IN VITRO AND *IN SITU* BIOMASS AND ARTEMISININ PRODUCTION IN WORM WOOD (*ARTEMISIA ANNUA* L. Var. Chiyong)

BY

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DECLARATION

I declare that the work in this Thesis entitled *In vitro* and *in situ* biomass and Artemisinin production in Worm wood (*Artemisia annua*L) has been carried out by me in the Department of Plant Science. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Thesis was previously presented for another degree or diploma at this or any other Institution.

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CERTIFICATION

This Thesis entitled *IN VITRO* AND *IN SITU* BIOMASS AND ARTEMISININ PRODUCTION IN WORM WOOD (*ARTEMISIA ANNUA* L) by Salisu Muhammad TAHIR meets the regulations governing the award of the degree of Doctor of Philosophy in Plant Physiology of the Ahmadu Bello University, and it is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION.

This Thesis is dedicated to my late parents MalamTahir Muhammad SalisuDankama and MalamaAisha AdamuBabaleDandago for the care and proper upbringing they rendered to me.

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ABSTRACT

*Artemisia annua*has been identified for its anti- malarial properties and millions of Nigerians are at high risk of being infected by the disease. Its propagation is hindered by shortage of seeds, germination problem and effective protocol for*in vitro* and *in situ* propagation. The effect of some plant growth regulators (PGRs) on the *in vitro* regeneration of *Artemisia annua* using seed, leaf primordial, node explants and callus initiation were investigated in the Biotechnology Laboratory of the Department of Plant Science Ahmadu Bello University, Zaria. Explants were surface sterilized and inoculated on Murashige&Skoog basal media supplemented with varying concentrations of plant growth regulators. Results of Analysis of Variance for the *in vitro* experiment indicated significant difference among the treatments ($P<0.05$).*In situ* experiment was conducted on the farm of the Institute for Agricultural Research (IAR) ABU, Zaria. during the 2012 rainy season and 2013 dry season to determine the effect of some physical, chemical and hormone treatments as well as levels of organic and inorganic fertilizers on the growth, yield and artemisinin content in *A. annua*. Results of Analysis of Variance (ANOVA) for the i*n situ* experiment indicated no significant difference between the seasons with respect to germination, shoot and root lengths ($P\geq0.05$). Experiment with GA_3 and BAP, concentrations 1.5 mg/l of GA_3 and 0.5 mg/l BAP recorded the shortest days to *in vitro* germination. Highest regeneration percentage was observed at 2.0 mg/l of GA_3 and 0.5 mg/l of BAP. A concentration of 2.0 mg/l of GA_3 and 0.5 mg/lof BAP had the best vigor. Highest shoot height was observed at 2.0 mg/l concentration of GA_3 and 0.5 mg/l of BAP. GA_3 produced a significant effect on the seed, leaf and nodal explants with respect to vigour shoot length, days to regeneration and percentage regeneration except on the leaf's percentage regeneration. Also, BAP had significant effect on all the parameters studied on all the explants except on seed's percentage regeneration. Using BAP and NAA, best vigour was observed with 0.5 mg/l of BAP and 0.5 mg/l of NAA. Highest shoot height was recorded at 0.5 mg/l of BAP and 0.5 mg/l of NAA. However, lowest days to regeneration were observed with equal concentrations of 0.5mg/l of both BAP and NAA. Similarly, highest percentage regeneration was observed at equal concentrations of 0.5

mg/l of both BAP and NAA. However, with GA_3 and NAA plant growth regulators, concentrations of 1.0 mg/l of GA_3 and 0.5 mg/l produced the highest vigour. Also, a concentration of 1.5 mg/l of GA_3 and 0.5 mg/l of NAA had the best shoot height. However, 0.5mg/l of GA_3 and 0.5 mg/l of NAA had the lowest days to regeneration. Highest germination percent of 96 % was observed during the rainy season trial with 10 % Sulphuric acid treatment. Application of 6 t/ha of poultry manure during the raining season had the highest plant height followed by 4 t/ha of poultry manure during the irrigation trial.Warm water treatments in both rain and irrigation trials at 40 ^{0}C for 3 minutes and 60^{0}C for 3minutes produced the best vigor. Similarly, rain season trial had the highest number of branches with 50 kg/ha of NPK followed by same quantity of NPK during the irrigation trial. Similarly, rain trial had the best leaf fresh weight after flowering with 6 t/ha of poultry manure followed by irrigation trial with 4 t/ha of poultry manure. The best leaf dry weight before flowering was observed with 25 kg/ha of NPK during the rain trial. It was followed by the same quantity of NPK during the irrigation trial. Similarly, rain had the highest leaf fresh weight before flowering with 2 t/ha of poultry manure followed by the same quantity during the irrigation trial. Also, the rain season had the best result with respect to leaf dry weight after flowering with 8 t/ha of poultry manure followed by the same quantity during the irrigation trial. Treatment 6 t/ha of poultry manure had the highest artemisinin content of 2.1 % followed by treatment 75 kg/ha with 1.51 %. Lowest artemisinin content of 0.4 % was recorded with the control. Plant growth regulators and the organic and inorganic manure had significant effect on the*in vitro* and *in situ* regeneration and artemisinin production of *A. annua*.

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CHAPTER ONE

1INTRODUCTION

Artemisia annua L (Wormwood) belongs to the family Asteraceae (Ferreira, and Janick, 2002), whichconsist of about 400 species (Bailey and Bailey, 1976; Bennett *et al*., 1982; McVaugh, 1984 El-haq *et al.,* 1991; Klayman, 1993; Jaime and Da silver, 2003).The family is characterized by extreme bitterness of all parts of the plant (Tripathi *et al,* 2000; Tripathi *et al.,* 2001; Ferreira and Janick, 2009).

Artemisia annua is a new crop in Nigeria. Its propagation started in 2003 while artemisinin extraction commenced in 2005. As of August 2007, over 1,500 ha were prepared for planting with seeds from Brazil, China and local breeds (Anonymous, 2007; National Academics, 2007). Cultivation of *A. annua* spread from China to Africa, mainly Kenya, Tanzania and Nigeria in response to the call by the World Health Organization for the use of Artemisinin-Combination Therapies (ACT).The most concentrated areas of *Artemisia* production are Asia, Europe, and USA and recently East Africa (Klayman, 1993). However, global estimates for *A. annua* revealed a gradual increase in production area of 2,000 ha in 2003, 3000 ha in 2004, 9,500 ha in 2005, 26.000 ha in 2006, 14,500 ha in 2007, 4,500-5,000 ha in 2008, 6,000 ha in 2009, 15,000 ha in 2010, 17,500 ha in 2011 and 16,500 ha in 2012 (Artepal, 2008; Hiey, 2010; Ut, 2011). Production in 2013 is very uncertain due to labour shortages, competition from other crops and currency devaluation, but above all due to the reductions in artemisinin prices, market uncertainties and the introduction of semi-synthetic artemisinin (Pilloy, 2008; Roll Back Malaria, 2009: Antony, 2010). In China, production rate of 2,000 ha in 2004, 6,000 ha in 2005 and 9,000 ha in 2006 were reported (Tan, 2006). Vietnam is also a major producer but was reported to have

dropped from 10,000 ha in 2006 to 3,000 ha in 2007, 1,000 ha in 2008, 700 in 2009, 500-700 in 2010, and then expanded to 1,500 ha in 2011 (Artepal, 2008; Hiey, 2010; Ut, 2011). India has the potential for *Artemisia* cultivation but no estimates of production is available (Sharma, 2006; Cutler, 2008). In 2005, Kenya, Uganda, and Tanzania recorded an annual production of 1,650 ha. The yield rose in 2007 to 3,500 and 4,000 ha (Advanced Bio-Extracts, 2007).

1.1Soil and Climate

Artemisia grows very well in slightly alkaline loamy and well drained soils.The plant grows well in places exposed to good sunlight throughout the day. *Artemisia* is quite tolerant to drought and low moisture conditions (Ferreira and Janick, 2009). In fact, plants grown in poor and dry soils do produce a high aromatic quality. However, *Artemisia* tolerates temperatures that are as low as -5° C (Bui *et al*., 2011). Another notable feature of all the plants in this genus is their resistance to the honey fungus - a common fungus that affects many plants. At the same time, plants in this genus are rarely troubled by animals. *Artemisia annua* is a determinate short-day plant that is very responsive to photoperiodic stimulus.The critical photoperiod is about 13.5 hours, but there are likely to be photoperiod × temperature interactions. It thrives in many temperate to sub-tropical ecologies, but the plant is not adapted to the tropics because flowering will be induced when the plants are very small with the possible exceptions of high altitude plateaus or regions with a pronounced cool period (Klayman, 1993).

1.2 Economic Importance

Despite its anti-malaria properties, the effectiveness of *A. annua* has been demonstrated in the treatment of skin diseases and it has also been shown to be an effective non-selective herbicide (Duke *et al.*, 1987; Paniego and Giulietti, 1994). The anti-bacterial and anti-oxidant properties of

the plant's extracts and essential oil are a welcome development in the livestock industry. (Xiao and Catto, 1989; Allen *et al.,* 1997; Xiao *et al.,* 2000;Lescano *et al.,* 2004; Arab *et al.,* 2006; Brisibei *et al.,* 2008). The plant has also been used to impair the growth and development of insects that attack stored grains (Tripathi *et al.,* 2000;Tripathi, 2001).Three common derivatives found in *Artemisia* include artesunate, artemether and artemisinin (Duke *et al*., 1987; Kindscher, 1992). Artemisinin contains two oxygen atoms linked together in an 'endoperoxide bridge', which react with iron atoms to form free radicals and the artemisinin becomes toxic to malaria parasites. When it reacts with the high iron content of the parasites, it generates free radicals, which damage the parasite (Luo and Shen, 1987). By this same mechanism, artemisinin becomes toxic to cancer cells, which sequester relatively large amounts of iron compared to normal, healthy human cells. Tests conducted revealed that artemisinin causes rapid and extensive damage and death in cancer cells and yet has relatively low toxicity to normal cells (Luo and Shen, 1987).

