1.23	0.321
Residual	24	2647.3	110.3		
Total	39	7019.7			

Tables of means

Variate: Plantht2

Grand mean 79.5

LOC	Roodeplaat	LC
	70.9	88.1

LINE	1	2	3	4	5
	84.8	78.3	75.6	79.4	

LOC	LINE	1	2	3	4	5
Roodeplaat		79.7	65.7	69.8	68.6	
LC		89.9	90.9	81.5	90.3	

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	20	10	5
e.s.e.	1.85	3.32	4.47
d.f.	8	24	31.00
Except when comparing means with the same level(s) of			
LOC			4.70
d.f.			24

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	20	10	5
l.s.d.	6.05	9.69	12.89
d.f.	8	24	31.00
Except when comparing means with the same level(s) of			
LOC			13.71
d.f.			24

Stratum standard errors and coefficients of variation

Variate: Plantht2

Stratum	d.f.	s.e.	cv%
Loc.REP	8	4.15	5.2
Loc.REP.*Units*	24	10.50	13.2

Fisher's protected least significant difference test

LINE

Warning 5, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LINE is not significant.

Fisher's protected least significant difference test

LOC.LINE

Warning 6, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LOC.LINE is not significant.

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	79.65	154.5	89.91	64.0
2	65.65	64.7	90.88	123.1
3	69.76	44.7	81.53	158.1
4	68.56	115.3	90.28	75.0
5	*	*	*	*
Margin	70.91	109.0	88.15	104.0

LOC	Margin	
	Mean	Variance
LINE		
1	84.78	126.3
2	78.27	260.2
3	75.64	128.6
4	79.42	215.6
5	*	*
Margin	79.53	180.0

LOC	REP	COL	LINE	Planth2	FITTED	RESIDUAL
Roodeplaat	1	1	3	70.53	71.83	-1.304
Roodeplaat	1	2	4	62.57	70.64	-8.069
Roodeplaat	1	3	2	74.75	67.73	7.022
Roodeplaat	1	4	1	84.08	81.73	2.351
Roodeplaat	2	1	1	68.60	79.20	-10.602
Roodeplaat	2	2	2	61.69	65.20	-3.510
Roodeplaat	2	4	4	84.13	68.11	16.018
Roodeplaat	2	5	3	67.40	69.31	-1.906
Roodeplaat	3	2	1	64.85	73.12	-8.270

Roodeplaat	3	3	4	64.58	62.03	2.551
Roodeplaat	3	4	3	61.73	63.22	-1.494
Roodeplaat	3	5	2	66.33	59.12	7.212
Roodeplaat	4	1	4	57.07	68.64	-11.572
Roodeplaat	4	3	3	69.00	69.84	-0.836
Roodeplaat	4	4	2	71.20	65.73	5.470
Roodeplaat	4	5	1	86.67	79.73	6.938
Roodeplaat	5	1	2	54.29	70.48	-16.195
Roodeplaat	5	2	3	80.13	74.59	5.539
Roodeplaat	5	3	1	94.07	84.49	9.583
Roodeplaat	5	5	4	74.47	73.40	1.073
LC	1	1	2	105.20	94.76	10.436
LC	1	2	4	102.93	94.17	8.763
LC	1	4	3	73.93	85.42	-11.485
LC	1	5	1	86.08	93.79	-7.714
LC	2	1	3	82.00	76.20	5.800
LC	2	3	1	84.14	84.58	-0.438
LC	2	4	4	80.07	84.95	-4.883
LC	2	5	2	85.07	85.55	-0.479
LC	3	1	4	90.14	86.88	3.263
LC	3	2	1	100.13	86.50	13.626
LC	3	3	2	83.92	87.47	-3.553
LC	3	5	3	64.79	78.13	-13.335
LC	4	2	2	100.20	93.55	6.654
LC	4	3	3	95.60	84.20	11.402
LC	4	4	1	82.40	92.58	-10.176
LC	4	5	4	85.07	92.95	-7.880
LC	5	1	1	96.79	92.09	4.702
LC	5	2	3	91.33	83.71	7.619
LC	5	3	4	93.20	92.46	0.738
LC	5	4	2	80.00	93.06	-13.059

Analysis of variance

Variate: Freshmass

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	0.30	0.30	0.02	0.880
Residual	8	99.44	12.43	0.82	
Loc.REP.*Units* stratum					
LINE	3	5.76	1.92	0.13	0.943
LOC.LINE	3	3.55	1.18	0.08	0.971
Residual	24	361.91	15.08		
Total	39	470.95			

Tables of means

Variate: Freshmass

Grand mean 14.46

LOC	Roodeplaat	LC
	14.54	14.37

LINE	1	2	3	4	5
	14.90	14.33	13.90	14.69	

LOC	LINE	1	2	3	4	5
Roodeplaat		15.48	14.13	13.84	14.72	
LC		14.31	14.53	13.96	14.67	

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	20	10	5
e.s.e.	0.788	1.228	1.698
d.f.	8	24	31.80
Except when comparing means with the same level(s) of			
LOC			1.737
d.f.			24

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	20	10	5
l.s.d.	2.571	3.584	4.893
d.f.	8	24	31.80
Except when comparing means with the same level(s) of			
LOC			5.069
d.f.			24

Stratum standard errors and coefficients of variation

Variate: Freshmass

Stratum	d.f.	s.e.	cv%
Loc.REP	8	1.763	12.2
Loc.REP.*Units*	24	3.883	26.9

Fisher's protected least significant difference test

LINE

Warning 7, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LINE is not significant.

Fisher's protected least significant difference test

LOC.LINE

Warning 8, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LOC.LINE is not significant.

