ENHANCEMENT OF ARTEMISININ IN *ARTEMISIA ANNUA L.* THROUGH INDUCED MUTATION

BY

MUTAI RAYMOND

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DECLARATION AND APPROVAL

Declaration

I, Mutai Raymond, declare that the work presented in this thesis, to the best of my knowledge and belief is original, except as acknowledged in the text. The material has not been submitted, or being considered for submission, either in whole or in part, for another degree at this or any other University.

Signed ..... Date……..

Mutai Raymond (Reg No.AGR/PGB/09/10)
Department of Biotechnology,
School of Agriculture and Biotechnology,
University of Eldoret, Kenya.

Approval

This thesis is submitted with our approval as supervisors:

Signed ..... Date……..

Prof. Miriam Kinyua,
Department of Biotechnology,
School of Agriculture and Biotechnology,
University of Eldoret, Kenya.

Signed ..... Date ……..

Dr.Oliver Kiplagat,
Department of Biotechnology,
School of Agriculture and Biotechnology,
University of Eldoret, Kenya.

DEDICATION

To my parents Mr. and Mrs. Birgen, siblings Doro, Evans, Judy, Oliver, Vivian, Edimond and my wife Clara.

ABSTRACT

Artemisia annua is the source of artemisinin, an antimalarial drug which is effective against multidrug-resistant strains of plasmodium, the malarial parasite. Malaria has serious effects on morbidity and mortality thus negatively impacting on agricultural production and food security. Although artemisinin has been found to be a useful medicine; its production is very low in comparison with what is actually needed to treat the worldwide threat of malaria. On the other hand, the lower content (0.01–0.8%, dry weight) of artemisinin found in leaves and flowers of *A. annua* has seriously limited its commercialization Currently there are only two varieties of *A. annua* varieties present in Kenya hence there is a need to increase its diversity shown to lead to variability.In the present study, induced mutation was used to create variation in agronomical characteristics and artemisinin production. The other objectives were to determine the effect of mutation on agronomical traits and artemisinin production by parents and mutant (M_2) plants in two Agroecological zones. Seeds of two varieties of *Artemesia annua var artemis* and *varanamed* were sent to Vienna Austria for irradiation at the International Atomic Energy Agency at a dosage of 150 gray. The M_1 seeds were multiplied at the University of Eldoret farm. The harvested seeds were planted in replicate at the University of Eldoret and Njoro (KALRO). Seventy one single plants were selected from the preliminary evaluation of *Artemisia annua* mutants for high artemisinin production. These lines were again replanted at the University of Eldoret as M_3 in a RCBD design with three replicates. The results showed that mutation had significant effect on agronomical traits (P-value<0.05). Mutant varieties and lines showed wide variation in terms of agronomical traits (Crown Length, Stem Length, Plant height and Stem Diameter) and yield of artemisinin content. The following lines showed superiority in Artemisinin production; Artemis line 1, 2 and 9 with an average mean of 58.8, 58.4 and 69.2µg/l respectively while Anamed line 2, 3, 5 and 8 with an average of 56.3, 51.7, 53.5 and 54.4µg/l respectively. The effect of mutation on both agronomical traits and production of the Artemisinin content appeared to occur randomly and was also dependent on environmental factors in the different ecological zones. Higher means in agronomical traits was observed in UoE while production of artemisinin content was enhanced in Njoro. The artemisinin yield in *A. annua* crops was negatively correlated with leaf traits, shoot and stem characteristics. Leaf traits had positive correlations with shoot and stem Characteristics. It is recommended that superior lines be advanced in generations for further stability and evaluation of its efficacy in treatment of malaria.

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LIST OF ABBREVIATIONS

UoE	University of Eldoret
KALRO	Kenya Agricultural and Livestock Research Organization
ICRAF	International Centre for Research in Agroforestry
ART	Artemisinin
IAEA	International Atomic Energy Agency
DNA	Deoxyribonucleic acid
SED	Standard error of difference
EMS	Ethlymethane sulfonate
EABL	East Africa Botanicals Limited
WHO	World Health Organisation
ACT	Artemisinin Based Combination Therapy
LET	Linear Energy Transfer
MAE	Microwave assisted extraction
CIMAP	Central Institute of Medicinal and Aromatic Plants
Gy	Gray

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Artemisia annua L. is also referred to as annual wormwood, sweet wormwood and sweet annie. It is a highly aromatic annual herb of Asian and eastern European origin. It is widely dispersed throughout the temperate region (Simon *et al.,* 1984). *A. annua* is the source of artemisinin, an antimalarial drug which is effective against multidrug resistant strains of plasmodium, the malarial parasite (Luo and Shen, 1987).

Malaria is thought by some to be the oldest of human diseases (Bray 1996). It has had long serious effects on morbidity and mortality. About 3.2 billion people remain at risk of malaria. In 2015 alone, there were an estimated 214 million new cases of malaria and 438 000 deaths. Millions of people are still not accessing the services they need to prevent and treat malaria (WHO, 2015).Approximately 80% of malaria deaths are concentrated in just 15 countries, mainly in Africa(WHO, 2015).In Kenya, malaria remains a major cause of morbidity and mortality with more than 70 percent of the population at risk of the disease withchildren under age 5 and pregnant women being the most vulnerable to infection (MOH 2014).

Malaria is an infectious disease caused by protozoa of the genus *Plasmodium*, which are carried by mosquitoes of the genus *Anopheles*. There are fourparasites that cause malaria, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium falciparum*. However, only *Plasmodium falciparum* causes severe, potentially fatal malaria (CDC, 2004).

Artemisinin and its derivatives, artemether and artesunate, have been studied for their efficacy as antimalarial agents. In *in vitro* trials conducted in China (WHO, 1981), all three compounds were effective against the erythrocyte stages of two chloroquine resistant Hainan strains of *Plasmodium falciparum*, the malarial parasite, at lower minimum effective concentrations than chloroquine, the most commonly used drug. Artemisinin and its derivatives have effectively treated malaria and cerebral malaria in human subjects with no apparent adverse reactions or side effects (Klayman, 1985).It is now universally accepted that this family of compounds is among the most powerful antimalarial drugs ever discovered. The pharmacological and clinical evidence is well documented (Wright and Warhurst, 2002; Greenwood *et al*., 2005; Haynes *et al.,* 2006).

Artemisinins have demonstrated therapeutic potential against several important infectious diseases, besides malaria. The patients of *schistosomiasis* disease, caused by the protozoan species *Schistosoma japonicum, Schistosoma mansoni* and *Schistosoma haematobium*, which afflicts 200 million people and causes 1.5 million disabilities, have been found to respond well to artemether (Utzinger *et al.,* 2001; Shuhua *et al*., 2002; Mishina *et al.,* 2007). Arteether has been found to block the function of quinolone resistant DNA gyrase in *Eschrichia coli*,*Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (Kumar *etal*., 2002; Khanuja *et al.,* 2002; Srivastava, 2002). It has also been found to have effect on *Cryptosporidiosis, Amoebiasis, Giardiasis, Clonorchiasis, Leishmaniasis* (Mu *etal*., 2003), and cancer (Efferth, 2006). Moreover, artemisinin has been recently indicated as a potential and effective compound against a number of viruses including hepatitis B, C and others (Efferth *etal*., 2008). Artemisinin has also been reported to be a potent plant inhibitor

with potential as a natural herbicide (Duke *etal*., 1988). In view of the applications arising from array of their activities, the artemisinin related drugs would be in future required in commensurate huge amounts.

The artemisinin yield from *A. annua* crops is expected to depend on the inherent artemisinin content of the cultivated genotype and agronomy of cultivation. The artemisinin content of the various *A. annua* genetic resources has been reported to vary from ≤0.01 to >1.0% (Liersch *etal*., 1986; Singh *etal*., 1988; Charles et al., 1990; Woerdenbag *et al.,* 1994; Kawamoto *etal*., 1999; Gupta *etal*., 2002). The maximum reported yield of artemisinin from the field grown crops of *A. annua*is about 25 kg per ha (de Magelhaes *etal*., 1999). To improve the economics of production of artemisinin and antimalarial or antimicrobials semi-synthesized from artemisinin, there is need to increase the yields of artemisinin from the field grown *A. annua.* Since the total chemical synthesis of the molecule is complex and uneconomical (Yadav *etal*., 2003), field production of *A. annua* is therefore; recommended as the only commercially viable method to produce artemisinin. However, due to the rapid population increase in Kenya and increased pressure on land resulting in reduced fallow period, and continuous cropping, most soils are depleted of their nutrients (Ofori *etal*., 1993).

Induced mutation has great potentials and serves as a complementary approach in genetic improvement of crops for greater yield and quality traits (Ahloowalia and Maluzynski 2001). The generation of genetic variability by induced mutagenesis provides a base for strengthening plant improvement programs (Rekha and Langer 2007). For any mutation breeding program, selection and efficient mutagen is very essential to recover high frequency of desirable mutation (Solanki and Sharma, 1994).

The mutagenesis approach is alternative and alternate method for enhancement of artemisinin in vivo as well as in vitro. The main advantage of mutation breeding is the possibility of improving one or two characters without changing the rest of the genotypes. Induced mutation breeding, which has been recognized as a valuable supplement to conventional breeding in crop improvement has been least, applied in *A.annua*. Mutation breeding can induce enormous variability in the *A.annua* through physical and chemical mutagenesis because genetic variation is the starting point of any breeding program. Produced new mutant plants have great potential to be incorporated in further breeding programs for upgrading new productive cultivars.