Artemisinin has also been shown to regulate plant growth by inhibiting lateral root growth (Duke *et al*., 1987). Artemisinin has been more effective than glyphosate, when tested as a herbicide in the mung bean (*Vigna radiata*) (Chen *et al*., 1991).

1.3 Propagation constraints of *Artemisia annua*

Some factors preventing the normal propagation of *A. Annua* through the seeds include shortage of the seeds, presence of Phenolics on the surface of the seeds which was linked with germination inhibition and dormancy of the plant (Farouk *et al*., 2008). Similarly, the devastating decline in the natural habitats due to destruction of plant resources as a result of Man's economic and social activities, lack of sufficient training, difficulty to meet quality standards for processing

and extraction, steady market and profitable price to attract farmers, weed control, harvesting and drying techniques (Advanced Bio-Extracts, 2006). Artemisinin content and production is influenced by climatic conditions together with the time of planting and method of harvesting of the plant (Ram *et al.*, 1997; Wallaart *et al*., 2000). Cost of production of metabolites from cultured plant cells are some barriers which must be overcome to make their commercial regeneration a reality (Whitaker and Hashimoto, 1986; Giles and Songstad, 1990; Razdan, 2002)

1.4 Justification

In the year 2013, 123-283 million malaria infections and deaths in the range of 367, 000–755, 000 were reported globally,while 437, 000 African children were reported dying annually due to malaria infection (WHO, 2014). In 2014, malaria cases were reported in 97 countries and territories. An estimated 3.3 billion people were at risk of malaria, out of which 1.2 billion are at high risk. In Nigeria, malaria is endemic and 97% of the population is at risk of infection. The World Health Organization estimated 100 million malaria cases with over 300,000 deaths annually and child's mortality rate at 729 per 100, 000 (WHO, 2014).

Between 2010 and 2013, 53 countries of the world reported incidence of mosquito resistance to at least one insecticide especially Pyrethroids which is most frequently used in the control of malaria vector (Misawa, 1985). Similarly, reported cases of parasite resistance to anti-malarial medicines and mosquito resistance to insecticides rendered efforts of controlling malaria ineffective and triggered a rise in global malaria mortality undermining malaria control measures (WHO, 2014). At the moment, the only safe and affordable treatment for malaria to which the *Plasmodium* parasite is yet to develop resistanceis artemisinin- based combination therapy (ACT). It is necessary to develop reliable and reproducible methods for the analysis of

artemisinin and its derivatives. This will checkmate the aggravation of the incidence and burden of malaria in the tropics (WHO, 2014).

In situ production of *A. annua* is presently the most commercially feasible approach to produce artemisinin and related compounds, but farmers face the challenge of access to good-quality seeds,germination inhibition and dormancy of the plant, decline in the natural habitats due to destruction of plant resources as a result of Man's economic and social activities, lack of sufficient training, difficulty to meet quality standards for processing and extraction, steady market and profitable price to attract farmers, weed control, harvesting and drying techniques (Advanced Bio-Extracts, 2006). However, *in vitro* propagation is independent of seasonal changes and is a fast method of propagation which produces hhealthy plant material and requires small space.

There is also the need to enhance the cultivation of the plant by the development of effective protocol using *in vitro* and *in situ* culture techniques and improve the artemisinin extraction methods (Trigiano and Gray; 2000). This may be overcome through the use of *in vitro* facilities which can be installed in any part of the globe or the use of efficient *in situ* techniques for year round plant propagation. This is expected to bring about increased production of *Artemisia* and Artemisinin, createjob opportunities and increase in the livelihood of small scale growers of *Artemisia*. This will also supplement other efforts made through several studies and will help towards improving health services in Nigeria.

The aim of this work therefore, is to enhance the level of production of *A. annua* and artemisinin using *in vitro* and in *situ* cultures of *Artemisia* plant.

The specific objectives are as follows:

i. To develop an effective protocol for *in vitro* biomass and artemisinin production.

ii. To determine the most effective treatment for *in situ* germination of *A. annua*using seed.

iii. To determine the effect of organic and inorganic fertilizer on biomass and artemisinin production*in situ*.

iv. To compare the relative effectiveness of *in vitro* and *in situ* artemisinin production.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Artemisia annua

Artemisia (*Artemisia annua* L.) wormwood is a herb of Asiatic (Probably China) and Eastern European origin and it is the source of the traditional Chinese herbal medicine which has been used for over 2000 years to alleviate fevers (Antony, 2010). More than 400 species were identified and it is widely dispersed throughout the temperate regions of the world and in high-altitude regions with a pronounced cool period. Other *Artemisia* species are aromatic perennials and are also used for medicinal purposes (Klayman *et al*., 1984).

The plant has become naturalized and is cultivated in many countries including Argentina, Spain, the United States and Africa, mainly Kenya, Tanzania and Nigeria to support the production of the anti-malaria Artemisinin (Bailey and Bailey, 1976; Klayman 1993; Ferreira *et al*., 1997). Cultivation of *A. annua* in Africa was in response to the call by the World Health Organization to the adoption of Artemisinin Combination Therapies (ACT) as a replacement of Artemisinin monotherapy in the fight against multi-drug resistant *Plasmodium falciparum*. Large scale and commercial propagation of *Artemisia* in Africa is limited to Kenya and Tanzania. However, current demand for artemisinin has led to a significant increase in the large scale propagation, both in established and new areas (Ferreira *et al*., 1997).

2.1.1 The Morphology of *A. annua*

Artemisia annua, is an annual, short day plant with a critical photoperiod of 13.5 hr (Ferreira *et al*. 1995).The chromosome number of *A. annua* is 2*n* = 36 (Bennett *et al*., 1982). The plant

usually reaches about 2 m in height with alternate branches and alternate, deeply dissected, aromatic leaves ranging from 2.5 to 5.0 cm in length. Tiny yellow flowers (capitula) only 2 or 3 mm across are displayed in lose panicles containing numerous, greenish or yellowish, bisexual central (disc) florets containing little nectar and pistillate marginal (ray) florets.The central flowers are perfect and can be either fertile or sterile (Ferreira *et al*., 1995). Ovaries are inferior and unilocular and each generates one achene, 1 mm in length and faintly nerved. The pistillate marginal florets in the capitulum produce numerous achenes without pappus. The pollen is tricolpate and smooth, typical of anemophilous species, and has vestigial or no spines (Stix, 1960). It has an internal, complex, columellae-tecta configuration in the exine, which is common to all taxa of the tribe Anthemideae and varies from two to three layers in *A. annua*. The plant is naturally cross-pollinated by insects and wind action, which is unusual in the Asteraceae. Leaves alternate, dark green or brownish-green, rolled and crumpled. Odour is characteristically aromatic and taste bitter (Ferreira *et al*., 1995).

Non-glandular T-shaped trichomes and 10-celled biseriate glandular trichomes occur on leaves, stems, and inflorescences. The morphology and origin of the glandular trichomes has been described for leaves (Duke and Poul, 1993) and capitula (Ferreira and Janick, 1995) using light and/or scanning electron microscopy. The fruit of *A. annua* is an achene with a single seed inside. The seeds are approximately 1 mm in length, oblong, yellow-brownish with a lustrous surface marked by vertical furrows; seed endosperm is creamy white in colour and fatty in content.The seeds weigh approximately 0.03 g per 1000 (WHO, 2006)



Plate i: *Artemisia annua* on the field. **Source: Eric Sawyer, 2012**

2.2 Botany of *Artemisia annua*

2.2.1 Taxonomy

Artemisia belongs to the tribe Anthemideae of the Asteroideae, a subfamily of the Asteraceae. The genus *Artemisia* was sub-divided into various subgeneric sections; *A.annua* has been considered in the sub-section *Absinthium* or in a combined sub-section of *Absinthium* and *Abrotanum*. Greatest number of species was reported in Asia, with 150 accessions in China, 174 in the former U.S.S.R. and about 50 were reported in Japan, 57 species were identified in the European region and 30 species were documented in the New World (Colin, 2002).

The first natural classification of the genus was given by dividing the genus into four sections based on fundamental differences in the floral structure. *Abrotanum* and *Absinthium* were considered as the most primitive sections, while *Dracunculus* and *Seriphidium* being the most advanced (Colin, 2002).

Some authorities have elevated Besser's sections to the subgenus level and reduced the number of sub-genera to three, by combining *Abrotanum* with *Absinthium* to form the subgenus *Artemisia*, which was further divided into three sections: *Artemisia*, *Abrotanum* and *Absinthium*.The shrubby members of the subgenus *Seriphidium* endemic to North America have been recognized as being closely related, distinct from the Old World *Seriphidium* and grouped together in the section Tridentatae of the subgenus *Seriphidium*. Many authors have not accepted this assignment and have argued that the New World Tridentatae and the rest of the New World *Seriphidium* species should be considered as separate taxonomic entities. A similar close relationship among different species of the *Abrotanum*, was recognized and grouped them in the subsection Vulgares (Colin, 2002).