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	15.48	5.95	14.31	16.58
2	14.13	25.03	14.53	7.56
3	13.84	4.55	13.96	36.78
4	14.72	4.06	14.67	14.84
5	*	*	*	*
Margin	14.54	8.75	14.37	16.02

LOC	Margin	
	Mean	Variance
LINE		
1	14.90	10.39
2	14.33	14.53
3	13.90	18.37
4	14.70	8.40
5	*	*
Margin	14.46	12.08

LOC	REP	COL	LINE	Freshmass	FITTED	RESIDUAL
Roodeplaat	1	1	3	11.80	14.22	-2.422
Roodeplaat	1	2	4	13.90	15.10	-1.203
Roodeplaat	1	3	2	17.50	14.51	2.987
Roodeplaat	1	4	1	16.50	15.86	0.637
Roodeplaat	2	1	1	13.40	16.80	-3.400
Roodeplaat	2	2	2	15.60	15.45	0.150
Roodeplaat	2	4	4	17.30	16.04	1.260
Roodeplaat	2	5	3	17.15	15.16	1.990
Roodeplaat	3	2	1	12.75	15.22	-2.475
Roodeplaat	3	3	4	16.00	14.46	1.535
Roodeplaat	3	4	3	14.30	13.59	0.715
Roodeplaat	3	5	2	14.10	13.88	0.225
Roodeplaat	4	1	4	12.05	15.79	-3.740
Roodeplaat	4	3	3	13.80	14.91	-1.110
Roodeplaat	4	4	2	17.85	15.20	2.650
Roodeplaat	4	5	1	18.75	16.55	2.200
Roodeplaat	5	1	2	5.60	11.61	-6.013
Roodeplaat	5	2	3	12.15	11.32	0.828
Roodeplaat	5	3	1	16.00	12.96	3.038
Roodeplaat	5	5	4	14.35	12.20	2.147
LC	1	1	2	16.95	13.34	3.613
LC	1	2	4	16.00	13.48	2.523
LC	1	4	3	10.00	12.77	-2.767
LC	1	5	1	9.75	13.12	-3.369
LC	2	1	3	15.75	12.67	3.083

LC	2	3	1	13.45	13.02	0.431
LC	2	4	4	11.15	13.38	-2.227
LC	2	5	2	11.95	13.24	-1.287
LC	3	1	4	18.65	13.90	4.748
LC	3	2	1	16.75	13.54	3.206
LC	3	3	2	13.20	13.76	-0.562
LC	3	5	3	5.80	13.19	-7.392
LC	4	2	2	18.00	14.36	3.638
LC	4	3	3	17.09	13.79	3.298
LC	4	4	1	11.66	14.14	-2.484
LC	4	5	4	10.05	14.50	-4.452
LC	5	1	1	19.95	17.73	2.216
LC	5	2	3	21.16	17.38	3.778
LC	5	3	4	17.50	18.09	-0.592
LC	5	4	2	12.55	17.95	-5.402

Analysis of variance

Variate: Drylfmass

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	0.34225	0.34225	12.49	0.008
Residual	8	0.21914	0.02739	0.32	
Loc.REP.*Units* stratum					
LINE	3	0.07195	0.02398	0.28	0.836
LOC.LINE	3	0.19211	0.06404	0.76	0.528
Residual	24	2.02594	0.08441		
Total	39	2.85139			

Tables of means

Variate: Drylfmass

Grand mean 0.966

LOC	Roodeplaat	LC
	1.058	0.873

LINE	1	2	3	4	5
	0.971	0.899	1.017	0.975	

LOC	LINE	1	2	3	4	5
Roodeplaat		1.034	0.960	1.228	1.010	
LC		0.908	0.838	0.806	0.940	

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	20	10	5
e.s.e.	0.0370	0.0919	0.1185

d.f.	8	24	28.47
Except when comparing means with the same level(s) of			
LOC			0.1299
d.f.			24

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	20	10	5
l.s.d.	0.1207	0.2682	0.3429
d.f.	8	24	28.47
Except when comparing means with the same level(s) of			
LOC			0.3793
d.f.			24

Stratum standard errors and coefficients of variation

Variate: Drylfmass

Stratum	d.f.	s.e.	cv%
Loc.REP	8	0.0828	8.6
Loc.REP.*Units*	24	0.2905	30.1

Fisher's protected least significant difference test

LINE

Warning 9, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LINE is not significant.

Fisher's protected least significant difference test

LOC.LINE

Warning 10, code UF 2, statement 159 in procedure AMCOMPARISON

Fisher's protected LSD is not calculated as variance ratio for LOC.LINE is not significant.

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	1.0340	0.08618	0.9080	0.01817
2	0.9600	0.08290	0.8380	0.08877
3	1.2280	0.14582	0.8060	0.06053
4	1.0100	0.01055	0.9400	0.06835
5	*	*	*	*
Margin	1.0580	0.07941	0.8730	0.05265

LOC	Margin	
	Mean	Variance
LINE		
1	0.9710	0.05079
2	0.8990	0.08043
3	1.0170	0.14118
4	0.9750	0.03643
5	*	*
Margin	0.9655	0.07311