1.2 Statement of the problem

Artemisinin and its derivatives have been found to be effective against all stages of resistant strains of *P. falciparum* (Balint, 2001). Although artemisinin has been found to be a useful medicine; its production is very low in comparison with what is actually needed to treat the worldwide threat of malaria. On the other hand, the lower content of artemisinin found in leaves and flowers of *A. annua* has seriously limited its commercialization (Van Agtmael *etal*., 1999). The World Health Organization (WHO) estimated that at least 130 million treatments would be needed in 2006, requiring 330 tons of artemisinin (WHO, 2004). This presents a problem because of the very low production levels of artemisinin in the native plant. One tonne of dry *A. annua* leaves produce only 6 kg of artemisinin (Van Geldre *et al.,* 1997). The complex biosynthetic pathway for artemisinin has prevented it from being produced by organic synthesis (Abdin *et al*., 2003; Yadav *etal*., 2003). Increasing the content of artemisinin in *artemisia* plant remains one of the remaining alternatives in the production of high yielding *A. annua*varieties. As it has been currently there are only two varieties of *A. annua* present in Kenya there is need to increase its diversity and

variability. Recent work has also included engineering pathways in both yeast and *E. coli* to produce precursors to artemisinin (Ro *et al.,* 2006). Mutational breeding has an important role for the improvement of yield and quality traits of the crops including medicinal plants (Khan *etal*., 2010), for instances gamma irradiation has been used for the enhancement of artemisinin in *A. annua* (Koobkokkruad *etal*., 2008).

1.3 Justification

The World Health Organization recommends Artemisinin based combination therapys (ACTs) in regions where multi drug resistance is prevalent. In line with this, a large proportion of the government of Kenya's annual budget is allocated to the importation of ACTs. These costs could however be reduced by production of artemisinin from *A. annua.* A substantial increase in the content of artemisinin is required to make artemisinin available on a large scale.Mutation breeding is the genetic improvement of crop plants for various characters through the use of induced mutation.Mutation breeding has been demonstrated as a viable technique in improving single traits in plants. It will also lead to the development of many different kinds of the *A. annua* varieties hence increasing the biodiversity of the plant. The main strategy in mutation based breeding is to upgrade the welladapted plant varieties by improving a few desirable major yield and quality traits (Ahloowalia *et al*., 2004).Besides the increased yield and enhanced quality of novel varieties included, several other traits such as breeding, improved harvest index from heterosis in hybrid cultivars, response to increased agronomic inputs and consumer preference are enhanced.Mutational breeding will result in increased artemisinin in *A. annua*whichwill give insight to possibly steer drug formulation for treatment and control of malaria and as well as have a positive economic impact to Kenya.

1.4.0 Objectives

1.4.1 General objective

To enhance Artemisinin content in *Artemisia annua* varieties through induced mutation.

1.4.2 Specific objectives

1. To determine the effect of mutation on agronomical traits in *A. annua* mutant population.
2. To determine Artemisinin production in parent and mutant (M_2) plants withintwo Agroeconomical zones.
3. To determine the effect of mutation on agronomical traits in segregating *A. annua* population at M_3.

1.5.0 Hypotheses

1. There is no effect of mutation on agronomic traits in *A. annua* mutant population.
2. Artemisinin production in parent and mutant (M_2) plants within the two Agroeconomical zones are similar.
3. There is no effect of mutation on agronomical traits in segregating *A. annua* population at M_3

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and botany of *Artemisia annua*

Artemisia annua (Asteraceae) is native to China, where it is known as qinghao (green herb) and has been used for over 2,000 years to treat symptoms associated with fever and malaria. It is known in the United States as sweet Annie, annual or sweet wormwood (Ferreira *etal.*, 1997). It has been introduced to many other countries, in Europe, North America, and the Tropics (Laughlin *etal*., 2002). The name of the plant is derived from that of the Greek goddess Artemis, daughter of Zeus, twin sister of Apollo, and credited with "healing diseases and averting evil". In antiquity, plants of the genus Artemisia were used to control the pangs of childbirth, to regulate women's menstrual disorders, and as an abortifacient (Riddle and Estes, 1992).

In 1969, the Chinese screened their medicinal plants in search of an effective antimalarial. A diethyl ether extract of *A.annua* was found to be effective against *Plasmodium* sp., and in 1972 the active ingredient, artemisinin, was isolated and identified by the Chinese. *A. annua* is so named because it is almost the only member of the genus with an annual cycle. It is a shrub, often growing over 2 m high (Ferreira *et al*., 1997). Varieties adapted to lower altitudes have been bred, and cultivation has been successfully achieved in many tropical countries, for example, in the Congo (Mueller *et al*., 2000), India (Mukherjee, 1991), and Brazil (Milliken, 1997).

The crop grows to a height of 1 – 3 m and 1m in width and it is an annual plant with a growth cycle of about 180 days (80 days in the nursery and 100 days on the field) (Ferreira and Janick, 1995). The plants are generally longer lived, more hardy and

aromatic when grown in poor dry soil. *A.annua* is a large shrub with a single-stem and alternate branches and the leaves are aromatic; fern-like and ranges from 2.5cm to 5cm in length (Whipley *etal*., 1992).

The plant has a short tap root and aggressive fibrous root (Laughlin, 1994). Mitchell (1975) noted that artemisia has flowers which are greenish yellow and about 2 to 3 mm in diameter. He further found out that the pollen has no spines but is extremely allergic. The most valuable parts are the leaves and the flowers where artemisinin is concentrated (Ferreira and Janick, 1995). Glandular trichomes are more prominent in the corolla and receptacle florets. There is strong evidence that artemisinin is sequestered in the glandular trichomes (Duke and Paul, 1993).

2.2 Ecology and distribution of *Artemisia annua*

The plant is native to China but is currently found in many countries. *A. annua* occurs naturally as part of vegetation in the northern parts of Chahar and Suiyuan provinces (40N, 109E) in Northern China, at 1000m to 1500m above sea level (Wang, 2005). The plant grows in many countries, such as Argentina, Bulgaria, France, Hungary, Romania, Italy, Spain, USA and former Yugoslavia (Klayman, 1993). The crop is grown in China and Vietnam as a source of artemisinin and cultivated on small scale in the USA as source of aromatic wreaths (Klayman, 1993).

The geographic range of *A.annua* is paramount in determining areas for potential cultivation. Although *A.annua* originated in relatively temperate latitudes it appears it can grow well at much lower tropical latitudes with lines which are either found in these areas or which have been adapted by breeding (Magalhaes, *etal*., 1996). The current availability of late flowering clones makes it possible to cultivate *A.annua* in

areas, which were previously considered unsuitable due to their proximity to the equator, and short photoperiod. The high artemisinin concentrations (0.5– 1.5%) in the leaves of some of these clones could allow high artemisinin yields in tropical latitudes, such as Vietnam, Madagascar and sub-Saharan Africa, even though the leaf biomass may not be as high as some strains of *A. annua* grown in temperate latitudes (Delabays *etal*.,1993). The influence that higher altitudes have on the production of *A.annua* at tropical latitudes is a principle that could be applied to parts of tropical Africa and elsewhere. Currently, with the opportunity offered by the availability of late flowering clones and the world demand for artemisinin, several international agencies are carefully analyzing the possibility of cultivating *A.annua* in tropical countries including Kenya and Tanzania (Technoserve, 2004).

2.3 Distribution in Kenya

Artemesia annua was introduced in Kenya in 1990s (EABL, 2005) although it has not been extensively embraced in Western parts of the country where malaria prevalence is at its highest (Kenya Demographic and Health Survey, 2008-2009). The plant is cultivated in parts of Central and Rift Valley provinces on approximate total of 10,000ha land coverage and gives artemisinin content by assay in the range 0.85-1.2%. It is mainly grown for commercial purposes in Thika, Nakuru, Eldoret and Kitale (EABL, 2005).

2.4 Genotypes

Artemisia annua L. is not an indigenous crop in Africa and most of the germplasm came from China, Vietnam, the Americas and mediplant in Indiana, Center for new crops in Switzerland (Technoserve, 2004). The germsplasm include different populations representing great diversity, which are then further improved in the national breeding programmes to suit local conditions (Ferraira and Janick, 1995).

The polymorphism determines a large number of species and different varieties producing artemisinin and essential oil with varying chemical composition (Hussain *etal*., 1988). The genus *Artemisia* includes about 400 species worldwide (Heywood and Humphries, 1977). Some of the species of *Artemisia* includes *A. annua, A. vulgaris, A. arborescens, A. campestries, A. maritima, A. pontica* and *A. verlotiorum* (Heywood and Humphries, 1977). Harvesting wild *A. annua* before flowering stage has led to depletion of the seeds, which is a threat to the germplasm resources. Therefore, priority has been put on investigation, characterization and collection of *A. annua* germplasm (Ferreira *etal*., 2005).

The genetic base of *Artemisia*is restricted and needs to be broadened. F1 hybrid seeds still remain expensive and F2 populations are still being used for cultivation although yield turns to be low (Delabays *etal*., 2001). Brazil has developed 3 hybrid seeds 2/3 9 x IV, IV x 2/39 and Chx viet 55 in collaboration with Mediplant, Switzerland to increase seed availability (Magalhaes *et al.,* 1996). CIMAP (Central Institute of Medicinal and Aromatic Plants) recently developed a new variety of *A. annua* "Jeevanraksha" which contains high levels of artemisinin (Tandon *et al.,* 2003). Anamed (Action for Natural Medicine) coordination in Germany has committed to making hybrid seeds readily available. Hybrid plant named "*A. annua anamed*" or"A3" is now widely available for cultivation (Delabays *etal*., 2001). In Turkey three ecotypes; Aduana, samankaya and serinyol that produce high artemisinin yield have been developed compared to other strains (Delabays *etal*., 2001).

Central Institute of Medicinal and Aromatic Plants is using molecular breeding techniques with *Agrobacterium tumefaciens* to enhance the production of artemisinin.

*Agrobacteriumtumefaciens*mediated system of high efficiency of genetic transformation and regeneration of *A. annua* has been established (Delabays *etal*., 1993). The process of identifying a few more genes in *A. annua* that, if transplanted into *Escherichia coli* could enable the bacterium to go a few extra steps in the chemical process and produce artemisinic acid, a precursor of artemisinin has been investigated (Srivastava, 2002).