2.3 The significance of *Artemisia annua*

2.3.1 The use of *Artemisia annua* in the treatment of malaria fever

Artemisia annua has received considerable attention because of its anti-malarial properties. The plant activity has been established to be due to artemisinin (qinghaosu) a cadinanetype sesquiterpene lactone endoperoxide, present in the aerial parts. Its semi-synthetic derivatives, artemether; arteether and artesunate are effective against multi-drug resistant malaria caused by *Plasmodium falciparum* and against the life-threatening complication, cerebral malaria (Klayman *et al.*, 1984).

The World Health Organization estimated that in 2010 malaria caused 219 million clinical episodes, and 660,000 deaths. An estimated 91 % of deaths in 2010 were in the African Region, followed by the South-East Asian Region (6 %), and the Eastern Mediterranean Region (3 %). About 86 % of deaths globally were in children **(**Malaria Fact Sheet United States Embassy in Nigeria, 2011).

The two oxygen atoms of artemisinin react with iron atoms of the *Plasmodium* parasite to form free radicals thereby making the artemisinin toxic hence damaging the malaria parasites (Luo and Shen, 1987).

Currently, efforts are being made to make these drugs available world-wide and in order to meet the industrial demand for it, many research programmes have started focusing on the selection and cloning of high-artemisinin yielding chemotypes of *A. annua*. A review of recent agricultural techniques to improve yields from cultivated plants is also available (Laughlin, 1994). At the present time, the total synthesis of artemisinin is not yet economical for large-scale production, and so alternative approaches for improving the economics of drug production are under study

through the extraction of artemisinin related sesquiterpenes from *A. annua* plants and their subsequent conversion into artemisinin semisynthetically. Among these, artemisinic acid is the most promising (Laughlin, 1994).

Use of the drug by itself as a monotherapy is explicitly discouraged by the World Health Organization as there have been signs that malarial parasites are developing resistance to the drug. Therapies that combine artemisinin with some other anti-malarial drug are the preferred treatment for malaria and are both effective and well tolerated in patients. The drug is also increasingly being used in *P. vivax*malaria as well as being a topic of research in cancer treatment (WHO, 2002).

2.3.2 The use of *Artemisia annua* in livestock industry

The uses of *Artemisia annua* and artemisinin in the livestock industry are currently in expansion based on current reports of its anti-protozoal, anti-bacterial and antioxidant activities of its extracts and essential oil on *Babesia* Eimeriaor coccidiosis (Allen *et al*., 1997; Arab *et al*., 2006; Brisibe *et al*., 2008), others include trematodal blood fluke *Schistosoma* spp (Xiao and Catto, 1989; Xiao *et al*., 2000; Lescano *et al.*, 2004). Recently, different tissues of the plant have been analyzed for its potential use in animal feed and scored high values for anti-oxidant capacity and as source of amino acids, with negligible amounts of anti-nutritive components such as phytates and oxalates (Lescano *et al.*, 2004).

2.3.3 The use of *Artemisia annua* in the treatment of Cancer

Another possible use of artemisinin is in the treatment of cancer. Its anti-malarial activity is thought to be due to its interaction with iron, present in very high concentrations in the malarial parasite. Since some cancer cells, particularly leukemia cells, also have high iron concentrations,

they may also be killed by artemisinin, as has been demonstrated in some initial studies with cancer cells in tissue culture (Luo and Shen, 1987). The potential of artemisinin and its derivatives as cancer chemotherapeutic agents is being actively investigated in a variety of anticancer screens. The combination of a high demand for artemisinin-based anti-malarials and limited commercial-scale production of *Artemesia annua* (in only a few locales in China and Vietnam) has left artemisinin-based therapies in short supply (Chen e*t al.*, 1991). The World Health Organization has stepped in to develop a plan to bolster production (Duke *et al*., 1987; Chen *et al.*, 1991).

The same mechanism of action of artemisinin on *Plasmodium* parasite was observed on cancerous cells, which sequester relatively large amounts of iron compared to normal, healthy human cells. Tests conducted shown that artemisinin causes rapid and extensive damage and death in cancer cells and yet has relatively low toxicity to normal cells (Luo and Shen, 1987). Cancer is a general term used to describe the uncontrolled proliferation of somatic cells, which can develop into a tumor or spread around the body affecting one or more vital systems. In the United States alone, cancer kills over 500 thousand people a year, being the second killer after heart disease (Brown, 1990). Among the types of cancer, lung cancer kills the most, indistinctive of sex, followed by prostate cancer in man and by breast cancer in women. Cancer is an economic burden to the United States, costing over £585 million just in health care, not to mention disability and productivity losses. Traditional treatment includes radiation therapy or chemotherapy; the latter uses synthetic or semi-synthetic drugs that kill cancer cells as well as healthy cells. The chemotherapy treatment also causes undesirable side effects such as loss of appetite, nausea, and depression (Luo and Shen, 1987).

2.4 *In vitro* propagation of *Artemisia annua*

Artemisia annua is easily propagated *in vitro* by standard protocols. Cytokinins increase shoot proliferation but decrease rooting and increase vitrification. *Artemisia annua* can be grown and propagated by microcuttings in a hormone-free medium (Hamish, 1998). Artemisinin is produced in shoots *in vitro* and is enhanced by the presence of roots. No trace of artemisinin is found in roots or callus. There is no evidence that *in vitro* production of artemisinin will ever be commercially feasible. However, researchers have succeeded in producing artemisinic acid (a precursor) in yeast cultures (Dorsen, 2009) and artemisinin production through its precursor, in bioreactors might be a future avenue to explore (Razdan 2002, Hamish, 1998).

In vitro micro propagation is a suitable technique for the mass production of genetically uniform and disease free plantlets in limited time and space (Zobayed and Saxena, 2003). *In vitro* propagation of plants through tissue culture technique has been achieved by using varying concentrations andcombinations of plant growth regulators (Murashige, 1990; Uranbey *et al*.,

2005).

In vitro germination of *A. annua* seeds was reported to commence from the fifth to seventh day of inoculation, and 80 to 90% mature seeds produced full plantlets. The addition of growth hormones to culture medium did not necessarily shorten the duration or increase percentage of germination of *A. annua (*anamed)as reports have shown that, germination can occur at about 6th to 7^{th} day when grown under field conditions (Jamaleddine *et al*., 2011). This does not conform with other studies like that of Nikolic *et al*. (2006), who reported that, different hormones concentrations on seeds of *Lotus coniculatus* stimulated the percentage seed germination at least two fold in optimum concentrations.The growth response of germinating plantlets of *A. annua*

was apparently influenced by the combination of hormones. Well developed single shoots were recorded for most treatments with the exception of BenzylaminopurineBAP (0.025 mg/l) Naphthaleneacetic acidNAA (0.01 mg/l) and BA (0.10 mg/l)/NAA (0.01 mg/l). Similarly, plantlet growth on hormone free Murashige and Skoog (MS) medium was estimable with the addition of plant growth regulators (PGRs) having significant effect on the growth of *in vitro* cultures. The mean shoot and root lengths of plantlets on MS fortified with kinetin increased as concentration level increased (Elhaq, 1991). Moreover, the combination of 0.1 mg/l kinetin and 0.0 1mg/l NAA was considered optimal for elongation. Kinetin treatments produced thicker and elongated stems but with less number of nodes. It was experiential that cultures on auxin free medium supplemented with smaller amounts of BAP, showed better growth response with regards to number of nodes. Highest number of nodes was recorded with 0.025 mg/l BAP, indicating that BAP can be superior to kinetin in the provision of more nodes (Jamaleddine *et al*., 2011).

Mahmoodzadeh *et al.* (2010) investigated the effect of hormones and sucrose level on *in vitro* germination of *Zinnia elegans* plants. This indicated that, it is possible to improve the production of plantlets from seeds using different cytokinins and carbon sources. Similarly, Rashmi *et al*. (2010) reported that concentration of various PGRs increased the root and shoot length of plantlets germinating from bamboo seeds as compared to control. If properly planned, growth regulator can bring in very quick, rapid and distinctive changes in the target plants (Dorsen, 2009).

No multiple shoots were produced in *vitro* from seed explants. Moreover, an increase in BAP to 0.075 mg/l led to the formation of callus. Compact basal callus became more prominent without

emergence of any new shoots. In another study on *A. annua*, callus was induced on medium containing Murashige and Skoog inorganic salts and Gamborg B5 vitamins, as a basal medium and was maximal at 1.0 mg/L 2, 4-D and 0.1 mg/l kinetin (El-haq *et al*., 1991).

Aseptically growing shoots were induced from mono-nodal stem segments on MS medium supplemented with different concentration of BAP (0.1–1.0 mg/l) to evaluate the effect of cytokinin on *A. chamaemelifolia* shoot multiplication. Shoots were developed directly from all media variants. Increased number of shoots and decrease of their length was observed with increasing BAP concentration in the culture medium (Hristova *et al*., 2013).

The effect of BAP on stimulating shoot formation was reported for several species from the genus *Artemisia.* And about 50% explants were observed to form multiple shoots at 0.3, 0.4 and 0.9 mg/lconcentration. (Holobiuc and Blindu, 2007; Zia *et al*., 2007).