LOC	REP	COL	LINE	Drylfmass	FITTED	RESIDUAL
Roodeplaat	1	1	3	1.8900	1.3525	0.5375
Roodeplaat	1	2	4	0.9300	1.1345	-0.2045
Roodeplaat	1	3	2	1.0500	1.0845	-0.0345
Roodeplaat	1	4	1	0.8600	1.1585	-0.2985
Roodeplaat	2	1	1	0.6600	0.9385	-0.2785
Roodeplaat	2	2	2	0.9200	0.8645	0.0555
Roodeplaat	2	4	4	1.1400	0.9145	0.2255
Roodeplaat	2	5	3	1.1300	1.1325	-0.0025
Roodeplaat	3	2	1	1.0000	1.0360	-0.0360
Roodeplaat	3	3	4	1.0900	1.0120	0.0780
Roodeplaat	3	4	3	0.9000	1.2300	-0.3300
Roodeplaat	3	5	2	1.2500	0.9620	0.2880
Roodeplaat	4	1	4	0.9900	1.0920	-0.1020
Roodeplaat	4	3	3	1.1100	1.3100	-0.2000
Roodeplaat	4	4	2	1.0900	1.0420	0.0480
Roodeplaat	4	5	1	1.3700	1.1160	0.2540
Roodeplaat	5	1	2	0.4900	0.8470	-0.3570
Roodeplaat	5	2	3	1.1100	1.1150	-0.0050
Roodeplaat	5	3	1	1.2800	0.9210	0.3590
Roodeplaat	5	5	4	0.9000	0.8970	0.0030
LC	1	1	2	1.2100	0.8775	0.3325
LC	1	2	4	1.1500	0.9795	0.1705
LC	1	4	3	0.5900	0.8455	-0.2555
LC	1	5	1	0.7000	0.9475	-0.2475
LC	2	1	3	0.9800	0.7655	0.2145
LC	2	3	1	0.8900	0.8675	0.0225
LC	2	4	4	0.6800	0.8995	-0.2195
LC	2	5	2	0.7800	0.7975	-0.0175
LC	3	1	4	1.2800	0.8870	0.3930
LC	3	2	1	0.9600	0.8550	0.1050
LC	3	3	2	0.5500	0.7850	-0.2350
LC	3	5	3	0.4900	0.7530	-0.2630
LC	4	2	2	1.0800	0.9050	0.1750

LC	4	3	3	1.0100	0.8730	0.1370
LC	4	4	1	0.9200	0.9750	-0.0550
LC	4	5	4	0.7500	1.0070	-0.2570
LC	5	1	1	1.0700	0.8950	0.1750
LC	5	2	3	0.9600	0.7930	0.1670
LC	5	3	4	0.8400	0.9270	-0.0870
LC	5	4	2	0.5700	0.8250	-0.2550

Analysis of variance

Variate: Artemisinin

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Loc.REP stratum					
LOC	1	0.196000	0.196000	87.79	<.001
Residual	8	0.017860	0.002232	0.62	
Loc.REP.*Units* stratum					
LINE	3	0.064290	0.021430	5.91	0.004
LOC.LINE	3	0.107780	0.035927	9.91	<.001
Residual	24	0.086980	0.003624		
Total	39	0.472910			

Tables of means

Variate: Artemisinin

Grand mean 0.3865

LOC	Roodeplaat	LC
	0.3165	0.4565

LINE	1	2	3	4	5
	0.3610	0.4340	0.3350	0.4160	

LOC	LINE	1	2	3	4	5
Roodeplaat		0.3780	0.3140	0.2500	0.3240	
LC		0.3440	0.5540	0.4200	0.5080	

Standard errors of means

Table	LOC	LINE	LOC LINE
rep.	20	10	5
e.s.e.	0.01057	0.01904	0.02560
d.f.	8	24	30.95
Except when comparing means with the same level(s) of			
LOC			0.02692
d.f.			24

Least significant differences of means (5% level)

Table	LOC	LINE	LOC LINE
rep.	20	10	5
l.s.d.	0.03446	0.05557	0.07384
d.f.	8	24	30.95
Except when comparing means with the same level(s) of			
LOC			0.07858
d.f.			24

Stratum standard errors and coefficients of variation

Variate: Artemisinin

Stratum	d.f.	s.e.	cv%
Loc.REP	8	0.02362	6.1
Loc.REP.*Units*	24	0.06020	15.6

Fisher's protected least significant difference test

LINE

	Mean	
2	0.4340	a
4	0.4160	ab
1	0.3610	bc
3	0.3350	c

Fisher's protected least significant difference test

LOC.LINE

	Mean	
LC 2	0.5540	a
LC 4	0.5080	a
LC 3	0.4200	b
Roodeplaat 1	0.3780	bc
LC 1	0.3440	bc
Roodeplaat 4	0.3240	cd
Roodeplaat 2	0.3140	cd
Roodeplaat 3	0.2500	d

======= Summary of data =======

LOC	Roodeplaat		LC	
	Mean	Variance	Mean	Variance
LINE				
1	0.3780	0.008220	0.3440	0.003530
2	0.3140	0.000880	0.5540	0.001330
3	0.2500	0.000550	0.4200	0.004950
4	0.3240	0.001130	0.5080	0.005620
5	*	*	*	*
Margin	0.3165	0.004445	0.4565	0.010129

LOC Margin

	Mean	Variance
LINE		
1	0.3610	0.005543
2	0.4340	0.016982
3	0.3350	0.010472
4	0.4160	0.012404
5	*	*
Margin	0.3865	0.012126