2.5 Climatic and agronomical requirements for Artemisia

The environment includes all micro climatological and physical factors such as water, radiation, temperature, evaporation, soil conditions, human management, economic and political considerations. Laughlin (1993) indicated that genetic improvement of the crop plant alone would not meet the world demand for artemisinin. For crop productivity to be increased, the planting of high yielding cultivars must be combined with improved practices of irrigation, fertilization, pests and disease control. *Artemisia* is grown in temperate and sub-tropical climates. The plant is not adapted to the tropics because flowering will be induced when the plants are very small but grows easily in temperate areas and tropical areas at higher altitude (Klayman, 1993 and Hirt and Lindsey, 2000). On the other hand, it has also been reported that the crop can be grown in the tropics at 1000 – 1500 metres about sea level (Duke and Paul, 1993). It grows well on well-drained sandy loam soils and prefers soils with pH 5.0 – 8.0 with good water holding capacity (Laughlin, 1993and Hirt and Lindsey, 2000). Once established, the plants are drought tolerant. It thrives in temperate to sub-tropical climates but not very well in the tropics (Ferreira *et al*., 1997 and Laughlin *et al*., 2002). Marchese *et al*., (2002) indicated that depending on genotype and geographical origin, *Artemisia* present variations in the flowering behavior under the same photoperiod and temperature condition.

Water is required at the start of the planting season for good establishment of seedlings but dry weather conditions are needed at harvest for drying. Moisture stress also induces early flowering and reduce yield. The plant requires an average rainfall between 1000-1500mm with a minimum rainfall of 600mm during the growth period (Marchese *et al*., 2002). Irrigation is needed to avoid the negative effect of drought (Technoserve, 2004). Temperature has an important bearing on the productivity of *Artemisia*. A study has revealed that suitable temperature for *Artemisia* cultivation and production ranges between 10 – 17ºC with the optimum temperature between 13 – 29ºC (Liu *et al*., 2003). The plant grows well between longitude 105º-115ºE and latitude 25º-35º N by producing high biomass and artemisinin content (Duke and Paul, 1993). Similar findings have been reported from Vietnam, where the artemisinin content was high in the high altitude north than in the low-altitude south (WHO, 2003). *A. annua* is a short day plant with a photoperiod requirement of 13.5 hrs. (Ferreira *etal*., 1995a) and a chromosome number of 2n = 2x= 18 (Bennett *etal*., 1982). The plant is naturally cross pollinated by insects and wind (McVaugh, 1984). Ferreira *etal*., (1997) reported that self-pollination is not only rare but difficult to achieve which infers the presence of self-incompatibility. The plant at present does not seem to have any particular insect or disease problems.

2.6 Artemisinin production

Artemisinin is an odourless, non-volatile compound, which is purified as white crystals with a melting point of 156–157°C (Lin *et al.*, 1985). Its molecular weight is 282.1742 kg/mol, with an empirical formula of $C_{15}H_{22}O_5$.The chemical name is 3R, 5aS, 6R, 8aS, 9R, 12S, 12aR-Octahydro-3, 6, 9-trimethyl-3, 12-epoxy-12H-pyrano (4.3-j)-1, 2-benzodioxepin-10(3H)-one (Lin et al., 1985).

Artemisinin is the main active ingredient of *A. annua* effective against malaria. Derivatives of artemisinin include arteether, artemetther and sodium artesunate. Among the artemisinin derivatives, the compounds arteether artisunate, artinulate and di-hydro- artemisinin have been found to be highly potent anti-malarials (Jains *etal*., 2000). At present, a number of formulation of artemisinin and its derivatives are being marketed. The most widely available preparations are artemether, artesunate+amodiaquine, artesunate+ pyrimethamine (WHO, 1994).

The compound is isolated from the shrub *A. annua* which is used in traditional Chinese medicine. Artemisinin, used in the semi synthesis of related compounds in Artemisinin Based Combination therapies (ACTs), are found mainly in the leaves and flowers of *A. annua*, little artemisinin is found in the stems, and none is found in seeds or roots(Acton *etal*.,1985). Not all shrubs of this species contain artemisinin. Apparently, it is only produced when the plant is subjected to certain conditions (Charles *etal*., 1990). Artemisinin compounds have been predominately found in the upper parts of the *A. annua* plant, with the concentration of artemisinin said to peak just before or during full flowering, the difference being attributed to climatic conditions, plant variety, or other, yet undetermined factors (Charles *etal*.,1990). The leaves from the same plant have different artemisinin contents according to their localization along the stem with upper leaves containing significantly more artemisinin than middle and lower ones (Charles *etal*., 1990; Laughlin, 1995). The plant content of artemisinin also varies during the season (Delabays *etal*., 2001).

Artemisinin and its precursor, artemisinic acid, have been shown to be localised in the glandular trichomes on the leaf surface. The main consequences of this are that; it

may not be necessary to mechanically crush the plants prior to extraction for reasons other than to increase the packing density and the artemisinin content depends on the age of the leaf, since in older leaves the glands often ruptured (Mehrotra *etal*., 1990).

The demand for ACTs is increasing and increased from a million in 2003 to 30 million in 2004 (WHO, 2004). The world market price for artemisinin was USD 200-300/kg in 2002, increased to USD 400 – 500/kg and USD 600 – 800/kg in 2003 and 2004 respectively, with the price expected to stabilize around 250/kg (Technoserve, 2004).

EABL (2005) compared yield of A*rtemisia* and noted that artemisinin content is highly variable; natural population may be as low as 0.1% and improved cultivars as high as 1.3% with the range in East Africa by assay between 0.85% - 1.2%. Artemisinin can also be obtained from artemisinic acid which occurs in concentration as much as 10-fold higher than artemisinin (Acton *etal*., 1985).

The essential oils of *A. annua* contain at least 40 volatile compounds and several non-volatile sesquiterpenes, of which artemisinin and related compounds are the ones of most interest due to their anti-malarial properties (Charles *etal*., 1990). Some of the major constituents of the essential oils include (in relative % of total essential oil) alpha-pinene (0.032%), camphene (0.047%), ß-pinene (0.882%), myrcene (3.8%), 1, 8-cineole (5.5%), artemisia ketone (66.7%), linalool (3.4%), camphor (0.6%), borneol (0.2%), and ß-caryophyllene (1.2%) (Chen *et al*., 1991). The plant also yields 0.3% essential oil used in the perfumery and flavouring industries (Chen *et al*., 1991).

Artemisinin also has phytotoxic activity and a candidate as a natural pesticide (Chen *et al*., 1991).

The screening of *A. annua* germplasm for both artemisinin and essential oil content in one operation is a useful strategy to increase cost benefit ratio of the extraction (Laughlin, 1994). This is confirmed by Vonwiller *et al*., (1993) who reported that the extraction method which makes possible the extraction of both compounds from the same plant material increases the final production of artemisinin. Vries *et al.,* (1998) found out that substances in the aqueous extract protect the extract against resistances from the malaria strains. Clinical trials have shown artemisinin to be 90% effective than standard drugs (Blanke *et al.,* 2008). Simon *et al.,* (1990) indicated that the levels of artemisinin and its derivatives achieved are linked to inherent genetic factors and agronomy of cultivation. Commercial production of artemisinin in Africa has largely been limited to Kenya and Tanzania. However, current demand for artemisinin has led to a significant increase in the commercial production, both in established as well as new areas (EABL, 2005).

2.7 Biotechnological production of artemisinin

Due to relatively low level of artemisinin in *A. annua* (0.01-0.8 %) (Ferreira et al., 1997; Bhakuni *et al.,* 2001), several strategies have been adopted to increase artemisinin production to meet the demand in the medical market. This includes plant tissue culture, chemical synthesis, biotransformation and genetic engineering of *A. annua* (Abdin *et al.,* 2003). Tissue culture of shoots, callus suspension culture and hairy roots have been investigated (Weathers et al., 1994; Liu *et al*., 2004). However, the artemisinin content from tissue cultures is inconsistent and non-reproducible in some cases (Jaziri *et al.,* 1995).

Attempts to increase artemisinin production by adjusting the nutrients, hormones, growth condition, elicitors, and stresses were also reported (Wang and Tan.2002, Liu, 2003). Chemical synthesis of artemisinin is possible but complicated and not feasible for large-scale production due to the complex structure of artemisinin (Jung *et al.,* 2004; O'Neill, 2005; Jefford, 2007). Heterologous expression of artemisinin metabolic pathway in microorganisms, such as *Escherichiacoli* and *Saccaromyces cerevisiae* is an attractive way due to the low cost carbon source and feasibility of large scale preparation (Chang and Keasling, 2006; Ro *et al*., 2006). Genetic engineering of *A. annua* has become attractive since the transformation of *A. annua* was established (Banerjee *et al*., 1997; Ghosh et al; 1997; Hans *etal*., 2005), but both methods are reported to be complex (Hans *etal*., 2005).Singh (2001) however indicated that to improve the economics and commercial production of artemisinin and anti-malarials, there is the need to increase the yields of artemisinin from the field grown *A. annua* through the application of fertilizers.

2.8 Effect of Mutation breeding on Artemisinin and agronomical traits

Mutation is defined as the sudden and heritable change in the character of an organism, which does not arise due to segregation or recombination. Mostly they are large and easily detectable but sometimes they are so small that one may not be able to notice them. Mutations cause variationswhich are not always useful but in a few cases they are of a great advantage to the breeder. A mutagen is a chemical or physical agent that changes the genetic information, usually DNA of an organism thus increasing the number of mutations above the natural level. Muller (1927) working with Drosophila and Stadler (1930) working with cereals demonstrated that mutation frequency could be enhanced with the help of mutation agents such as X-rays. Auerbach and Robison (1946) reported the use of chemicals such as mustard gas to be

highly mutagenic. Since then, a number of mutagenic agents have been discovered. The main mutagenic agents used to induce mutations include physical mutagens such as UV, electro-magnetic waves such as X-rays, gamma rays and cosmic rays, fast moving particles such as alpha particles, beta particles and neutrons. Chemical agents include alkylating agents, intercalating agents and base analogs. X-rays are produced by X-rays machines by bombarding tungsten or molybdenum with electrons in a vacuum, whereas gamma rays are produced from radioisotopes like Cobalt 60 and Caesium 137 in the gamma chamber. The UV radiations possess limited tissue penetrating ability (low linear energy transfer) and therefore cause little damage except after prolonged exposure as a result of which their use is limited to pollen grains (Kovacs and Keresztes, 2002).