There was a trend of increase of the fresh weight of the *in vitro* propagated explants along with the increase of theBAP concentration. However, this might be due to hyperhydracitycaused by vitrification with 0.4 mg/l BAPwhere the weight of the explants reduced drastically up to 11 times folds after drying. The vitrification negativelyaffects the leaves causing abnormal morphology and physiology and this leads to ineffective micropropagation in long-term perspective. Another negative aspect was the riskof necrosis and retained development of the explants. Explants treatedwith 0.5, 0.6 and 0.7 mg/l BAP showed such an influence overhalf of the set explants (Ziv, 1991).

Moderate concentration of cytokinin (BAP) is highly sufficient towards the successful production of quality shoots. Several studies indicated that, when the concentration of BAP is

increased gradually, the multiplication rate is reduced to a greater extent. Similar results were obtained with medium fortified with BAP (1.0 and 2.0 mg/l) alone and in combination with NAA (0.5 mg/l). The media enriched with MS+IBA 1mg/l showed optimum rooting and this is also in line with the previous reports in *Artemisia vulgaris* L. (Ananthi *et al*., 2011). Auxin such as IAA was used for root induction in various crops like *Arachis stenosperma* and *A. villosa*. Increasing the concentration of IAA to 0.5 mg/l and above gradually led to the reduction of root induction. Similar result was observed in *Artemisia vulgaris*L (Gopinath*et al.,* 2014).

Callus is induced either by injury or by the addition of growth regulators usually under a proper ratio of auxin and cytokinin (Sugimoto *et al*., 2011). Lowest concentration of BAP applied alone was reported to cause callogenesis. It is probably an inherited ability of the species to form callus and a genotype dependent feature. The callus is the starting material for cell suspension cultures, which are widely used for bioreactor cultivation of plants (Paniego and Giulietti, 1994).

The number of days required for callus induction from the leaf explants of *Artemisia annua* was observed to varied from 10 to 30 days according to the combinations and concentrations of the growth regulators *viz.,* BAP, NAA, 2,4-D, IBA and TDZ in the MS medium (Ananthi *et al.,* 2011). MS medium fortified with NAA at 0.9 mg/l initiated 98.66 % of leaf discs for callus formation followed by 0.7 mg/l of NAA which initiated 84 % of leaf discs for callusing and 0.5 mg/l of NAA and TDZ each initiated 73 % of discs for callusing. Other combinations and concentrations of growth hormones in the medium initiated only 20 to 50 % of leaf discs of *A.annua* for callus formation. Baskaran and Jayabalan (2005) explained that, the differential response of same or different explants for callus formation could be due to the nature of tissue, degree of totipotency and composition of medium with respect to micronutrients and hormones.

Similarly, the variation in response of discs in terms of callus initiation may be due to the variation in distribution of endogenous level of growth regulators as observed in many other plants (Senthilkumar and Paulsamy, 2010). NAA alone or in combination with TDZ had the highest callus initiation percentage (>50 %) from leaf explant. Effective callus formation was reported from the leaf explants in a medium containing high quantity of NAA using *Ageratum conyzoides* species (Senthilkumar and Paulsamy, 2010). Similarly, Mariani *et al.* (2011) confirmed the significance of cytokinins and TDZ for effective callus formation in the ornamental plant, *Aglaonema sp.*Green and brown calli were obtained from different combinations and concentrations of the growth regulators.BAP alone (>1.5 mg/l) or in combination with GA_3 have enhanced shoot formation by 90-98 % from the subcultured calli (Jamaleddine *et al*., 2011).

Higher shoot length of 10 cm was obtained in the MS medium fortified with BAP alone at 2 mg/l. This indicates that BAP is an essential growth regulator for effective shooting of *A.annua*. This is supported by the fact that cytokinin is a major growth hormone involved in shoot formation in many plant species (Vijaykumari *et al.,* 2001; Roy *et al.,* 2008; Senthilkumr and Paulsamy, 2010).

The IBA concentration at 0.9 mg/l initiated 85% shoots which were used for root formation followed by 0.7mg/l which produced 80% and 0.5 mg/l had 71% shoots formation rate. The number of roots per shoot was also observed to be higher in the MS medium fortified with 0.9mg/l IBA. Similarly, the root length was greater (5.1cm) with subcultured shoots on MS medium with IBA at 0.9 mg/l. This confirmed the concept that auxins are the plant hormones endogenously or exogenously inducing root formation in majority of plant species (Mallikadevi

and Paulsamy, 2009; Mahesh *et al.,* 2010; Loc and Kiet, 2011; Mungole *et al.,* 2011; Rajput *et al.,* 2011).

2.5 Plant Growth Promotors

A plant hormone (phytohormone) is an organic compound synthesized in one part of a plant and translocated to another part, where in very low concentrations it causes a physiological response. Plant hormones are identified as promotors (auxin, gibberellin, and cytokinin), inhibitors (abscisic acid, xanthoxin, and violaxanthin), ethylene, and other hypothetical growth substances such as florigen etc. (Syed, 2001).

The mechanism by which hormones trigger a response is still not well understood. Specific receptors have been proposed, but no proof for their function in mediating hormone action has been given. There is, however, considerable evidence that gene expression is controlled by these hormones, but how it is done biochemically is largely unknown (Kawaguchi *et al.,* 1993). These hormones are found in all actively growing plant parts; young leaves and apical buds are particularly high in auxin, whereas young roots are high in gibberellins and cytokinins. Fruits and seeds are generally rich in all growth hormones (Syed, 2001).

2.5.1 Auxins

Auxin is a generic term for chemicals that stimulate cell elongation and influence a wide range of growth and development response. The existence of growth-regulating chemicals that control plant growth, and the interrelations between their parts, was the outcome of experiments on root and shoot responses to external stimuli (Syed 2001).

Besides IAA, plants contain three other compounds that are structurally similar and elicit many of the same responses as that of IAA: 4-chloroindoleacetic acid (CIIAA), phenylacetic acid (PAA), and indolebutyric acid (IBA). However, their physiological significance and transport properties remain obscure at present. Four additional compounds—indoleacetaldehyde (IAALD), indoleacetamide (IAM), indoleacetonitrile (IAN), and indole ethanol—are also found in a range of plants, but they are readily converted to IAA *in vivo*. The enzymes aldehyde dehydrogenase and indoleacetamide hydrolase, which catalyze the conversion of IAALD and IAM, respectively, to IAA, are active in plant tissues in which workers have detected IAALD or IAM. Similarly, IAN, found in the Cruciferae and Graminae families, is also accompanied by the enzyme nitrilase, which is involved in the conversion of IAN to IAA. These similar circumstances, in a range of plants, indicate that IAA is the true active free auxin in plants. Furthermore, free auxin forms are probably the most immediately utilizable by plants in their growth processes (Kawaguchi *et al.*, 1993, Syed, 2001).

Biological Activity

Biological activities of the applied auxin are so diverse and a number of responses at the molecular, cellular, organ, and whole plant levels have been described which are known to be influenced by the exogenous application of IAA. But to what extent these are under the control of endogenous IAA has not been established unequivocally. However, there are a few examples, such as control of the elongation of stamen filament of *Gaillardia grandiflora* and the photoinhibition of mesocotyl growth, that correlate well with the endogenous IAA levels (Bandurski and Nonhebel, 1984).

Auxin response is related to concentration, which is normally extremely low. In plants, free IAA is on the order of 108 g/kg fresh weight. The endogenous level of auxin is important in determining the course of development. Changing concentration can convert root meristem to shoot meristem and vice versa. A high concentration is inhibitory, while a low concentration is stimulatory, and both are important (Syed, 2001).

Apical Dominance.

In a pioneering work using *Vicia faba*, it was demonstrated that auxin diffused in agar blocks from the excised shoot apices, *Rhizopus* filterate. When the shoot apex of 'Wintex' barley (*Hordeum vulgare*) was destroyed, tillering was profuse unless the apex destroyed was replaced by an auxin. These observations indicated that, auxins from the apex exerts an influence on *Vicia* lateral bud growth as well as on barley tillering (Kawaguchi *et al.,* 1993). Studies on inhibiting auxins emanating from an apical bud, using inhibitors such as TIBA or morphactin, have shown that the lateral bud growth was effectively promoted. In intact tobacco, *Petunia*, and *Arabidopsis thaliana* plants, use of transgen emediated auxin and/or ethylene deficiencies, along with mutants insensitive to auxin or ethylene, supports the idea that apical dominance is the result of the auxin/cytokinin ratio rather than auxin-induced ethylene production. (Tamas, 1987).

Root Formation.

The most apparent auxin control of cell division is the formation of roots. Early evidence indicating the presence of active buds on cuttings to promote root development below led to the identification of auxin as the root-forming hormone (Kawaguchi *et al.,* 1993). The ease with which roots can form on cuttings varies enormously; shoot cuttings from some plants produce

roots simply if their basal cut end is left in water, whereas other species do so only rarely. Root formation shows polarity and always occurs at the morphological basal end, even if the cuttings are inverted upside down. Because auxin moves basipolarly, it was logical to believe that root formation at the basal end is a consequence of the movement of auxin to the lower tissues (Tamas, 1987). Removal of rich sources of auxin (i.e., buds and young leaves) reduces the number of lateral roots formed. This capacity is restored, however, if auxin is substituted for these organs. Tissue culture studies have provided further support by showing that a higher auxin/cytokinin ratio induces root formation and that changing it converts shoot meristem to root meristem and vice versa. To preserve genetic homogeneity, vegetative propagation (cloning) is important in horticulture, floriculture, forestry, and in the conventional breeding and/or biotechnology of higher plants. Therefore, basal treatment with indolebutyric acid (IBA) and synthetic auxin naphthaleneacetic acid (NAA) is commonly used to induce adventitious root formation in hard-to-root species (Tamas, 1987, Syed, 2001)

2.6 Gibberellins

Gibberellin was discovered in 1926 by Kurosawa, from cell-free fungus (*Gibberella fujikuroi*) filterate that promoted growth of rice (*Oryza sativa*) seedlings. Gibberellins (GAs) posses the unique property of increasing the growth of plants by greatly elongating their cells (Takahashi *et al*., 1986; Luo and Shen, 1987).