LOC	REP	COL	LINE	Artemisinin	FITTED	RESIDUAL
Roodeplaat	1	1	3	0.2500	0.2385	0.01150
Roodeplaat	1	2	4	0.3700	0.3125	0.05750
Roodeplaat	1	3	2	0.3200	0.3025	0.01750
Roodeplaat	1	4	1	0.2800	0.3665	-0.08650
Roodeplaat	2	1	1	0.5200	0.3990	0.12100
Roodeplaat	2	2	2	0.2700	0.3350	-0.06500
Roodeplaat	2	4	4	0.3200	0.3450	-0.02500
Roodeplaat	2	5	3	0.2400	0.2710	-0.03100
Roodeplaat	3	2	1	0.4000	0.3865	0.01350
Roodeplaat	3	3	4	0.3100	0.3325	-0.02250
Roodeplaat	3	4	3	0.2900	0.2585	0.03150
Roodeplaat	3	5	2	0.3000	0.3225	-0.02250
Roodeplaat	4	1	4	0.2800	0.3100	-0.03000
Roodeplaat	4	3	3	0.2300	0.2360	-0.00600
Roodeplaat	4	4	2	0.3400	0.3000	0.04000
Roodeplaat	4	5	1	0.3600	0.3640	-0.00400
Roodeplaat	5	1	2	0.3400	0.3100	0.03000
Roodeplaat	5	2	3	0.2400	0.2460	-0.00600
Roodeplaat	5	3	1	0.3300	0.3740	-0.04400
Roodeplaat	5	5	4	0.3400	0.3200	0.02000
LC	1	1	2	0.5000	0.5900	-0.09000
LC	1	2	4	0.6100	0.5440	0.06600
LC	1	4	3	0.4800	0.4560	0.02400
LC	1	5	1	0.3800	0.3800	0.00000
LC	2	1	3	0.3100	0.4160	-0.10600
LC	2	3	1	0.3700	0.3400	0.03000
LC	2	4	4	0.5300	0.5040	0.02600
LC	2	5	2	0.6000	0.5500	0.05000
LC	3	1	4	0.4500	0.4665	-0.01650
LC	3	2	1	0.2400	0.3025	-0.06250
LC	3	3	2	0.5700	0.5125	0.05750
LC	3	5	3	0.4000	0.3785	0.02150
LC	4	2	2	0.5500	0.5750	-0.02500
LC	4	3	3	0.4800	0.4410	0.03900
LC	4	4	1	0.3500	0.3650	-0.01500
LC	4	5	4	0.5300	0.5290	0.00100
LC	5	1	1	0.3800	0.3325	0.04750
LC	5	2	3	0.4300	0.4085	0.02150
LC	5	3	4	0.4200	0.4965	-0.07650
LC	5	4	2	0.5500	0.5425	0.00750

End of Riana Kleynhans (Francois Kruger) - VOPI Project 060202. Current data space: 1 block, peak usage 74% at line 84.

GenStat 64-bit Release 15.1 (PC/Windows 7) 21 November 2012 07:57:23
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Appendix B: Weather table showing average temperatures and rainfall for UP