Mutagenic agents vary in their mode of action.UV light is absorbed by pyrimidines in DNA,causing adjacent bases on the same DNA strand to bond covalently to form pyrimidine dimmers and subsequently cause errors during DNA replications.X-rays,gamma rays and cosmic rays act directly on the critical targets in the cell (DNA),or interact with other atoms or molecules in the cell, particularly water hence forming radicals that break DNA strands and alter purine and pyrimidine bases (Kovacs and Keresztes, 2002).This radiolysis of water may affect temperature,pH and dilution of solutions by the presence or absence of oxygen (Diehl,1990).

Changes in DNA may include disruption of continuity of one or both strands, removal of a base and chemical alteration of a base which changes its pairing properties causing gene mutations. Several mechanisms may be responsible for the occurrence of mutations after DNA damage. One mechanism may involve an alteration in the

specificity of base pairing such as deamination of cytosine to uracil. The resulting mispairing leads to errors during replications and a mutation may be induced opposite to such lesions. Another mechanism may involve the loss of base pairing potential, leading to mutations (Kovacs and Keresztes, 2002).Fast moving particles such as neutrons have much higher LET (Linear Energy Transfer) and can physically punch holes in DNA (Waugh *et al*.,2006)

Chemical mutagens have three modes of action. Base analogues such as 2-amino purine and 5-bromouracil with different hydrogen properties get incorporated into DNA during replication. Intercalating agents such as Ethidium Promide, Proflavin and Acridine Orange slip between adjacent base pairs in DNA, reducing the fidelity of DNA replication and causing insertions, deletions and additions that induce frame shifts. Alkylating agents such as ethyl methane sulfonate (EMS) and N-ethyl-N-nitrosourea, deaminating agents such as nitrous acid and nitrosoguanidine and hydroxylating agents such as hydroxylamine are base modifying agents.

One of the first steps in mutagenic treatment is estimation of the appropriate dose of mutagen to apply. The unit of the dose of radiation energy absorbed is Gy (Gray), which is equivalent to 1 J kg-1 or equivalent to 100 rad. The choice of the dose of mutagen to apply for the highest probability of success for useful mutant rescue is left to the breeder’s experience on the specified plant material, its genetics and physiology. Radio-sensitivities of plant materials varied with species and cultivars, physiological conditions of plant and plant organs, as well as with manipulation of the irradiated plant material before and after treatment with the mutagen. Correlations between the physiological status of the plant and its radio-sensitivity is often

determined by water content of the plant tissue, since water molecules are most frequently become primary targets of the ionizing radiation. To avoid negative effects of mutation, most researchers preferred the use of relatively low doses of irradiation (Predieri, 2001).

For breeding purposes, the mutagen used need to have low somatic but strong genetic effects to the plant. After treatment, however, growth of the culture needs to be sustained as to overcome primary irradiation injuries and to allow for evaluation of treatment efficiency. The culture can either be irradiated during the growth proliferation or then transferred rapidly to a fresh medium to avoid the formation to toxic compound (Ahloowalia, l998).

2.9 Artemisinin extraction, detection and quantification

The techniques available for the extraction of artemisinin from *A. annua* include solvent extraction where a non-aqueous polar solvent, for example ethanol, methanol, dichloromethane, acetone, methylisobutyl or n-hexane is used as liquid phase, microwave assisted extraction (MAE), Soxhlet method and Supercritical Fluid Extraction (SFE) using CO as supercritical solvent (Klayman, 1985; Kohler *et al*., 1997; Christen and Veuthey, 2001). Solvent extraction is the most convenient to adopt though not efficient as compared to the other three methods.

After extraction, several methods for detection and quantification can be employed including high performance liquid chromatography with electrochemical detector (HPLC-EC), HPLC with ultra violet detection (HPLC-UV) after derivatisation to a compound which absorbs at 260nm, HPLC with a photodiode array detecter (HPLC-DAD), HPLC with evaporative light-scattering detector (HPLC-ELSD), gas

chromatography with mass spectro-metric detection (GC-MS), GC with flame ionization detection (GC-FID) and enzyme-linked immunosorbant assay (ELISA) (Zhao and Zeng, 1985; Sipahimalani, 1991; Woerdenbag *et al*., 1991; Melendez, 1991; Fereirra and Janick, 1996; Avery *et al*., 1999). HPLC-UV is fast (approximately 11min per sample running time), more selective and sensitive and that it provides a more uniform response. A DAD detects the absorption in UV to VIS region. While a UV-VIS detector has only one sample-side light-receiving section, a DAD has multiple photodiode arrays to obtain information over a wide range of wavelengths at one time, which is a merit of the DAD. Furthermore, a wide variety of substances can be detected that absorb light from 190nm to 900nm. Sensitivity depends strongly on the component and spectrum can be confirmed for each component (Christen and Veuthey, 2001).

For analysis of artemisinin content in the leaf, acetonitrile or ethyl acetate are the best to be used as solvent. Exhaustive extraction from plant material is confirmed by sequential extraction to quantify the residual amount in the leaf after first extraction. Stability of artemisinin in the extract must be taken into account and therefore the extracts should be analysed immediately without prolonged storage. The HPLC-ELSD method is used for quantification of artemisinin in the leaf on the basis of exhaustive extraction. If only UV detector is available, retention time of the artemisinin peak must be confirmed on the same day when extract is being analysed. The detector wavelength set at 210-216 of UV or DAD and C18 column of 250 mm length is used, for example Betasil C18 5 µm 250 x 4.6 mm at column flow-rate 1.0 mL min-1 gives consistently good results. Best mobile phases for analysis of extracts

are acetonitrile: water 75:25 % v/v or acetonitrile: water: methanol 50:30:20 % v/v (Jansen and Soomro, 2007).

Freshly prepared standards of artemisinin and its precursors are used to calibrate HPLC-UV or HPLC-DAD and to test the instrument linearity and accuracy. The calibration curves obtained are used to quantify the compound in the samples (Snyder *et al*., 1997; Peng *et al*., 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Site description

The study was conducted at Kenya Agricultural and Livestock Research Organisation (KALRO) Njoro and the University of Eldoret (UoE), (0 30' 0'' North, 35^{o} 15' 0'' East), 10 Km from Eldoret town, in Uasin Gishu county, Kenya. It is located at an altitude of 2180 m above sea level in the agro-ecological zone Lower Highland 3 (LH3). The University of Eldoret receives a unimodial rainfall which begins in March. The average annual range is between 900 mm and 1100 mm and mean annual temperature of 16.6^{o}C. The soils are shallow, ferralsol, well drained, non humic cambisols with low nutrient availability and moisture storage (Jaetzol and Schimidt, 1983).

Kenya Agricultural Livestock Research Organization, Njoro is located in Nakuru County, (0^{o} 20'S 35^{o} 56'E), located in the lower highlands (LH3), at an altitude of 2166 m above sea level. The temperature ranged between 18-28^{o}C during the period of study, while the average annual rainfall is about 1000 mm. the soils are deep, well drained, fertile *vitric Andosols* (Jaetzold and Schimdt, 1983)

3.2 Mutant seed development

One gram of M_0 (non-mutated seeds) of both *Artemisia annua var artemis* and *Artemisia annua var anamed*, obtained from ICRAF (World Agroforestry Centre, Nairobi), Kenya, were sent to International Atomic Energy Agency (IAEA) in Vienna Austria and subjected to gamma radiation at an irradiation dose of 150 gy (gray) to obtain M_1(mutated seeds that gives rise to the first generation of mutants). This is the first generation of irradiated seeds which was designed as M_1.

3.2.1 Determination of LD_{50}

Irradiated seeds were sown under controlled conditions. Germination rates of the seeds were determined from the 7^{th} day after sowing until the 14^{th} day. The seeds were exposed to irradiation of between 50gy and 450 at intervals of 50. The effective dose was determined by measuring the dose that decreases germination rate down to 50% hence 150 gy.

3.3 Multiplication and selection

The nurseries were prepared in the greenhouse and the seeds spread and left uncovered according to the World Agroforestry Centre recommendations. The seeds germinated after 7 days although some could be seen germinating after 4 days. The seedlings were transplanted in poly-tubes (75 by 140 by 37 mm) after the appearance of four leaves. After 7 weeks the seedlings were hardened off through the exposure to full sunlight for a week before transplanting to the experimental field site. The field site was prepared in advanced. The seedlings were planted at spacing of 1m by 1m between and within row. The field were kept weed free until the Artemisia were fully established. At harvest the chimeric and deformed plants were not selected. The harvested seeds from each plant were put into individual envelope and labeled. One group was planted at the university of Eldoret experimental field while the corresponding groups were planted at Njoro. The seeds from each plant were designated as M_2.

3.4 Genotypes

Seventy one M_2single plants were selected from the preliminary evaluation of *Artemisia annua* mutants for high production of artemisinin at Njoro and University of Eldoret fields. They were then used as the genotypes for further evaluation at M3.

Concentration levels of artemisinin that were above 60µg/l, is the commercially viable level (Delabays *et al.*, 1993; Abdin *et al*., 2003). The control was also included.