The concentration of GAs is usually highest in immature seeds, reaching up to 18 mg/kg fresh weight in *Phaseolus* species. However, it decreases rapidly as the seeds mature. In general, roots contain higher amounts of GAs than the shoots, and vegetative tissues contain a comparatively

low level of GAs, depending on the types of tissues and their stages of development (Bandurski and Nonhebel, 1984)

2.6.1 Biological Activity

The physiological properties of these highly active compounds are wide ranging, but extensive growth and enzyme synthesis are the most significant. The GAs act synergistically with other hormones in a system approach. The best known response is the stimulation of internode growth of dwarf maize, pea, and bush bean, which after treatment with GA attains the normal height. In some cases, but not all, dwarfism does in fact seem to be correlated with endogenous GA deficiency (Takahashi *et al.,* 1986). The most detailed analysis, at the molecular level, has been done with dwarf mutant of maize, known as dwarf- 5 (d5) (Takahashi *et al.,* 1986). The height of the dwarf-5 mutant is about one fifth that of its parent, due to a single gene mutation causing a deficiency of GA. When treated with GA, the mutant attains the height of normal maize. The action of much GAs is similar to that of IAA, including cell elongation, promotion of cambial activity, induction of parthenocarpy, and stimulation of nucleic acid and protein synthesis. The GAs varies greatly in their biological activity, and GA_3 and GA_7 are considered to have the widest range. In ferns, algae, and fungi, GAs has also been shown to influence growth and development. The content of GAs varies depending on the types of tissues and their stages of growth. Tissues other than seeds usually contain very low amounts (e.g., 0.3 g/kg in young bamboo shoots). Roots are considered to be the richest source of GAs, and most GAs, as such or in bound form, are supplied to the shoot (Takahashi *et al.,* 1986).

Buds and seeds of many plants show the ability to retain viability while having limited metabolic activity and no observable growth during an unfavorable season. This physiological condition is

commonly known as dormancy, and plants that grow in regions with a pronounced season usually adopt this strategy in late summer or early fall. Buds in dormant conditions are relatively more cold and drought tolerant than are actively growing buds. Similarly, seeds of many noncrop plant species remain dormant when they mature and will not germinate even if favorable conditions are provided. Dormancy of buds and seeds must be broken at a time when conditions are suitable for their growth and germination, respectively, during the spring. Long-day or brief red-light exposures have been found to break seasonal dormancy in many species. Gibberellins have also been found effective in overcoming both kinds of dormancy in buds as well as in seeds. Treatment with gibberellins has been observed to substitute effectively for long-day, low-temperature, or red-light exposure requirements. (Syed, 2001).

2.6.2 Growth Promotion

The isolation of gibberellins from plants, combined with their physiological responses to applied GAs, suggests that they do play a role in the regulation of various phases of their development. At the same time, if a process fails to respond to a certain GA, it could not be used as evidence that GA is not required. It may be that a different GA is required to elicit the response (Bandurski and Nonhebel, 1984). In *Silene*, GA_3 fails while GA_7 induces flowering under noninductive conditions, suggesting the involvement of gibberellin in this process. Several species of conifers show little or no elongation to GA_3 treatment, but they do respond to a mixture of GA_4 and GA_7 (Pharis, 1989).

During the vegetative growth phase, mitotic activity in subapical meristem is regulated by gibberellins. A reduction in its level causes a severe imbalance between internode and leaf growth, resulting in a form of growth called a *rosette,* first noted in *Hyoscymus niger* and later in

many other plants (Rood *et al.,* 1990). In plants such as cabbage (*Brassica oleracea capitata*), leaf development is profuse and internode growth is retarded during the vegetative phase. But before the start of reproductive growth, a marked elongation of the internode, called *bolting*, takes place. When treated with GA_3 during their rosette phase, such plants bolt and flower, whereas non-treated plants remain rosetted. There is evidence that endogenous gibberellins levels are higher in the bolted plants than in the rosetted plants (Pharis 1989). In addition, higher concentrations have been found in the bolted long-day *Rudbeckia speciosa* and cold-requiring *Chrysanthimum morifolium* cv. Shuokan than in their nonbolted forms. Thus, it appears that, the influence of gibberellin in such a response includes the stimulation of cell division as well as cell elongation. (Rood *et al.*, 1990).

2.7 Cytokinins

The discovery of cytokinins was an outgrowth of tissue culture research by Skoog and associates. Although, kinetin does not occur naturally, its discovery greatly supported the concept of the existence of a cell division factor is generally credited as the pioneer in providing experimental evidence for the hypothetical cell division factor. Phloem diffusates could cause cell division in potato (*Solanum tuberosum*) parenchyma cells. Similarly, cell division induced by wounding was prevented if the cut surface was washed and that leaf juice spread over the washed cut surface would restore it. It was observed that coconut milk could sustain the growth of isolated *Datura* embryo. According to (Koshizima and Iwamura, 1986), subsequent work by Stewart and his associates established that coconut milk markedly stimulated the growth of carrot (*Daucus carota*) root explants by cell division. They did succeed in isolating the growth-inducing factor, but it was a mixture rather than a single compound that was effective in their carrot bioassay system. According to Koshizima and Iwamura (1986), Skoog and associates, in the late 1940s,

using aseptically isolated slabs of non-dividing mature stem piths of tobacco plants observed cell division in the presence of vascular tissues (Syed, 2001).

2.7.1 Biological Response

Cytokinins are known to evoke a diversity of responses when applied exogenously to whole plants, plant tissues, or plant organs. Like other hormones, cytokinins have been used as a tool to investigate their role as endogenous controllers of plant growth and development. Like other hormones, cytokinins influence a multitude of morphological and physiological processes, among them seed germination, cell division and cell elongation, promotion of cotyledonary and leaf growth, control of apical dominance, delayed senescence, and morphology of cultured tissues (Koshizima and Iwamura 1986). Deficiency of a hormone must exist either experimentally or genetically to show that, adding hormone has an effect. Because cytokinins occur in all meristematic as well as in potential growing tissues and organs, it is not possible to create experimental deficiency. Genetically engineered cytokinin-overproducing tobacco and *Arabidopsis* plants have been used to study the phenomena of apical dominance. But in the absence of comparison with cytokinin-deficient mutants, the conclusion is equivocal. Thus, clear evidence is yet lacking which shows that specific physiological processes in plants and crops are under the control of endogenous cytokinins (Syed, 2001).

2.7.2 Organ Development

The totipotency of plant cells was demonstrated in a classical paper by Skoog and Miller in 1957 showing that a balance between cytokinin and auxin controlled bud and root formation in tobacco pith explant. At high concentrations of both hormones, cells often grow amorphously without differentiation. But a high cytokinin/auxin ratio causes induction of shoots, whereas a

high auxin/cytokinin ratio enhances root formation (Syed, 2001). Lateral development of axillary buds is inhibited by the presence of apical bud. This was shown by excision of the apical bud to remove correlative inhibition. But in the presence of apical bud, soaking the entire shoot in cytokinin enhanced lateral bud growth to a large extent (Koshizima and Iwamura 1986). Treatment with hadacidin, an inhibitor of purine synthesis, inhibited axillary bud growth following decapitation. Because adenine treatment could not reverse this inhibition, the inhibitor may not be specific. In the absence of cytokinin deficient mutants in higher plants, there is only indirect evidence that an endogenous cytokinin level regulated the development of axillary buds using genetically engineered cytokinin-overproducing tobacco and *Arabidopsis* plants, observed that the most significant morphological change of high cytokinin levels was that, it caused extensive growth of the axillary buds. Thus, there is strong evidence that cytokinin and auxin balance is important in organ differentiation and its further development (Syed, 2001).

2.8 In situ Propagation of *Artemisia annua*

Field production of *A. annua* is presently the only commercially viable method to produce artemisinin because the synthesis of the complex molecule is uneconomic. Currently used selections reach the peak in artemisinin before flowering and at the end of vegetative growth, allowing maximal biomass accumulation of artemisinin before harvest. The most important management problems involve planting, the achievement of uniform stands, weed control, and post-harvest drying of the crop. The plant is extremely vigorous and essentially disease and pest free. Most researchers transplant seedlings but direct seeding and mechanical transplanters have been used in commercial production. Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world (WHO, 2002). Natural products have always played an important role in medicine. It is well

known that secondary metabolites are a source of biologically active natural products with various functions, including anti-bacterial, anti-fungal, anti-viral, anti-neoplastic and anti-cancer activities, acting also as inhibitors and plant growth promoters (Syed, 2001).

2.8.1 Organic and inorganic Manure and the Propagation of *A. annua*

The significance of using manures and compost as soil additives are a well established and ancient agricultural practice used by small and large scale farmers alike. Since poultry manure is organic, it is able to have good effect on the physical and chemical properties of the soil (Asafu-Agyei *et al*., 1997). The purpose of organic fertilization besides the addition of nutrients to the soil is to improve the soil organic matter content. It is also known to improve soil aeration, water holding capacity, permeability and soil resistance to erosion. The importance of using manures and compost as soil additives is a well established and traditional agricultural practice used by both small and large scale farmers (Muchena, 1986).