Date	Maximum Air Temperature (°C)	Maximum RH (%)	Minimum RH (%)	Average Wind Speed (m/s)	Total Solar Radiation (MJ/m2)	Daily ETo (mm)	Total Rainfall (mm)	Average VPD (kPa)
01-Feb-12	31.2408905	78.3486481	16.76435661	1.1752313	20.51113701	5.0065	0	1.6546119
02-Feb-12	32.13222504	78.6218414	19.03389549	1.0909852	17.73975372	4.6193	0	1.8329278
03-Feb-12	31.75829315	71.0730057	16.81930351	1.375295	18.66166687	5.1023	0	1.7876078
04-Feb-12	32.14443207	68.4982147	15.20758057	1.4286375	17.60778427	5.0896	0	1.9283013
05-Feb-12	25.57849121	88.0922394	40.6685257	1.3710736	5.545752525	2.2529	50.29985	0.7101634
06-Feb-12	28.26927948	79.8138428	37.23903275	1.0152535	3.485346556	1.171	0	1.2697022
07-Feb-12	28.91030121	84.3223953	28.86296082	0.6141272	0.969273806	0.4841	0	0.9779007
08-Feb-12	32.35657501	84.8169098	21.95972252	0.8478585	15.3936367	3.9778	0	1.3816053
09-Feb-12	27.21463013	82.7747726	40.62578964	0.9699462	7.896492958	2.5867	0	0.9745892
10-Feb-12	24.08428192	87.4313736	59.85352325	0.8014079	1.778124094	0.5555	8.099995	0.626444
11-Feb-12	27.64045715	91.1920624	35.26406479	1.1549463	10.85431767	3.1331	0.9	0.7357115
12-Feb-12	29.39717865	57.577877	10.72039795	1.556901	20.63027954	5.3675	0	1.9453034
13-Feb-12	31.36756897	77.844986	14.06136322	1.1079302	16.78574371	4.4517	0	1.5744317
14-Feb-12	31.50798798	84.14077	16.93835258	0.9462224	16.51263809	4.2252	0	1.3535802
15-Feb-12	31.3965683	64.4765396	20.55099106	0.8670331	3.928511381	1.4277	0	1.9593811
16-Feb-12	32.8709259	78.9896698	19.46124649	0.9860695	17.59841919	4.4963	0	1.5851548
17-Feb-12	32.53362274	76.9750214	19.65202713	1.3943805	13.88768578	4.4448	0.5	1.5928957
18-Feb-12	32.08490753	84.8657455	15.86844635	1.7915312	15.82945251	5.0682	4.399998	1.5897915
19-Feb-12	30.89137268	89.1010971	25.49757385	1.0175879	15.09124756	3.9139	10.1	1.0950431
20-Feb-12	29.61695099	74.3010254	29.60624695	1.278712	3.531024218	1.351	0	1.6176866
21-Feb-12	31.05010986	85.9631195	27.09403419	1.2746367	13.13092041	3.9016	3.499999	1.0259008
22-Feb-12	32.18411255	85.3678818	20.2991581	1.831062	17.86970329	5.2509	4.399999	1.4252948
23-Feb-12	33.66763306	82.6694641	7.600737095	1.5762087	19.36643219	5.5675	0	2.188591
24-Feb-12	29.38191223	83.0082932	27.53817558	1.4679303	12.43375683	3.8669	0	1.0756761
25-Feb-12	30.55712128	86.5919418	24.88554764	1.3772486	15.98576164	4.3322	2.7	1.1006895
26-Feb-12	31.06078339	83.8080521	17.40843582	1.4421206	16.70269394	4.6573	0	1.6723813
27-Feb-12	30.41213226	76.4881439	17.54427147	1.3523045	15.84720325	4.4547	0	1.6132317
28-Feb-12	30.29918671	83.7851563	9.38950634	0.9096825	19.12024117	4.3377	0	1.7616128
29-Feb-12	30.73722839	71.0852127	17.5351162	0.9806298	15.40449524	4.0146	0	1.7017846
01-Mar-12	29.70089722	78.2677536	18.28297997	1.5189923	13.57107067	4.2574	0	1.5440929
02-Mar-12	30.00614929	77.3672714	8.315023422	3.0810335	18.86157608	6.5791	0	1.8007264
03-Mar-12	30.65328217	83.5119629	3.467645645	2.3419354	19.61448288	6.1329	0	1.9966103
04-Mar-12	34.22776031	69.4231186	4.030833244	2.6452267	19.12040329	7.103	0	2.5717208
05-Mar-12	34.32239532	61.9231148	7.278697968	2.5066097	18.5411377	6.8216	0	2.3768973
06-Mar-12	28.24028015	78.9362488	27.30618286	1.3583027	9.381868362	3.3174	0	1.1245768
07-Mar-12	29.04308319	82.2314301	24.41393471	1.3735286	12.58608437	3.7797	4.099998	1.1772132
08-Mar-12	29.68258667	84.4399185	21.37364006	1.3975656	10.86362934	3.6912	0.5	1.2129538
09-Mar-12	31.63313293	86.1951141	16.58120728	1.4813939	11.63825989	4.1153	0.7	1.4858317
10-Mar-12	33.46311188	77.0009689	6.048538208	2.1834958	17.16936684	6.0155	0.2	2.1174433
11-Mar-12	31.379776	70.8730621	14.21398926	2.9559906	16.03761864	6.2473	0	1.5069251
12-Mar-12	29.61543274	86.0119553	14.81838512	1.331355	18.01529884	4.4696	0.3	1.3367771
13-Mar-12	31.38283539	86.735405	7.61447382	1.4959222	17.04229736	4.823	0	1.8231508
14-Mar-12	32.22226715	63.3287926	8.108979225	1.655805	10.62098408	3.4722	0	2.6210058
15-Mar-12								
16-Mar-12	22.04979324	89.5803375	52.95791245	0.9785888	4.205476284	1.4452	6.099997	0.4030342
17-Mar-12	28.9347229	90.2167892	11.9780302	1.4402316	14.00337029	4.0892	0	1.2600853
18-Mar-12	29.8428421	73.3471222	6.520150661	1.3054727	17.96384811	4.5597	0	1.8983444
19-Mar-12	27.17494965	84.069046	25.07785416	1.2641572	13.66559887	3.576	0	1.0297345
20-Mar-12	29.95272827	85.9524384	13.55006886	0.8621337	14.68997574	3.5695	0	1.5871063
21-Mar-12	29.4246521	85.6731262	13.23413563	1.4207512	15.49153233	4.244	0.6	1.3163699
22-Mar-12	31.31872559	48.0677948	10.86539173	0.492134	2.537311554	0.9637	0	2.1661956
23-Mar-12	30.17098236	64.1026077	12.85409832	1.092348	15.87994766	4.0697	0	1.8230245
24-Mar-12	30.40755463	73.8690948	14.9755888	1.4387654	15.55533886	4.3763	0	1.6775763
25-Mar-12	29.23234558	77.1413803	8.493595123	1.5930601	16.72515869	4.6228	0	1.6802175