3.5 Field operation

The nurseries were prepared in the greenhouse and the seeds spread and left uncovered. The seeds germinated after 7 days.The seedlings were transplanted in poly-tubes (75 by 140 by 37 mm) after the appearance of four leaves. After 7 weeks the seedlings were hardened off through the exposure to full sunlight for a week before transplanting to the experimental field site. A field experiment was then established. The land was disc ploughed suitable for *Artemisia annua* planting. The experiment was laid out in a randomized completely block design (RCBD) replicated three times. The spacing between and within plants was 1m by 1m. The experimental units were separated by 1.5m by 1.5m wide alleyways within and between blocks, respectively. The fields were kept weed free until the Artemisia were fully established.

3.5.1 Data collection

The agronomic traits for all the M_2 plants in the different zones were measured 4 months after establishment which included; Crown length, Stem length, Plant height, Branches and Stem diameter. For Artemisinin analysis the leaves found in the upper parts of the *Artemisia annua* plant, were harvested just before full flowering.

3.5.2 Artemisinin extraction and sample preparation

Leaves were obtained from the plant during the flowering stage and air dried at 27^{o}c. merceration was performed in Erlenmeyer with a magnetic stirrer speed of 700rpm using ethanol as a solvent. This process was done many times until the methanol layer was colorless. The extract was then evaporated using a rotavapor vacuum at a temperature of 40° C until the extract volume was 100ml.

3.5.3 Spectrophotometric quantification of Artemisinin was done following the method of Sreeviyda, T.V and Narayana, B., 2007

Reagents

All solutions were prepared with double distilled water. Chemicals were of analytical grade. Hydrochloric acid (5M), Potassium iodide (2%), Sodium acetate (2M) and 0.001% solution of Safranin were used.

Standard Artemisinin (ART) solution

A 1000µgml^{-1} standard drug solution was prepared by dissolving 0.1 g of ART in ethanol and diluting to the mark in 100ml calibrated flask.

Method

Determination of ART

Different aliquots (16.0- 112.0 µg/l) were prepared. 10ml of standard ART and sample extract was transferred to calibrated flasks by means of micro burette, then 1ml each of 2% potassium iodide and 5M HCL was then added followed by 2ml of 2M sodium acetate solution then the mixture was shaken for 5 mins. The contents were then diluted to the mark with distilled water and mixed well. The absorbance of each solution was then measured at 521 nm against the corresponding reagent blank. The reagent blank was prepared by replacing the analyte (ART) solution with distilled water. The absorbance of the corresponding to the bleached color, which in turn corresponds to the analyte (ART) concentration, was obtained by subtracting the absorbance of the blank solution by that of the test solution.

3.5.4 Data analysis

The data was analyzed using Genstat 13th edition and means separated using Tukeys Multiplerange test where applicable. Correlation was done using Genstat Pearson correlation Coefficients.

The following statistical model was used;

$X_{ijk}=\mu+t_i+\beta_j+e_{ijk}$

Where,

X=observation

μ=overall mean

t_i=treatment effect (mutant lines)

B_j=block effect

E_{ijk}=experimental error

CHAPTER FOUR

RESULTS

4.1 Effects of mutation on agronomical traits at M2

There was no significant difference in all agronomical aspects that were analyzed between the two agro-ecological zones in parents of both plant varieties (Artemis and Anamed) ($P<0.001$) (Table 1).In mutant Artemis plants, there was significant difference in Crown length, plant height, and stem diameter between UoE and Njoro sites. Crown length, plant height and stem diameter had means of 105.9, 136.3 and 3.0 in UoE and 84.3, 116.38 and 2.2 inNjoro respectively. In mutant Anamed plants, there was significant difference in Crown length, Stem length, plant height, and stem diameter between UoE and Njoro sites. Crown length, Stem length, plant height, and stem diameter had means of 127.6, 102.7, 145.6 and 3.1 for UoE and 80.9, 46.0, 11.2 and 2.1 in Njoro respectively.

In UoE Artemis plants, there was significant difference in Crown length, stem length and Stem diameter in Artemis plants with means of 67.4, 18.59 and 1.8 for parent plants and 105.9, 75.8 and 3.0 for mutant plants respectively, while in Anamed plants there was significant difference in Crown length, stem length, Plant height and Stem diameter with means of 77.9, 29.8, 103.25and 2.1 for parent plants and means of 127.6, 102.7, 3.1 and 145.6 for mutant plants respectively. None of the agronomical characteristics analyzed were significantly different at Njoro in the two plant varieties.

Table 1: Comparison of Means for Agronomical characteristics in parent and mutant plants in two plant varieties in UoE and Njoro sites (95.0 percent SED intervals, P-value 0.001)

TRAITS	SITE	MEAN/VARIETY(units in cm)		P-VALUE
		Par Artemis	**Mutant Artemis**	
Crown length	UoE	67.4±6.4	105.9±6.4	<.001
	Njoro	71.8±5.2	84.3±5.2	0.017
Stem length	UoE	18.4±14.2	75.8±14.2	<.001
	Njoro	42.5±12.7	62.8±12.7	0.019
Plant height	UoE	17.8± 1.5	15.5± 1.5	0.115
	Njoro	98.4±7.5	116.4±7.5	0.018
Branches	UoE	21.0±4.6	27.4±4.6	0.168
	Njoro	18.2±4.5	31.7±4.5	0.003
Stem diameter	UoE	1.8±0.2	3.0±0.2	<.001
	Njoro	2.0±0.2	2.2±0.2	0.179
		Par Anamed	**Mutant Anamed**	
Crown length	UoE	77.9±6.4	127.6±6.4	<.001
	Njoro	83.9±5.7	80.9±5.7	0.600
Stem length	UoE	29.8±14.9	102.7±14.9	<.001
	Njoro	44.4±10.2	46.0±10.2	0.875
Plant height	UoE	103.3±5.9	145.6±5.9	<.001
	Njoro	112.5±6.8	111.2±6.8	0.839
Branches	UoE	24.5±4.8	35.5±4.8	0.022
	Njoro	29.2±4.2	35.0±4.2	0.170
Stem diameter	UoE	2.1±0.2	3.1±0.2	<.001
	Njoro	2.2±0.2	2.1±0.2	0.410

The means of Agronomical characteristics per plant line at UoE and Njoro sites ($P<0.001$) are shown in Table 2. There was significant differencein stem diameter of Artemis lines at UoE only. Line 2 had the highest mean of 3.9 while Line 12 had the lowest mean of 2.2. In UoE Anamed lines, there was significant difference in Crown length and plant height. In Crown length, line 5 had the highest mean of 148.6 while line 6 had the lowest mean of 107.8. In plant height, line 1 had the highest mean of 165.4 while line 7 had the lowest mean of 128.4. In Njoro Anamed lines, there was a significant difference in stem diameter only with line 8 having highest mean of 2.7 while line 7 had the lowest mean of 1.6.

Table 2: means of Agronomical characteristics per plant line in UoE and Njoro sites (95.0 percent SED intervals, P-value 0.001)

Artemi	Njoro (units in cm)					UoE (units in cm)				
Lines	**C.Lgt**	**S.Lgt**	**P. hgt**	**branch**	**s dter**	**C. Lgt**	**S.Lgt**	**P. hgt**	**branch**	**s dter**
Parents	71.7a	42.5a	98.4a	18.2a	2.0a	67.4a	18.6a	17.8a	21.0a	1.8c
1	83.4a	68.8a	109.6a	33.1a	2.6a	102.4a	119.4a	137.4a	41.3a	3.5a
2	85.7a	38.0a	103.6a	29.3a	2.0a	109.9a	54.1a	132.6a	24.8a	3.0b
3	83.0a	52.1a	103.0a	30.0a	2.5a	99.2a	49.8a	132.2a	20.9a	3.1b
4	70.5a	48.6a	106.5a	24.0a	1.8a	102.9a	56.3a	136.6a	27.1a	2.6b
5	85.6a	51.3a	116.1a	32.6a	1.8a	113.3a	95.4a	136.8a	31.4a	2.6b
6	87.7a	60.5a	122.7a	34.7a	2.1a	97.4a	98.0a	137.8a	30.7a	2.4c
7	79.4a	79.2a	122.4a	31.8a	2.5a	105.1a	98.7a	122.0a	34.6a	2.7b
8	90.4a	81.8a	130.6a	38.3a	2.2a	91.1a	91.6a	146.9a	27.4a	2.2c
9	93.4a	75.9a	124.0a	37.3a	2.5a	125.9a	51.0a	145.1a	18.7a	3.9a
10	88.9a	48.0a	110.1a	31.7a	2.4a	100.2a	74.9a	105.4a	32.8a	3.1b
11	88.0a	65.1a	113.9a	26.4a	1.8a	122.8a	93.6a	147.7a	31.0a	3.7a
12	66.7a	69.1a	123.4a	25.1a	2.2a	102.0a	36.8a	151.7a	11.4a	3.0b
p-value	0.247	0.772	0.266	0.757	0.02	0.123	0.048	0.003	0.052	<.001
Anam	**Njoro**					**UoE**				
Parents	83.9a	44.0a	112.2a	29.2a	2.2b	77.9d	29.8a	103.3d	24.5a	2.1a
1	-	-	-	-	-	-	-	-	-	-
2	74.4a	33.5a	116.2a	38.7a	1.7c	146.1a	122.7a	165.4a	43.1a	3.2a
3	86.5a	76.9a	102.1a	39.6a	2.1b	120.7b	106.0a	136.0b	36.3a	3.3a
4	77.9a	52.1a	120.8a	33.6a	2.1b	120.7b	111.3a	147.6b	39.3a	3.3a
5	79.2a	27.8a	117.8a	43.6a	2.1b	142.1a	122.4a	159.6a	36.8a	3.9a
6	89.4a	57.2a	114.9a	32.9a	2.1b	148.6a	102.8a	146.9b	37.1a	2.9a
7	68.8a	35.1a	97.9a	21.8a	1.6c	107.8c	75.8a	140.6b	27.3a	2.9a
8	85.4a	40.7a	104.0a	29.2a	2.7a	109.1c	73.1a	128.4c	29.4a	2.8a
9	-	-	-	-	-	126.1b	107.2a	139.9b	36.3a	2.8a
p-value	0.519	0.04	0.162	0.06	<.001	<.001	0.49	<.001	0.648	0.003

Means followed by the same letter in a column are not significantly different

4.2 Comparison of Artemisinin production by parents and mutant (M2) plants

The results for Means of comparison of artemisinin production between UoE and Njoro sites(P-value 0.001) are shown in Table 3. There was significant difference in Artemisinin production between the two sites in both Mutant varieties (Artemis and Anamed). The Artemis plants from Njoro had the highest mean of 44.9while UoE had the lowest mean of 24.9. In Mutant Anamed, Njoro had the highest amount of artemisinin with a mean of 43.7while UOE had the lowest mean of 25.7. There was

no significant difference in Artemisinin production by parents of the two varieties (Artemis and Anamed) between the two sites.