Miller and Turk (1991) reported that, poultry manure promotes the growth of plants as a result of the presence of microorganisms, micronutrients, organic matter and regulating substances. According to Bandel *et al*. (1972), poultry manure contains appreciable amount of N, P and K based on the following percentages; 45% nitrogen, 2.5 % phosphorus and 2 % potassium. Ahn (1993) who found out that poultry manure is rich in nitrogen and the most concentrated farmyard manure increased yield. Kallah and Adamu (1988) conducted a study involving the use of different organic manures. It was reported that, the relative efficiency of organic manure in improving soil fertility followed the order; poultry manure > pig manure > farmyard manure. De Ridder (1990) reported significant yield responses from the application between 2.5 - 5.0 tonnes/ha of manure. The manure tonnage is high because the concentration of nitrogen in

animal manure varies for as much as four folds, depending on the type of feed fed to the animals. This is confirmed by Ofori *et al*. (1997) who reported a variation from 0.5 to 2.0 % nitrogen in manure. Liebhardt (1976) reported that, the addition of 5 t/ha of poultry manure doubled yield of *Artemisia* as compared to the control. Hileman (1971) also reported that, poultry manure enhances rapid releases of ammonia. Application of organic fertilizer increased the biomass yield and total essential oil yields of *Artemisia* (Parakasa, 1997). Poultry manure have good effects on the physical and chemical properties of the soil through increase in water infiltration rate, water holding capacity, cation exchange capacity and structure stability of the soil (Moore *et al.,* 1995). Bationoand Mokwunye (1992) also confirmed that, the addition and incorporation of organic materials either in the form of manures or crop residues have beneficial effects on the soil chemical, physical and biological properties.

According to Khalid and Shafei (2005) treating plants with different combinations of organic fertilizers and its rates resulted in a significant increase in growth, yield characters and artemisinin content extracted from the plants. Marculescu *et al.* (2002) observed that, a soil rich in macro and micro-elements enhanced by the use of organic fertilizers plays an essential role in plants growth and development and biosynthesis of the organic substances at all levels. The reason for increase in crude extract may be due to the influence of poultry manure in promoting vegetative growth, which resulted in increased herbage production, consequently the crude extract yield increased to a greater extent (Singh, 2001; Singh *et al.,* 2004). The possible reason for increased in shoot weight may be due to increase in length of shoot when poultry manure was applied due to its effect on photosynthetic efficiency of a crop resulting in the production of more number of leaves and stems or increase vegetative growth (Majbur Rahman *et al.,* 2003).

Inorganic manure is important for improving soil quality including organic carbon and total nitrogen. If applied, organic matter improves soil structure, aeration, greater root penetration, water percolation and

greater efficiency in utilizing irrigation water. The addition of 20 tons per hectare of well rotted manure has been recommended both as a short term crop stimulant as well as long term measure to improve soil fertility (Rodriquez, 1986). Organic fertilizer in addition to supplying nutrients also improves the physico-chemical condition of soils, enhance nutrient cycling and build the soil organic matter resources. Animal dung also has a favourable effect on artemisinin content. Land application of sewage sludge enhances microbial activity that may affect soil cycling and therefore influences the metabolism of the plant. Application of animal dung and other compost improve soil physical, biological and chemical properties and their promotive effect on artemisinin content may be attributed to the release of nutrients, mainly nitrogen which favours primary metabolism, growth, photosynthetic pigments and nutrient status (Gandhi *et al.*, 2000).

The kind of fertilizer to be applied is important not only for the economic returns the producer expects but for environmental reasons. Therefore, the need for soil test analysis has been recommended for predicting fertilizer needs (Sallah, 1991). Plants usually respond positively to N application, but this is not always true depending on the agro-ecology and the cropping history of the field. Adequate and balanced nutrients are necessary to obtain high yields. Rodriquez (1986) and Agboola (1986) in their report of improving maize grain yield suggested that response were found with up to 100 kg N/ha in the Sudan savannah and 150 kg N/ ha in the guinea savannah and even higher doses for the forest region. Thus, optimum application of the fertilizer is important in crop production but may vary from one agro-ecology to the other. Dennis (1990) observed that optimum application of fertilizer may be around 80 kg N/ha but

higher rates are needed on Savannah soils. Simon *et al*. (1990) reported that, both optimum essential oil of 85 kg/ha and fresh whole plant biomass yield of 35 t/ha were achieved at 67 kg N/ha at high plant density. However, the plants had a lower leaf - to- stem ratio. It was further observed that, fresh whole plant biomass of 270-750 g/plant was produced. Nitrogen deficiency was associated with a large decrease in artemisinin and leaf biomass yield (Fiqueira, 1996). Fiqueira (1996) observed that, the omission of nitrogen or phosphorus drastically reduced plant growth and dry matter production in hydroponic studies in Brazil. Magalhaes *et al*. (1996) conducted a study with four levels of nitrogen (0, 32, 64 and 97 kg N/ha) applied as urea. The results revealed that, the highest fresh whole plant biomass yield of 3880 kg/ha and artemisinin yield of 40.4 kg/ha was recorded. In the same study, no significant differences were found for leaf biomass and artemisinin yield when ammonium sulphate and ammonium nitrate were compared as a source of nitrogen. To obtain high efficiency of applied nitrogen, Rodriquez (1986) reported that, the nitrogen be applied in split and banded or side-dressed. Moll *et al*. (1982) defined nitrogen use efficiency (NUE) of a cultivar as yield produced per unit of available N (soil + compound fertilizer N). EABL (2005) reported that, 50 – 50 split application of N, 50% at planting and 50% when the crop reaches 50 cm tall increases leaf biomass and artemisinin content.

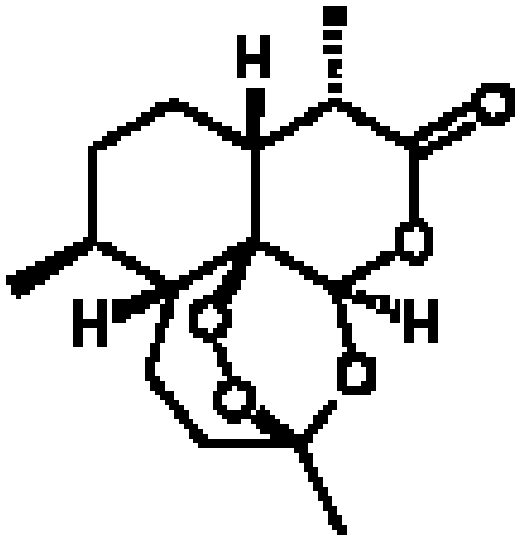
According to Ferreira *et al.* (1995), flowering could be delayed by cutting the apical meristem and providing nitrogen fertilizer. This causes the plant to branch out and potentially increase leaf biomass. WHO (1988) reported that, significant increase of total plant and leaf dry matter was obtained where a complete fertilizer mixture containing 100 kg N, 100 kg P and 100 kg K /ha was applied. Dry leaf yields of *Artemisia* between 6 – 12 t/ha were obtained in a mixed fertilizer containing 60 kg N, 60 kg P and 50 kg K/ha (Laughlin, 1994). Muchow (1988) reported that,

nitrogen supply to plants increase leaf area development and delays leaf senescence and increases the photosynthetic capacity of the leaf canopy. It was also noted that, maximum yield was obtained by the application of 83 and 95 kg N/ha in the forest and transition zones respectively. Response to P was non-significant. Wright (2002) noted that, nitrogen is a very mobile element and easily leached out of the root zone in tropical and sub-tropical conditions. He further observed that the yield of dry leaf per hectare varies from 1-40 t\ha.

Artemisia responds well to balanced fertilizers, and appears to be responsive to nitrogen but only a few data is available on the accumulation of artemisinin relative to fertility. It has also been noted that, nitrogen is the main constituent of protein and nucleic acid, which influences cell division, cell enlargement which might cause proliferation of roots and increase in shoot length resulting in better plant height (Gandhi, 1996). Veldkamp (1992) reported that, increase in shoot weight due to increase in N-levels may be attributed to adequate supply of plant nutrient needed for protein, amino acid, energy synthesis and improved metabolic activities. The number of branches increased substantially in treatments containing average level of manure or compound fertilizer additions (Ming, 1994). The number of branches per plant was significantly enhanced by the greater availability of chemical or organic nutrients due to their positive effect on shoot biomass production (Rao *et al.,* 1985). Magalhaes *et al.* (1996) reported that, nitrogen application increases the leaf dry yield about two fold. Martinez and Staba (1988) also observed that, N-fertilization stimulates the vegetative development of the plant, the greater the application of N, the greater the height of the plant. Plants with no manure or compound fertilizers additions were generally shorter and produced fewer nodes (Silva *et al.*, 1971).

2.9 Artemisinin

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 $C_{15}H_{22}O_5$(Fig. 1).



Source: Pandey *et al*., (1999)

Figure 1.The chemical structure of artemisinin.