26-Mar-12	30.82574463	76.4255676	11.90934753	1.0164213	15.16563416	3.8729	0	1.7618829
27-Mar-12	31.12947083	65.4838638	12.16728497	1.0413376	14.69744492	3.9086	0	2.0230832
28-Mar-12	27.67861176	50.7845268	16.77504158	1.7671697	9.467163086	4.0932	0	1.9819711
29-Mar-12	25.25798035	89.4170303	41.58885193	1.2863458	9.4706707	2.6686	19.00004	0.6729673
30-Mar-12	23.12274933	89.7299118	54.87182617	1.1601866	6.872755051	2.0143	8.999998	0.4312845
31-Mar-12	24.14992523	87.9960861	17.34433365	1.8796945	14.42959213	3.8764	7.099996	1.0565656
01-Apr-12	24.99393463	79.2033463	16.61631012	2.9253747	15.04734802	4.8755	0	0.9741322
02-Apr-12	24.59864044	64.8779373	17.41148949	1.5354507	10.38552284	2.8282	0	1.4623251
03-Apr-12	26.31719971	77.6969376	10.72650242	1.2673711	15.18030548	3.7325	0	1.226069
04-Apr-12	26.32330322	77.9609756	16.33395386	0.8964691	15.0663662	3.2461	0	1.2399763
05-Apr-12	27.71066284	77.2497482	11.57204723	1.7997154	14.99973679	4.381	0	1.4560907
06-Apr-12	24.22317505	71.8834381	15.72955799	1.5324016	15.4630537	3.7794	0	1.1673077
07-Apr-12	24.74210358	74.7756882	15.31136513	1.4387869	15.6762619	3.7291	0	1.1966517
08-Apr-12								
09-Apr-12								
10-Apr-12								
11-Apr-12								
12-Apr-12								
13-Apr-12								
14-Apr-12								
15-Apr-12								
16-Apr-12	26.88038635	50.3083344	9.536025047	1.4792464	11.61828518	3.2301	0	1.9479827
17-Apr-12	26.30498505	79.2277679	14.46124268	1.6401174	12.95076752	3.744	0	1.3491588
18-Apr-12	26.97348785	60.9569969	11.15232754	2.4432712	3.138320208	1.9683	0	1.8247312
19-Apr-12	25.15724945	84.259819	17.71216011	1.237323	12.12397003	3.1005	0	1.001058
20-Apr-12	26.78118134	85.6868744	16.52015686	0.7203056	13.26844788	2.7476	0	1.1188021
21-Apr-12	26.27751923	82.4008408	14.98779964	1.1537091	12.90010452	3.182	0.8	1.2890172
22-Apr-12	24.37123108	87.3169098	19.55282021	1.6827893	13.17637539	3.4255	3.099999	0.9188514
23-Apr-12	24.53759003	80.3861923	21.22101402	1.7097381	8.119882584	3.107	4.399999	0.8212976
24-Apr-12	20.87916183	81.230217	48.90571213	1.6096307	3.654345512	1.7425	0	0.4973542
25-Apr-12	17.68013382	87.9319839	53.88892365	0.9016687	3.756027699	1.1901	0	0.4053491
26-Apr-12								
27-Apr-12								
28-Apr-12								
29-Apr-12								
30-Apr-12								
01-May-12								
02-May-12								
03-May-12								
04-May-12								
05-May-12								
06-May-12	27.81903076	64.1071777	15.65629864	0.7877654	2.823629618	1.1003	0	1.6983917
07-May-12	28.84925079	67.8632889	16.19659233	1.2199823	11.42402649	2.9538	0	1.6848146
08-May-12	24.5177536	86.109642	24.20636559	0.9752494	9.498438835	2.393	0	0.9633215
09-May-12	26.5949707	81.7369308	11.63920116	1.1740659	9.076558113	2.8707	0	1.3331594
10-May-12	27.21463013	70.5220261	11.9276638	1.0091509	10.06101418	2.8038	0	1.5060241
11-May-12	26.88191223	57.9640198	5.686816692	1.8892382	10.41521835	4.0176	0	1.7692831
12-May-12	24.15755463	77.3001099	13.21582031	1.004794	9.925123215	2.5496	0	1.1515384
13-May-12	24.0949707	70.2518768	11.64683247	1.1353781	10.43102455	2.7509	0	1.1582825
14-May-12								
15-May-12								
16-May-12								
17-May-12								
18-May-12	24.33306885	29.6581364	3.199025393	1.6238214	2.154069662	1.4675	0	1.8109554
19-May-12	25.1679306	43.3470993	4.772592068	1.2089067	11.20838642	3.0777	0	1.5332454
20-May-12	23.32268906	51.779644	4.223140717	1.6706411	11.16173553	3.4617	0	1.4436883
21-May-12	21.87885666	75.4731903	6.637671947	2.0929608	11.31540585	3.5641	0	1.1347136
22-May-12	20.63190842	86.457634	20.49452019	1.5049695	10.91097832	2.6052	0	0.7129247
23-May-12	21.402668	88.8111038	17.91667747	1.1069957	10.79832649	2.3501	0	0.7786152
24-May-12	27.3260498	80.1694641	5.808917046	1.2132448	10.62469673	3.0701	0	1.499699
25-May-12	25.13435364	30.9386635	5.660870552	1.2121515	1.745488524	1.1559	0	1.7417969
26-May-12	25.25035095	69.5360641	10.02442646	1.1607791	10.65762329	2.7995	0	1.2368895
27-May-12	22.9396019	62.5961914	15.90202522	1.1483241	10.18523788	2.5651	0	1.0453143
28-May-12	20.7494278	78.1792297	20.80129623	1.1566064	9.121623993	2.2616	0	0.8725685
29-May-12	21.00125885	80.8730698	15.49298859	0.8744605	10.20494652	2.101	0	0.9157958
30-May-12	24.44143677	32.9014244	5.233520031	2.1164935	8.302819252	3.0255	0	1.9355615
31-May-12	21.72317505	58.8706131	15.13126659	1.156935	9.363365173	2.4693	0	1.1282818
01-Jun-12	22.53667068	67.4115219	12.22680855	1.3924677	8.246314049	2.2614	0	1.3488408
02-Jun-12	24.00340271	57.7243958	4.74206686	1.2680595	10.22451782	2.899	0	1.4542925
03-Jun-12	23.78361893	51.9734764	8.435598373	1.8971888	10.07133484	3.5734	0	1.3343853
04-Jun-12	21.46524048	81.9063416	13.40965462	0.9348063	10.08330154	2.1686	0	0.957549
05-Jun-12	20.38465118	48.1074791	7.73046875	0.9963269	1.369220853	0.7877	0	1.1632893
averages	27.58206125	74.7189511	17.67598972	1.3910101	12.0574169	3.4854	1.366989	1.4022596
totals							140.7999	