Table 3: Means for comparison of artemisinin amount production between UoE and Njoro sites (95.0 percent SED intervals, P-value 0.001)

Variety	UoE Mean ±SE (Units in µg/l)	Njoro Mean ±SE (Units in µg/l)	P- value
Par Artemis	21.8±2.0	22.4±2.0	0.783
Artemis	24.9±2.4	44.9±2.4	<.001
Par Anamed	23.7 ±2.0	22.8 ±2.0	0.644
Anamed	25.7±3.3	43.7±3.3	<.001

The amount of artemisinin in mutant plants in two plants grown in Njoro was more than that in the parent plants for both varieties. There was significant difference in Artemisinin production by Artemis and Anamed at Njoro($P<0.001$) (Table 4). Mutant plants had the highest mean of 43.1 and 45.5 while parent plants had the lowest mean of 22.4 and 22.8 for Artemis and Anamed respectively.

Table 4: Comparison of Means for Artemisinin production in parent and mutant plants in two plant varieties in UoE and Njoro sites (95.0 percent SED intervals, P-value 0.001)

SITE	MEAN/VARIETY (Units in µg/l)		P-VALUE
	Par Artemis	**Mutant Artemis**	
UoE	21.8±2.6	24.9±2.6	0.227
Njoro	22.4±5.8	43.2±5.8	<.001
	Par Anamed	**Mutant Anamed**	
UoE	22.665±3.6	25.908±3.6	0.375
Njoro	22.805±6.3	45.484±6.3	<.001

The results for means of Artemisinin production per plant line in UoE and Njoro sites ($P<0.001$) are shown in table 5. There was significant difference in production of Artemisinin by Artemis lines in Njoro only. Line 1 had the highest mean of 58.8 while Line 12 had the lowest mean of 25.9 for Atemisinin production. In Anamed there was significant difference in production of Artemisinin at both UoE and Njoro. At UoE, Line 2 had the highest mean of 39.2 while Line 5 had the lowest mean of 9.2; while at Njoro, Line 2 had the highest mean of 56.3 while Line 6 had the lowest mean of 20.2.

Table 5: Means of Artemisinin production per plant line in UoE and Njoro sites (95.0 percent SED intervals, P-value 0.001)

	Artemis (Units in µg/l)			Anamed(Units in µg/l)	
	(Line)	**UoE**	**NjoroLine**	**UoE**	**Njoro**
	Parents	21.8a	22.4d	parents	25.7b	43.7a
	1	23.6a	58.8b	1	-	-
	2	20.0a	58.4b	2	23.3b	56.3a
	3	28.6a	48.6c	3	39.2a	51.74a
	4	30.1a	47.3c	4	25.9b	27.0b
	5	17.3a	43.3c	5	24.5b	53.6a
	6	25.4a	29.8d	6	9.2c	20.2b
	7	19.9a	39.3c	7	27.6b	45.4a
	8	25.9a	47.6c	8	28.8b	54.4a
	9	24.0a	69.2a	9	27.2b	-
	10	30.1a	41.8c	10	-	-
	11	29.5a	44.2c	11	-	-
	12	24.7a	25.9d	12	-	-
P-value		0.1	<.001		0.001	<.001

Means followed by the same letter in a column are not significantly different

3.2 Effect of mutation on agronomical traits at M3 population.

There was significant difference in Crown length, Leaflength, and stem diameter at M3 ($P<0.001$) in Artemis variety (Table 6a).

Table 6a: Means of Agronomic traits in Artemis variety at M_3

Lines	Cr lengt	st length	l length	leaf width	pl height	No branches	stem dia
Parents	67.4c	18.6a	5.1b	3.7a	17.8a	21.0a	1.8c
1	144.0a	160.0a	45.8a	5.7a	165.7a	47.0a	5.4a
2	129.3ab	152.7a	7.1b	5.6a	161.3a	46.0a	5.1ab
3	128.7ab	147.7a	7.1b	5.5a	159.7a	45.0a	4.9ab
4	127.0ab	126.7a	7.1b	5.3a	154.3a	43.00a	4.8ab
5	126.3ab	125.0a	6.9b	5.2a	153.7a	42.7a	4.6ab
6	126.3ab	124.3a	6.9b	5.2a	153.3a	42.7a	4.3ab
7	124.3ab	116.5a	6.8b	5.2a	151.3a	42.7a	4.3ab
8	124.0ab	111.7a	6.8b	5.1a	150.0a	41.0a	4.3ab
9	123.3ab	111.0a	6.7b	5.0a	149.7a	38.3a	4.2ab
10	122.0ab	108.0a	6.5b	5.0a	149.0a	38.3a	4.1ab
11	121.3ab	106.7a	6.5b	4.9a	148.7a	36.7a	4.1ab
12	121.3ab	106.3a	6.4b	4.8a	148.3a	36.0a	4.0ab
13	120.0ab	105.3a	6.4b	4.8a	147.7a	35.0a	4.0ab
14	119.0ab	103.7a	6.3b	4.8a	146.0a	34.7a	3.7ab
15	118.7ab	102.3a	6.3b	4.8a	145.3a	34.7a	3.7ab
16	117.3ab	100.3a	6.2b	4.7a	145.0a	34.0a	3.7ab
17	115.0ab	97.0a	6.1b	4.6a	144.3a	33.7a	3.7ab
18	115.0ab	96.0a	6.0b	4.5a	144.0a	33.3a	3.7ab
19	114.3ab	95.0a	6.0b	4.5a	142.7a	33.0a	3.6ab
20	112.7ab	94.3a	5.9b	4.4a	140.7a	33.0a	3.6ab
21	111.3ab	93.5a	5.9b	4.4a	140.3a	32.7a	3.5ab
22	111.3ab	92.3a	5.9b	4.4a	140.0a	32.3a	3.4ab
23	111.0ab	91.3a	5.9b	4.3a	138.7a	32.0a	3.3ab
24	110.3ab	90.7a	5.9b	4.3a	138.7a	31.7a	3.2ab
25	110.3ab	88.0a	5.9b	4.3a	138.7a	31.0a	3.2ab
26	110.0ab	84.7a	5.8b	4.3a	138.3a	31.0a	3.2ab
27	109.0ab	84.5a	5.7b	4.2a	138.3a	30.7a	3.2ab
28	109.0ab	84.0a	5.6b	4.2a	137.7a	30.3a	3.13ab
29	107.7ab	83.7a	5.5b	4.1a	136.7a	30.0a	3.0ab
30	105.0ab	80.7a	5.5b	4.1a	134.7a	29.1a	3.0ab
31	103.3ab	79.3a	5.5b	4.0a	134.5a	29.3a	3.0ab
32	102.7ab	79.0a	5.5b	3.9a	133.0a	29.3a	3.0ab
33	102.0ab	70.0a	5.4b	3.9a	132.3a	26.3a	3.0ab
34	102.0ab	69.0a	5.3b	3.8a	131.5a	25.7a	2.9ab
35	101.3ab	68.7a	5.3b	3.8a	129.7a	24.7a	2.9ab
36	101.0ab	64.3a	5.3b	3.8a	129.0a	24.3a	2.8ab
37	99.7ab	63.7a	5.3b	3.8a	127.7a	23.7a	2.8ab
38	99.7ab	59.0a	5.3b	3.7a	126.7a	22.7a	2.8ab
39	98.7ab	58.8a	5.2b	3.7a	126.7a	21.7a	2.6ab
40	98.3ab	57.7a	5.3b	3.7a	126.7a	21.3a	2.6ab
41	97.7ab	57.0a	5.2b	3.6a	125.7a	21.3a	2.6ab
42	97.3ab	45.7a	5.1b	3.6a	124.0a	20.3a	2.5ab

43	97.3ab	44.7a	5.0b	3.5a	122.7a	20.0a	2.5ab
44	97.3ab	39.0a	4.9b	3.5a	122.0a	18.0a	2.4ab
45	91.3ab	36.3a	4.9b	3.5a	121.0a	17.0a	2.3b
46	90.0ab	27.3a	4.9b	3.4a	120.3a	12.7a	2.2b
47	83.3ab	24.3a	4.7b	3.3a	116.7a	12.0a	2.2b
48	83.3ab	23.3a	4.5b	3.3a	116.5a	10.3a	2.2b
49	82.3ab	23.3a	4.5b	3.2a	106.7a	10.0a	2.1b
50	64.7ab	22.7a	4.3b	3.0a	106.0a	8.7a	2.1b
Gr mean	108.7	82.3	6.6	4.3	136.6	29.2a	3.4
CV%	19.9	67.8	148.8	26.9	16.0	60.7	27.1

Means followed by the same letter in a column are not significantly different

There was significant difference in Crown length, plant height, and stem diameter at M3 at P-value 0.001 in Anamed variety (Table 6b).