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). It's physical properties include poor bioavailability that limit its effectiveness. As artemisinin cannot be synthesized chemically in an economically feasible way, *A. annua* is the only practical source of this valuable drug. Artemisinin is the new and promising drug which is also active against resistant Plasmodium malaria (McIntosh and Olliaro, 2004). Extensive work on the production of artemisinin from *A. annua* plants has been done (Acton and Klayman, 1985; El-Sohly, 1990; Jha *et al*., 1988; Ferreira and Janick, 1996; Ferreira and Simon, 1995; Titulaer *et al*., 1990). Artemisinin has also been reported from tissue culture of this plant (Fulzele *et al*., 1991; Jaziri, 1995; Martinez and Staba, 1988; Nair *et al*., 1986; Van Nieuwerburgh *et al*., 2006) and biochemical as well as molecular approaches for enhanced production of artemisinin from *A. annua* has also been tried (Abdin *et al*., 2003; Van Nieuwerburgh *et al*., 2006).The yield of artemisinin was higher (0.015%) in aerial plant parts in comparison to 0.001% of callus cultures (Singh et *al*., 2010).

Artemisinin is now available commercially as an anti-malarial drug that is efficacious against resistant strains of *Plasmodium* parasite. The demand for artemisinin has increased from 1 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 being expected to stabilize around 250/kg (Technoserve, 2004; Noorden, 2010). Estimate of yield per hectare varies significantly, but reports indicate a yield of 10-15 kg/ha from well-managed plantations in Africa (Wright, 2002).

Chemically, artemisinin is a sesquiterpene lactone containing an unusual peroxide bridge. It is believed that this peroxide is responsible for the drug's mechanism of action. No other natural compound with such a peroxide bridge is known (Duke *et al*., 1987; Chen *et al*., 1991,Ferreira *et al.*, 2005).

2.10 Artemisinin Production

The pathways for the synthesis of secondary metabolites may be influenced by factors such as nutrient levels, stress, and light and growth regulators. Most of the media constituents of plant cell culture affect the growth and subsequent production of secondary metabolites such as the artemisinin (Misava, 1985; Stafford *et al*., 1986; Ramachandra and Ravishankar, 2002).

The specific role of carbon sources in the production of plant secondary metabolite is not clear. However, shoot culture of *A. annua* produced high artemisinin content when the sucrose concentration was increased to 30 g/l (Liu *et al*., 2003). It was also reported that the application of 70 g/l sucrose improved artemisinin production in hairy root culture of *Artemisia* species (Liu *et al*., 1997). Weathers *et al*. (2004) suggested that, sugars might affect terpenoid metabolism not only as carbon sources, but also as signal molecules.Similarly, the ratio of nitrate to ammonia [NO_3NH_4 +] influences artemisinin content (Liu *et al*., 2003).

The biomass and artemisinin content for *A. annua* decreased with a higher ammonia to nitrate ratio. The optimum ratio of ammonia to nitrate was 1:3 for the production of artemisinin from *in vitro* shoot cultures of *A. annua* (Liu *et al*., 2003). Investigations in root culture showed that, a high ratio of nitrate to ammonia and a low total nitrogen concentration in the medium leads to a high secondary metabolite concentration (Ramachandra *et al*., 2002).

The influence of gibberellins and auxin on the growth and artemisinin production in a hairy root culture of *A. annua* was studied by Cai *et al*. (1995). He reported that an addition of 0.025 mg/l of NAA to the medium enhanced the hairy roots growth, but inhibited the artemisinin yield. On the contrary, supplementation of gibberellic acid (4.8 mg/l) stimulated both factors. Roots of *A. annua* incubated in a B5 medium supplemented with IAA inhibited the production of secondary metabolites more than those grown in synthetic auxins. A high concentration of IAA also stimulated the production of ethylene which inhibited the artemisinin production. Indole acetic acid IAA, Gibberellic acid GA_3 and Butyric acid BA significantly inhibited artemisinin production, when compared to cultures supplemented with NAA. Single roots grown in a B5 medium with GA_3 (0.029 mg) produced the highest values for a number of lateral roots, the length of primary roots, and the lateral root tip density.

The addition of 2-isopentenyladenine inhibited the root growth, but stimulated the artemisinin production more than twice in comparison to the controls. In shoot cultures of *A. annua*, various GA_3 concentrations influenced the growth and production of artemisinin (Weathers *et al*., 2005).

Shoot cultures were established on an MS basal medium which contained 30 g/l sucrose, 0.5 mg/l BA and 0.05 mg/l NAA. Using an optimized combination of sucrose (30 g/l), nitrate (45 μm), inorganic phosphate (200 mg/l), GA_3 (7 mg/l) and the ratio of nitrate to ammonia (1:3), artemisinin production reached 26.7 mg/l after 30 days (Liu *et al*., 2003).

The effect of amino acids: valine, cystine and growth regulators: BA and NAA on the production of artemisinin in callus of *A. absinthium* was also explored. Callus for the research was obtained from leaf explants which contained 223 μg of artemisinin per 1 g of fresh weight (FW). Very good results on callus growth and high concentrations of artemisinin were achieved when valine

(12.5 mg/l), or cystine (12.5 mg/l), and phytohormones: BA (2 mg/l), NAA (2 mg/l), were added to the medium. The amount of artemisinin in the callus culture was 3.1 μg/g after supplementation with valine, 2.8 μg/g following the addition of cystine. The amount of artemisinin in the callus cultures was 3.05 μg/g and 1.95 μg/g respectively, when BA (2 mg/l) and NAA (2 mg/l) were present in the medium (Zia *et al*., 2007).

Jing *et al*. (2009) showed that, the artemisinin content in *A. annua* plants treated with 10 mg ABA was 65 % higher than that in the control plants, up to an average of 1.84 % dry weight. This suggests a crosstalk between the ABA signaling pathway and the artemisinin biosynthetic pathway, and that CYP71AV1 (amorpha-4,11-diene C-12 oxidase gene), which was induced most significantly, may play a key regulatory role in the artemisinin biosynthetic pathway.

Banyai *et al*. (2011) observed enhancement of artemisinin content and leaf biomass in *A. annua* by exogenous GA_3 treatment. The increased artemisinin content was associated with an increased synthesis of key enzymes in the artemisinin biosynthesis pathway due to the GA_3 application (Zin *et al*., 2007). Interestingly, an exogenous GA_3 application continuously enhanced the artemisinin content from the vegetative stage to the flower initiation in both plant lines and gave a significantly higher leaf biomass. Consequently, the artemisinin yield in GA_3-treated plants was much higher than in the control plants (Mannan *et al*., 2008).

Singh and Sarin (2010) reported artemisinin content of 0.015% and 0.001% from both aerial parts and callus regenerated from the *in vitro* culture *A. annua*. Unorganized callus of *A. scoparia* was raised from the young nodal stem segments on an MS medium supplemented with 2,4-D (3 mg/l), kinetin (0.25 mg/l) and proline (100 mg/l).

Light exert stimulatory or inhibitory influence on the secondary metabolite accumulation in plant tissues. Moreover, it is involved in the regulation of the secretion mechanism for these products. Attempts were made to investigate the influence of light irradiation on the growth and accumulation of artemisinin in *A. annua*. Experiments showed that thirty days with 16 hours light exposition (3000 Lux, cool white fluorescent lamps) followed by an 8-hour-darkness, resulted in the highest levels of artemisinin for hairy roots culture (244.5 mg/l).Tests with darkness or continuous light conditions did not bring such good results (Liu *et al*., 2002).

A further effect of these factors on the hairy root culture included a low concentration of NAA (0.025 mg/l) which enhanced the growth of the roots, but inhibited the production of artemisinin. However, artemisinin production was higher after the addition of gibberellins to the medium (Mannan *et al*., 2008).

Artemisinin production by *A. annua* is usually in the range of 0.01 % to 0.4 % but some clones produce over 1 % (Delabays *et al*., 1993). Artemisinin can also be obtained from artemisinic acid which occurs at concentrations as much as 10-fold higher than artemisinin (Acton *et al*., 1985). Recently, Vonwiller *et al*. (1993) reported an extraction method which makes possible the extraction of both compounds from the same plant material, thus increasing the final production of artemisinin. Artemisinin compound was first extracted and identified as a potent anti-malarial drug by Chinese scientists in 1972. This was an outcome of a systematic examination of indigenous Chinese plants as sources of new medicines. Soluble derivatives developed from *Artemisia* include; artemether, artether, and artemotil. These medicines, in combination with other anti-malarials such as mefloquine, have proved highly effective in treating malaria, particularly *Plasmodium falciparum* which has become increasingly resistant to the first-line

treatments chloroquine and sulfadoxine-pyrimethamine—in Asia, South and Central America, and Africa. Given that malaria, despite intensive efforts by the world community, continues to kill between one and three million people each year, approximately three-fourths of whom are African children, and to cripple economies around the world, the importance of artemisinin and other effective anti-malarials cannot be overstated (Duke *et al*., 1987; Chen *et al*., 1991).

One possible solution to the supply problem may come from biotechnology. Scientists in California have recently produced the base structure of the chemical artemisinin in the bacterium *Escherichia coli*, and in yeast (*Saccharomyces cerevisiae*), by transferring the necessary genes from *Artemisia annua* into these microbes. For *E. coli* or yeast to become a viable source for artemisinin, the base structure would need to be modified, and the entire process would have to be scaled up to achieve commercial production levels. The use of Artemisinin as anti-malaria drug is currently the major hope in the fight against drug-resistant malaria. Artemisinin also known as Qinghaosu and its derivatives are a group of drugs that possess the most rapid action of all current drugs against *Plasmodium falciparum*. Treatments containing an artemisinin derivative (artemisinin-combination therapies, ACTs) are now standard treatment worldwide for *P. falciparum* malaria. The starting compound artemisinin is isolated from the plant *Artemisia annua*, a herb described in Chinese traditional medicine (WHO, 2002).