Appendix C: Weather table showing average temperatures and rainfall for ARC

Date	Maximum Air Temperature (°C)	Maximum RH (%)	Minimum RH (%)	Average Wind Speed (m/s)	Total Solar Radiation (MJ/m2)	Daily ETo (mm)	Total Rainfall (mm)	Average VPD (kPa)
01-Feb-12	32.07	87.3	27.1	0.58	32.66	6.51	0	1.49
02-Feb-12	32.97	86.4	28.59	0.58	27.04	5.61	0	1.75
03-Feb-12	32.84	81.4	26.01	0.53	30.72	6.25	0	1.63
04-Feb-12	33.51	72.6	26.62	0.47	27.82	5.73	0	1.76
05-Feb-12	27.72	91.3	52.62	0.5	9.25	1.94	25.9	0.67
06-Feb-12	29.25	92.3	39.16	0.18	18.26	3.67	0.2	0.81
07-Feb-12	30.54	91	35.42	0.24	22.7	4.63	0	0.98
08-Feb-12	32.23	90.5	32.65	0.49	26.34	5.56	0	1.27
09-Feb-12	28.43	89.3	48.05	0.53	15.86	3.3	0	0.96
10-Feb-12	28.7	90.1	35.55	0.72	14.28	2.99	4.2	0.72
11-Feb-12	29.07	93.4	48.65	0.58	16.98	3.49	26	0.64
12-Feb-12	30.13	91.3	21.8	0.57	32.27	6.38	0	1.37
13-Feb-12	31.12	89.2	27.2	0.43	26.67	5.33	0	1.25
14-Feb-12	30.75	90.5	28.22	0.27	25.01	4.96	0	1.13
15-Feb-12	32.16	89.4	26.52	0.33	26.6	5.49	0	1.34
16-Feb-12	32.91	90.2	31.77	0.35	27.23	5.54	0	1.37
17-Feb-12	33.73	86.6	30.45	0.66	21.75	4.7	2.5	1.33
18-Feb-12	32.73	90	25.51	0.82	28.94	5.83	4.9	1.57
19-Feb-12	30.1	92.1	37.7	0.18	22.9	4.46	19.1	0.9
20-Feb-12	29.61	92.6	44.91	0.18	25.65	5.02	18.1	0.88
21-Feb-12	31.13	91.7	38.34	0.88	24.46	5.19	0	0.97
22-Feb-12	33.38	90.7	28.96	1.27	29.18	6.22	0	1.51
23-Feb-12	33.64	88.1	17.66	0.77	30.04	6.28	0	1.82
24-Feb-12	29.18	84.9	40.14	0.43	20.43	4.09	0	1.02
25-Feb-12	31.19	91.9	35.36	0.88	27.24	5.57	3.2	0.98
26-Feb-12	31.19	89.6	31.09	0.5	27.07	5.37	0	1.34
27-Feb-12	31.15	88.8	23.45	0.32	26.91	5.39	0	1.37
28-Feb-12	31.36	91.3	14.11	0.37	31.02	6.01	0	1.47
29-Feb-12	31.96	87.3	26.02	0.48	27.65	5.51	0	1.47
01-Mar-12	30.82	86.1	29.23	0.44	24.22	4.9	0	1.3
02-Mar-12	31.9	84.2	16.07	1.3	30.53	6.35	0	1.8
03-Mar-12	32.07	88.3	11.13	1.31	31.7	6.66	0	1.85
04-Mar-12	35.7	87.2	11.33	1.63	30.68	7.24	0	2.2
05-Mar-12	35.09	84.5	15.59	0.92	29.74	6.26	0	2.14
06-Mar-12	28.59	81.5	37.72	0.49	12.51	2.68	0	1.04
07-Mar-12	30.4	91	32.52	0.51	23.16	4.51	5.4	1.1
08-Mar-12	31.9	91.9	28.59	0.56	22.41	4.54	4	1
09-Mar-12	32.07	92.7	25.75	0.84	26.86	5.32	0	1.6
10-Mar-12	33.4	77.9	19.32	1.22	24	5.03	0.3	1.85
11-Mar-12	31.29	82	25.95	1.41	27.14	5.62	5.5	1.35
12-Mar-12	30.29	92	26.56	0.71	28	5.52	0	1.15
13-Mar-12	32.58	93.2	18.18	1.13	27.42	5.91	0	1.46
14-Mar-12	32.79	83.1	18.61	1.09	28.33	5.81	0	1.58
15-Mar-12	23.21	92	57.18	0.52	6.98	1.49	3	0.46
16-Mar-12	19.56	93.6	80.2	0	4.78	0.99	7.7	0.2
17-Mar-12	30.37	94.5	20.04	1.21	24.13	5.14	0	1.07
18-Mar-12	31.17	90.3	11.74	0.67	28.53	5.79	0	1.59
19-Mar-12	27.58	88.2	35.48	0.42	19.64	3.86	0	0.88
20-Mar-12	30.39	92.2	25.56	0.37	19.86	3.94	0	1.29
21-Mar-12	30.05	92.6	23.82	0.97	25.72	5.25	0	1.23
22-Mar-12	31.73	88.4	20.91	0.28	23.87	4.66	0	1.39
23-Mar-12	30.44	88.4	24.6	0.5	26.96	5.27	0	1.43
24-Mar-12	30.7	84.8	26.02	0.56	24.85	4.9	0	1.41
25-Mar-12	29.52	90.5	16.11	0.74	27.24	5.38	0	1.41
26-Mar-12	31.17	91.4	21.55	0.42	24.34	4.78	0	1.38
27-Mar-12	31.63	82.5	23.45	0.58	24.01	4.92	0	1.61
28-Mar-12	28.53	70.4	27.43	1.45	17.39	4.17	0	1.68
29-Mar-12	25.11	93.8	51.97	0.42	11.33	2.2	26	0.59
30-Mar-12	23.6	93.5	62.03	0.33	10.86	1.96	13.1	0.36
31-Mar-12	24.59	93.7	24.52	1.07	23.9	4.46	16	0.87
01-Apr-12	25.6	89.4	29.03	1.39	23.33	4.19	0	0.89