Table 6b: Means of Agronomic traits in Anamed variety at M_3

Lines	cr length	st length	l length	l width	pla height	No. branches	stem dia
parents	77.9ab	29.8a	5.2a	4.8a	103.3c	24.5a	2.2ab
1	163.7a	159.00a	20.2a	16.9a	164.3a	52.3a	4.73a
2	153.7a	148.0a	7.9a	5.7a	164.0a	48.7a	3.9ab
3	150.3ab	137.8a	7.2a	5.1a	156.0ab	48.3a	3.9ab
4	146.7ab	136.3a	6.9a	5.0a	154.0ab	48.3a	3.7ab
5	146.0ab	133.7a	6.7a	4.9a	154.0ab	45.7a	3.7ab
6	143.3ab	129.2a	6.5a	4.6a	151.0ab	43.7a	3.6ab
7	139.7ab	125.3a	6.5a	4.6a	150.0ab	42.7a	3.5ab
8	138.3ab	121.7a	6.5a	4.5a	147.3ab	42.7a	3.5ab
9	135.3ab	118.7a	6.4a	4.4a	145.0ab	42.3a	3.4ab
10	132.7ab	115.0a	6.2a	4.3a	143.8ab	39.7a	3.2ab
11	130.7ab	112.8a	6.0a	4.3a	138.7ab	38.7a	3.2ab
12	118.7ab	109.7a	5.9a	4.3a	138.3ab	37.0a	3.2ab
13	118.3ab	105.3a	5.8a	4.2a	138.3ab	36.7a	3.2ab
14	117.3ab	100.3a	5.7a	4.1a	137.8ab	34.7a	3.1ab
15	114.7ab	93.3a	5.7a	4.1a	137.7ab	33.0a	3.1ab
16	113.0ab	84.7a	5.6a	4.1a	135.3ab	30.3a	3.0ab
17	112.0ab	74.6a	5.3a	4.0a	134.7ab	29.0a	2.9ab
18	106.3ab	69.7a	5.2a	3.9a	131.7ab	26.7a	2.8ab
19	103.0ab	68.7a	5.2a	3.7a	131.0ab	23.7a	2.7ab
20	100.3ab	60.3a	5.0a	3.6a	130.3ab	21.0a	2.4b
21	85.0ab	59.7a	4.9a	3.3a	121.0ab	19.3a	2.2b
Gr mean	126.5	104.7	6.6	4.9	141.6	36.5	3.3
CV%	16.8	56.6	82.8	100.7	10.6	48.8	19.8

Means followed by the same letter in a column are not significantly different.

4.4 Correlation analysis of agronomical traits and Artemisinin amounts

The estimated correlation among agronomical traits in mutant Artemis is represented in Table 7a. There was a significant positive correlation between crown length, stem length and stem diameter; Stem length, plant height, number of branches and stem diameter; Leaf width and stem diameter; plant height, number of branches and stem diameter; and number of branches with stem diameter. Artemisinin production had a significant negative correlation with Crown length, stem length, plant height and stem diameter.

Table 7a: Correlations Analysis of agronomical traits and Artemisinin in Mutant Artemis

1 crown length	-							
2 Stem length	**0.1563****	-						
3 Leaf length	-0.0372	0.0696	-					
4 Leaf width	0.0713	0.043	**0.1493****	-				
5 plant height	**0.5501***	**0.3718****	0.008	0.0745	-			
6 No. branches	0.0797	**0.7508***	0.0872	-0.0208	**0.2578****	-		
7 Stem diam	**0.6389***	**0.2322****	0.0333	**0.1534****	**0.5038***	**0.1223****	-	
8 Artemisinin	**-0.2828****	**-0.1492****	0.0709	0.0164	**-0.3642****	-0.0701	**-0.3599****	-
	1	2	**3**	4	5	**6**	7	8

** Significant at p<0.05; *Significant at p<0.01

The estimated correlation among agronomical traits in mutant Anamed is represented in Table 7b. There was a significant positive correlation between crown length, stem length, leaf width, plant height, number of branches and stem diameter; Stem length, leaf width, plant height, number of branches and stem diameter; plant height, number of branches and stem diameter; number of branches with stem diameter. Artemisinin production had a significant negative correlation with Crown length, stem length, leaf length and plant height.

Table 7b: Correlations Analysis of agronomical traits and Artemisinin in mutant Anamed

1 crown length	-							
2 Stem length	**0.5588***	-						
3 Leaf length	0.0307	**0.1031****	-					
4 Leaf width	**0.2003****	0.0380	0.0497	-				
5 plant height	**0.7896***	**0.5359***	0.0630	**0.1203****	-			
6 No. branches	**0.2256****	**0.6852***	-0.0139	0.0235	**0.2212****	-		
7 Stem diam	**0.7204***	**0.5273***	0.0912	0.2202**	**0.6384***	**0.1679****	-	
8 Artemisinin	**-0.2838****	**-0.2217****	**-0.1601****	-0.0167	**-0.3127****	-0.0499	**-0.1881****	-
	1	2	**3**	4	5	**6**	7	8

** Significant at p<0.05; *Significant at p<0.01

CHAPTER FIVE

DISCUSSION

5.1 Effects of mutation on agronomical traits and production of Artemisinin content

In the present study mutant varieties and lines showed wide variation in terms of agronomical traits (Crown Length, Stem Length, Plant height and Stem Diameter) and yield of artemisinin content. A total of108 Artemis and 72 Anamed plants were planted at UoE and KALRO Njoro sites respectively. At UoE site, only 3 mutant plants in each variety (Artemis and Anamed)had a production of more than 60µg/ml artemisin concentration while at Njoro,46 Artemis and 19 Anamed plants had a production of more than 60µg/l artemisin concentration. Parent Artemis had an average artemisinin production of 21.9 at UoE and 22.4 at Njoro while parent Anamed plants had an average artemisinin production of 22.7 at UoE and 22.8 at Njoro. Concentrations levels of artemisinin that are above 60µg/l, is the commercially viable level (Delabays *et al.*, 1993; Abdin *et al*., 2003). In some cases for example, mutant plants indicated higher performance in terms of Agronomical characteristics and/or artemisinin production, similarly some lines showed superiority over other mutant lines and control (parent plants) within and/or between the two agro economical zones where the study was undertaken. The following lines showed superiority in artemisinin production; Artemis line 1, 2 and 9 with an average mean of 58.8, 58.4 and 69.2 respectively while Anamed line 2, 3, 5 and 8 with an average of 56.3, 51.7, 53.6 and 54.4 respectively. Similar results have been observed by Mucci (1962) in wheat. Mutational breeding has important role for improvement of yield and quality of crops including medicinal plants for example Koobkokkruad *et al* (2008) used gamma irradiation for enhancement of artemisinin in *A. annua*. There was significant difference in production of artemisinin by Artemis lines at Njoro site only

where Line 1 had the highest mean of 58.8 and Line 12 with the lowest mean of 25.9 while in Anamed lines, significant difference in production of artemisinin was observed at the two study sites. At UoE, Line 2 had the highest mean of 39.2 while Line 5 had the lowest mean of 9.2; while at Njoro, Line 2 had the highest mean of 56.3 while Line 6 had the lowest mean of 20.2 (Table 5). The changes that were seen could have been due to the effect of mutation and judging from the results, the mutation could be variety and environmentally dependent. This result is consistent with the findings of Cox (1992) and Singh (2001) who reported that artemisinin content is influenced by genetic factors and therefore re arrangement of the gene sequences induced by mutation resulted in increase in artemisinin content production in some cases and a total decrease in others. Such unpredictable results may be due to random mutations at different loci in the genome. This effect could also be responsible for the differences seen in the agronomical characteristics which were also variety and environmentally dependent in their performance. Many authors agree that observed characteristics are also dependent on environmental factors (Khan *et al*., 2005) because the phenotype is the sum of factors genetic and environmental. There was significant difference in stem diameter of Artemis lines at UoE only while in Anamed lines, there was significant difference in Crown length and plant height at UoE and only stem diameter at Njoro. Basing on the results, the effect of mutation on both agronomical traits and production of the artemisinin content appear to occur randomly and is also dependent on environmental factors in the different ecological zones as higher agronomical traits was observed at UoE while production of artemisinin content was enhanced at Njoro even for the same plant lines and varieties. The physical mutagen gamma rays and its dose LD_{50} enhanced artemisinin content production from an average of 23.7 µg/l in original non irradiated seeds to 44.920 in

irradiated seeds. Koobkokkruad *et al* (2008) also found out that the Physical mutagen gamma rays (LD_{50}) enhanced artemisinin content from 0.03 to 0.70% (W/W). The pathway of artemisinin synthesis and the mechanism for its accumulation in leaves and inflorescences most probably determined the final artemisinin content in *A. annua* and the effect of other environmental factors such as nutrient availability might be incidental; a situation observed by Chalapathi *et al*. (2004). In mutant Artemis plants, there was significant difference in Crown length, plant height, and stem diameter while in mutant Anamed plants; there was significant difference in Crown length, Stem length, plant height, and stem diameter. Plants grown in UoE showed higher means in all the parameters that were analyzed as compared to Njoro (Table 1). There was significant difference in artemisinin production between the two sites in both Mutant varieties (Artemis and Anamed). In Artemis, Njoro had the highest mean of 44.9 while UoE had the lowest mean of 24.9. In Mutant Anamed, Njoro had the highest mean of 43.7 while UoE had the lowest mean of 25.7 (Table 3). There was no significant difference in artemisinin production by parents of the two varieties (Artemis and Anamed) between the two sites. Mutation may have been responsible for the difference in the agronomical characteristics and artemisinin production that was observed between the two agro- ecological zones and thus this is a clear indication that the changes induced by mutation is dependent on environmental influence. This observation was reinforced by the fact that in all the cases parents of both the plant varieties (Artemis and Anamed) did not show any significant difference in the agronomical characteristics analyzed and artemisinin production. Research conducted by Liu *et al.*,(2003)and Duke and Paul, (1993)has also shown temperature and longitude to have an important bearing on the productivity of *artemisia* by producing high biomass and artemisinin content. Similar findings have been reported

from Vietnam, where the artemisinin content was high in the high-altitude north than in the low-altitude south (WHO, 2003).Also there is evidence that mutagens (radiations) stimulate metabolic activity of plants such as respiration glycolysis and oxidative phosphorylation (Mergen and Johnson, 1964) and cytochrome oxidase and catalase activity which may ultimately influence and enhance synthesis of plant products. The lines exhibiting a total increase in artemisinin content and agronomical characteristics are the most suitable for high yield selection

At UoE Artemis plants, there was significant difference in Crown length, stem length and Stem diameter, while in Anamed plants there was significant difference in Crown length, stem length, Plant height and Stem diameter. None of the agronomical characteristics analyzed were significantly different at Njoro in the two plant varieties (Table 1). Similarly, there was significant difference in artemisinin production by Artemis and Anamed plants at UoE and Njoro (Table 4). Mutant plants have higher means in all cases thus indicating that mutation could have been responsible for the changes that were observed in both the agronomical characteristics and artemisinin production. The lines with pronounced agronomical traits and enhanced artemisinin content production could be positively selected for high yield. This explains why evaluation of leaf biomass accumulation or production by *A. annua* was more important, since it is a precursor for higher artemisinin yield (EABL, 2005). The study also reveals that the pattern of plant height, plant spread, number of branches per plant and dry leaf yield largely correspond to that of artemisinin yield obtained. This corroborates findings by Laughlin (1994) who stated that to achieve a commercially viable yield of artemisinin from *A. annua*, the evaluation of growth and

yield components is important. This implies that treatments that yield high morphological traits should be considered for higher artemisinin production.

5.2 Correlation Analysis of Morphological Traits and Artemisinin production

The coefficients of correlations between all the pairs of variables are presented in Table 7a and 7b. Artemisinin yield in *A. annua* crops was negatively correlated with Crown Length, Stem Length, leaf length, leaf width, Plant height, number of main branches and Stem Diameter. Besides, leaf traits (Crown Length, leaf length, and leaf width)had positive correlations with stem Characteristics (Stem Length, Plant height, number of main branches and Stem Diameter). This indicates that proliferated plant growth did not favor artemisinin accumulation in the leaves. The highly significant positive correlation between leaf traits on one hand and shoot or stem traits on the other hand and negative correlation of agronomical characteristics with artemisinin content of leaves indicate that high yields of artemisinin were realized when crops produced least possible leaf and stem tissue growth.The results suggested that the rate of leaf biomass yield kept pace with the rate of crop growth parameters. The major organ in artemisinin production is the foliage. An experiment has confirmed positive correlation between plant growth traits and biomass yield of *artemisia* (Dharm *et al*., 1996). Even though low proliferation of plant stem and leaf tissue resulted in higher Artemisinin content production, this could generally reduce the eventual expected yield and thus a plant with higher leaf yield will in turn have a higher leaf biomass which will then influence the total yield of Artemisinin.

The significant positive correlation of plant height, plant spread, number of branches with leaf and biomass yield indicates the expected influence of these growth parameters on the yielding ability of *A. annua.* It can therefore be inferred that leaf biomass yield and

artemisinin yield are largely dependent on the effect of the growth traits such as plant height and plant spread. Sushil, *et al*., (2004) indicated that there is a linear relationship between leaf biomass accumulation and artemisinin yield and therefore indirect selection via high leaf biomass production would generally be effective for high artemisinin yield.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- At UoE site, only 3 plants in each variety (Artemis and Anamed) had a production of more than 60µg/ml Artemisinin concentration while at Njoro, 46 Artemis and 19 Anamed plants had a production of more than 60µg/l artemisinin concentration. Plants producing artemisinin concentration ≥ 60µg/l are economically viable.
- Mutant varieties and lines showed wide variation in terms of agronomical traits (Crown Length, Stem Length, Plant height and Stem Diameter) and yield of artemisinincontent. Some lines showed superiority over other mutant lines and control (parent plants). There was significant difference in production of artemisinin by Artemis lines at Njoro site only where Line 1 had the highest mean of 58.843 and Line 12 with the lowest mean of 25.949 while in Anamed lines, significant difference in production of artemisinin was observed at the two study sites. At UoE, Line 2 had the highest mean of 39.2 while Line 5 had the lowest mean of 9.2; while at Njoro, Line 2 had the highest mean of 56.26 while Line 6 had the lowest mean of 20.19.
- The effect of mutation on both agronomical traits and production of the artemisinin content occur randomly. Environmental factors appear to have an influence in expression of mutated genes related to agronomical characteristics and artemisinin production. Higher agronomical traits was observed in UoE while production of artemisinin content was enhanced in Njoro

- Artemisinin yield in *A. annua* crops was negatively correlated with leaf traits and stem characteristics. Leaf traits had positive correlations with stem Characteristics.

6.2 Recommendations

From the study the following was recommended;

- Continual screening of the following lines as they may mutate further (Artemis lines 1, 2 and 9 and Anamed lines 2, 3, 5 and 8)
- Stabilization of these mutant lines through double haploid techniques and backcrossing to reduce the effects of mutations.
- Artemisinin product from the mutated plants should be tested for efficacy against Plasmodium parasites to check for possible change in their action.

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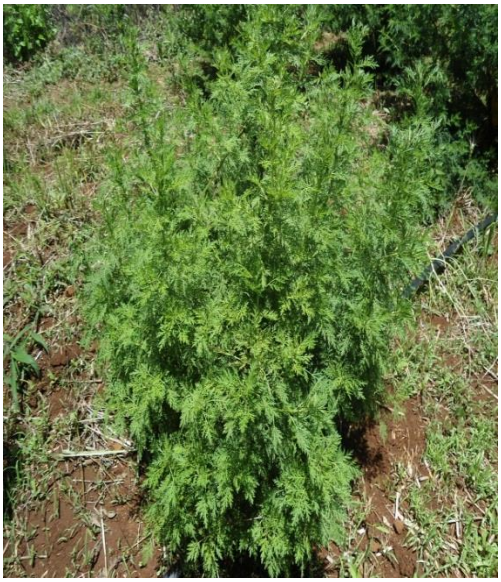
APPENDICES

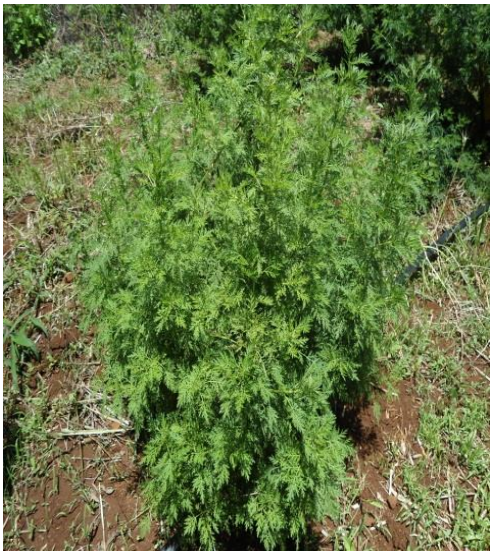
Appendix I: photographs showing *Artemesia annua* plants in the field

Artemis plant (good traits- UoE)



Anamed plant (reduced growth-Njoro)

Anamed plants (good traits UoE)



Anamed plant (reduced growth-UoE)

Artemis plant (Chimeric-UoE)

Anamed plant (Yellow green pigmentation-UoE)

Artemesia plants at UoE site

Appendix II: ANOVA Tables

Analysis of variance (UoE Artemis Lines)

Variate: ART

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	11	1733.4	157.6	1.50	0.144
Residual	91	9540.6	104.8		
Total	102	11274.1			

Tables of means

Grand mean 24.925

Line	1	2	3	4	5
mean	23.565	20.037	28.636	30.143	17.274
rep.	8	9	9	9	9
Line	6	7	8	9	10
mean	25.408	19.898	25.970	24.035	30.076
rep.	9	8	9	8	9
Line	11	12			
mean	29.526	24.748			
rep.	7	9			

Minimum standard error of difference	4.827
Average standard error of difference	4.956
Maximum standard error of difference	5.299

Analysis of variance (Njoro Artemis lines)

Variate: ART

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
line	11	14519.2	1319.9	3.71	<.001
Residual	82	29194.7	356.0		
Total	93	43713.9			

Tables of means

Grand mean 45.949

line	1	2	3	4	5
mean	58.843	58.393	48.648	47.293	43.253
rep.	9	7	7	9	8
line	6	7	8	9	10
mean	29.774	39.327	47.578	69.192	41.843
rep.	8	7	7	9	7
line	11	12			
mean	44.222	25.949			
rep.	5	11			

Minimum standard error of difference	8.481
Average standard error of difference	9.688
Maximum standard error of difference	11.048

Analysis of variance (UoE Anamed lines)

Variate: ART

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Line	7	4296.5	613.8	3.85	0.001
Residual	64	10201.4	159.4		
Total	71	14497.9			

Tables of means

Variate: ART

Grand mean 25.7

Line	1	2	3	4	5	6	7
	23.3	39.2	25.9	24.5	9.2	27.6	28.8

Line	8
	27.2

Standard errors of differences of means

Table	Line
rep.	9
d.f.	64
s.e.d. 5.95	

Analysis of variance (Anamed Njoro Lines)

Variate: ART

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
lines	6	10697.3	1782.9	4.91	<.001
Residual	48	17435.7	363.2		
Total	54	28133.0			

Tables of means

Grand mean 43.666

lines	2	3	4	5	6
mean	56.268	51.704	27.009	53.557	20.192
rep.	7	9	8	9	9
lines	7	8			
mean	45.401	54.448			
rep.	5	8			

Minimum standard error of difference	8.984
Average standard error of difference	9.780
Maximum standard error of difference	11.160