02-Apr-12	25.02	83.1	28.11	0.82	20.45	3.76	0	0.86
03-Apr-12	25.96	86.7	21.81	0.42	25.4	4.52	0	0.99
04-Apr-12	26.8	92.3	29.05	0.4	24.84	4.48	0	0.9
05-Apr-12	28.29	92.4	21.91	0.46	21.55	4.03	0	1.19
06-Apr-12	24.82	92.1	24.22	0.44	25.49	4.45	0	0.89
07-Apr-12	25.49	92.6	25.87	0.58	25.83	4.58	0	0.92
08-Apr-12	27.88	91.9	25.05	1	24.12	4.68	0.4	1.08
09-Apr-12	27.45	91.9	21.71	1.2	24.7	5.05	0	1.24
10-Apr-12	25.29	83.9	12.39	0.54	25.45	4.39	0	1.13
11-Apr-12	25.35	88.4	29.19	0.49	24.34	4.24	0	0.84
12-Apr-12	25.45	92.6	28	0.44	23.37	4.04	0	0.84
13-Apr-12	24.92	91.5	29.6	0.28	20.03	3.51	0	0.79
14-Apr-12	24.08	88.5	29.5	0.32	23.63	3.95	0	0.81
15-Apr-12	24.66	92.7	27.74	0.58	20.66	3.76	0	0.81
16-Apr-12	27.5	92	19.16	0.46	22.87	4.2	0	1.23
17-Apr-12	26.46	90.3	28.14	0.6	22.17	4.04	0	1.06
18-Apr-12	27.86	80.4	20.82	0.84	17.94	3.73	0	1.35
19-Apr-12	26.26	89.9	28.71	0.83	21.34	4.04	0	0.88
20-Apr-12	26.6	92.6	28.75	0.74	22.39	4.17	0	0.87
21-Apr-12	27.21	91.5	22.92	0.76	22.03	4.2	1.5	1.09
22-Apr-12	25.8	93.3	27.87	1.05	22.14	4.14	2.9	0.82
23-Apr-12	26.42	88	30.99	0.87	15.58	3.04	0.6	0.8
24-Apr-12	21.42	90.7	57.35	0.72	7.29	1.5	0	0.47
25-Apr-12	20.35	92.6	54.17	0.35	6.64	1.24	0	0.43
26-Apr-12	25.32	94.6	29.22	0.38	21.48	3.74	0.1	0.72
27-Apr-12	27.38	94.1	29.9	0.8	21.11	4.1	0	0.88
28-Apr-12	29.93	91.6	21.12	0.87	20.35	4.22	1	1.26
29-Apr-12	30.8	90.1	16.42	0.69	21.06	4.3	0	1.4
30-Apr-12	31.3	82	18.04	0.5	20.7	4.15	0	1.46
01-May-12	31.53	88	17.09	0.43	20.66	4.06	0	1.45
02-May-12	30.5	83.7	19.12	0.56	20.55	4.09	0	1.44
03-May-12	30.09	88	21.66	0.28	20.55	3.81	0	1.26
04-May-12	31.19	87.4	15.46	0.34	20.48	3.89	0	1.39
05-May-12	32.14	83.6	12.82	0.31	20.56	3.95	0	1.61
06-May-12	28.91	85.7	27.11	0.43	19.96	3.71	0	1.14
07-May-12	28.74	89.7	27.21	0.22	19.53	3.49	0	1.05
08-May-12	25.78	91.7	32.13	0.22	18.44	3.26	0	0.79
09-May-12	27.74	91.2	25.32	0.41	16.59	3.07	0.9	0.84
10-May-12	28	87.4	21.93	0.21	18.47	3.35	0	1.14
11-May-12	27.75	82	12.29	0.55	18.09	3.5	0	1.54
12-May-12	25.62	88.9	23.03	0.24	18.05	3.12	0	0.97
13-May-12	25.63	89.5	23.06	0.52	17.04	3.21	0	0.98
14-May-12	25.96	82.6	19.51	0.46	18.38	3.34	0	1.03
15-May-12	25.4	83.1	11.55	0.95	19.49	3.82	0	1.22
16-May-12	23.98	88.4	14.9	0.49	19.35	3.23	0	0.93
17-May-12	24.66	81.6	14.67	0.34	18.69	3.22	0	0.96
18-May-12	25.9	89.3	10.3	0.82	19.55	3.76	0	1.13
19-May-12	26.61	79.4	11.61	0.58	18.94	3.59	0	1.22
20-May-12	25.2	80.7	9.65	0.85	18.94	3.72	0	1.22
21-May-12	22.3	83.4	12.94	0.82	19.15	3.02	0	1.01
22-May-12	21.49	92.2	28.9	0.51	18.42	2.93	0	0.62
23-May-12	22.24	93.3	26.53	0.55	18.23	3.06	0	0.63
24-May-12	27.5	91.3	12.63	0.77	17.94	3.44	0	1.25
25-May-12	26.6	75.4	12.39	0.42	18.2	3.29	0	1.2
26-May-12	25.35	88.1	17.4	0.19	18.09	2.99	0	0.95
27-May-12	23.57	84.1	28.65	0.44	17.39	2.9	0	0.78
28-May-12	21.68	90.6	30.74	0.21	13.34	2.16	0	0.67
29-May-12	22.59	92.1	27.78	0.42	17.87	3.04	0	0.72
30-May-12	25.56	88.6	12.19	0.38	17.02	2.99	0	1.15
31-May-12	23.14	81.3	26.79	0.49	16.45	2.83	0	0.85
01-Jun-12	23.08	93	26.66	0.57	15.43	2.77	0	0.75
02-Jun-12	24.62	90	12.09	0.65	17.45	3.21	0	1.04
03-Jun-12	24.72	86.4	15.17	0.58	17.17	3.06	0	1.02
04-Jun-12	22.19	90.2	24.32	0.22	17.22	2.69	0	0.73
05-Jun-12	22.67	90.1	17.82	0.33	17.81	2.88	0	0.77
averages	28.18484127	88.5230159	26.3410317	0.6011111	21.680873	4.201746	1.527778	1.130079
totals							192.5	