Undifferentiated and differentiated *in vitro* tissue culture techniques have been recently developed. The main efforts have been devoted to the *in vitro* selection of high yielding clones and cell lines, producing secondary metabolites, with pharmacological and industrial applications. In this regard major attention has been paid to *Artemisia annua* L., owing to its anti-plasmodial properties and moreover for its value as an aromatic plant, employed in

fragrances, perfume and cosmetic production. Micropropagation techniques have been applied to *A. annua* in order to obtain *in vitro* multiple shoot-cultures from which desirable clones may be selected and conserved (El-haq *et al*., 1992).

Much interest has also been devoted to the close relationship which exists between *in vitro* plant morphology and Artemisinin production, i.e. the correlation of the biosynthesis of artemisinin and related compounds with flowering which is typical of mature plants (Laughlin, 1994,Razdan, 2002).

Artemisinin is produced by differentiated shoot cultures (Martinez and Staba, 1988; Fulzele *et al*., 1991; Whipkey *et al*., 1992; Ferreira and Janick, 1996) but occur only in trace levels, if at all, in shoots without roots (Martinez and Staba, 1988; Jha *et al.*, 1988; Fulzele *et al*., 1991; Woerdenbag *et al*., 1993; Paniego and Giuliette, 1994). Brown (1994) reported 0.0038 % of artemisinin being produced by callus cultures bearing shoots but did not specify whether or not they had roots. Most workers (Martinez and Staba, 1988; Tawfiq *et al*., 1989; Fulzele *et al*., 1991; Kim *et al*., 1992) did not detect artemisinin in roots, although Nair *et al*. (1986) and Jha *et al*. (1988) reported trace amounts. Weathers *et al*. (1994) reported high levels (0.4%) of artemisinin in hairy root cultures of *A. annua* transformed with *Agrobacterium rhyzogenes*, but this was not confirmed by Jaziri *et al*. (1995). With the exception of Jha *et al*. (1988), most authors (He *et al*., 1983; Nair *et al.*, 1986; Tawfiq *et al*., 1989; Fulzele *et al*., 1991; Kim *et al*., 1992; Woerdenbag *et al*., 1993; Brown, 1994; Paniego and Giuliette, 1994; Ferreira and Janick, 1996) either reported no artemisinin or only trace amounts produced from callus, cell, or the spent liquid media from these cultures. Ferreira and Janick (1996) presented evidence that, artemisinin production in shoots is enhanced by the presence of roots. The highest level of

artemisinin (0.287% DW) was obtained in hormone-free medium when the root production was maximized.

Ferreira *et al*. (1995) by studying *A. annua* in greenhouse, tissue culture and open field, found that artemisinin content was 4- to 11-fold higher in flowers than in leaves and that favourable results could be expected by intercrossing selected high artemisinin-yielding clones which had been grown in the greenhouse with prolonged lighting. Valuable metabolites have also been obtained in undifferentiated callus and cell suspension cultures of *A. annua*; including the coumarin, scopoletin and artemisinin from callus grown on MS medium, supplemented with 2,4-dichlorophenoxy-acetic acid and naphthalene-acetic acid. However, sometimes suspension cultures failed to accumulate any of the terpenoids found in the parent plant (Wang *et al.,* 2007). Other than callus and multiple shoot cultures producing terpenoids *in vitro*, protocols for plantlet cultures in bioreactors have been developed for different *A. annua* geographical varieties, thus resulting in the production of camphor, 1,8-cineole and P-caryophyllene (El-haq *et al*., 1991).

There are several reports on the biosynthetic pathway leading to artemisinin as well as biotechnological approaches for the production of this sesquiterpene lactone *in vitro* (El-haq *et al*., 1991). Artemisinin production has been obtained in transformed roots of *A. annua* after infection with *Agrobacterium rhizogenes*, in percentages suggesting a feasible commercial production of the active compound. Transformed shoot and root cultures of *A. annua* have also been established by infection with *Agrobacterium tumefaciens* and were shown to produce artemisinin during prolonged subculturing (Wang *et al.,* 2007, Razdan 2002).

A protocol was developed to preserve the hairy roots of artemisinin producing ***A.*** *annua* plants by cryopreservation in liquid nitrogen Ferreira and Janick (1995) developed a method for the immune-

quantitative analysis of artemisinin using polyclonal antibodies. Besides its anti-malarial characteristic, artemisinin also exhibits potent phytotoxic properties by inhibiting germination and reducing growth of many weeds and crop plants (Duke *et al*., 1987).

Callus cultures and protoplast isolation have also been obtained from other Artemisia species such as *A. absinthium* and *A. sphaerocephala*. Various environmental factors, such as artificial light quality, sucrose concentration and hormonal culture media supplements have been shown to be effective in promoting in vitro essential oil accumulation, as occurred in the test-tube plants of *Artemisia balchanorum.* Growth medium-related oil production was also found for *Artemisia alba* as well as for other species of the Belgian flora, where the aim of the research was to develop a conservation programme based on micropropagation (Wang *et al.*,2007, Razdan, 2002).

Micropropagation techniques have also been applied in the flavour industry, for the isolation and growth of *Artemisia* clones of the "genepi-group" (*A. umbelliformis* Lam. and *A. genipi* Weber), having a high degree of variability in their essential oil GC patterns and organoleptic characteristics (Duke and Paul, 1993).

2.10.1 Site of Artemisinin Synthesis

The lipophilic substances produced by the genus *Artemisia* are synthesised and accumulated in secretory structures that can be generally classified into two categories: glandular trichomes and secretory ducts. Glandular trichomes are epidermal structures whilst ducts are composed of secretory tissue present below the epidermal layers occurring in many organs of the whole body of the plant. The biology and chemistry of glandular trichomes has been reviewed by several authors however, only a limited number of species of the genus *Artemisia* have been studied.

In general, glandular trichomes are made from a series of differentiated cells with different functional properties (Wang *et al*., 2007). With regard to terpenoid production, the essential oil accumulates between the cuticle and the cellulosic wall of the secretory cells, whose position is always at the top of the structure. Below secretory cells are the so called stalk cells, whose function is to connect secretory cells to the plant body. The base of the trichome is made by a foot (basal) cell, which is a modified epidermal cell that upholds the entire trichome structure. The number of secretory, stalk and foot cells may vary from plant to plant (Duke and Paul, 1993). In the genus *Artemisia*, the ontogeny of glandular trichomes is described as the appearance of a protodermal cell that undergoes anticlinal and periclinal division to yield a couple of basal and stalk cells, and three couples of secretory cells (Colin, 2002).

During the early stages of trichome development, all cells are cytologically active and the secretion of oil components and oleoresins is transferred to the subcuticular layer that starts enlarging until rupture occurs. After this period, the trichome degenerates beginning from the secretory cells down to the stalk cells. In *A. campestris* maritima, the glandular trichomes are embedded in a dense layer of oleoresins which cover the leaf surfaces. The removal of the oleoresins reveals the presence of numerous biseriate trichomes which are mostly present on adaxial surfaces (Duke and Paul, 1993).

An ultrastructural study on A. annua carefully describes the structure of the biseriate 10-celled glandular trichomes (Duke and Paul, 1993). Three apical cell pairs represent the secretory head, the first two top pairs contain amoeboid plastids without thylakoids, whereas in the two cell pairs below, ameboid chloroplasts show the presence of photosynthetic thylakoids. Stalk cell plastids containing starch grains and thylakoids were occasionally present in basal cells. An osmophilic gradient from the stalk to the uppermost secretory cells indicates the path of secretion and the related secretory activity of the cells . The same situation was found for the glandular trichomes of *A. coerulescen*. In *A. umbelliformis*, besides essential oils, the subcuticular space accumulates some sesquiterpene lactones, like cytotoxic

hydroperoxyeudesmanolides, that can be localized histochemically, whereas in *A. nitida* the glandular trichome histochemistry reveals the presence of terpenoids as well as steroids and catecol tannins (Wang *et al.*, 2007). Sesquiterpene lactones have been localized in the glandular trichomes of *A. nova* (Duke and Poul 19930, whereas in *A. annua* the combined used of scanning electron microscopy and HPLCEC of the flowering branches provided corroborating evidence that, the biseriate trichomes that cover the epidermises are the site of sequestration of Artemisinin (Ferreira and Janick, 1995). Finally, the isolation and purification of glandular secretory cells has been obtained in *A. tridentata* vaseyana by a Percoll density gradient centrifugation (Wang *et al.*, 2007).

Another important secretory structure present in the genus *Artemisia* is represented by resin ducts. The composition of the secreted substances in these structures varies from terpenes to polysaccharides to several other products. In *A. campestris*maritima, the resin ducts are distributed in the cortical parenchyma near the vascular bundles, running longitudinally in petioles and stems. The duct lumen is surrounded by secretory cells and contains oleoresins which stain with histochemical reactions typical of lipophilic substances such as terpenoids. The capability of secreting oleoresins inside the ducts appears to be related to the presence of a great amount of endoplasmic reticulum. The latter is found in association with plastids with few thylakoids contained in the epithelia1 and sub-epithelia1 cells that surround the duct cavity. The same results have been obtained from *A. nitida*, where the oleoresin content of the ducts is similar to that of the glandular trichomes (Colin, 2002, Wang *et al.*,2007).

Artemisinin content (% DW) was shown to be 4 to 11 times higher in the inflorescences as compared to leaves and the presence and development of glandular trichomes in the inflorescences was associated with artemisinin production based on extraction studies (Ferreira and Janick, 1995). The glandular trichomes are more prominent in the corolla and receptacles florets than in leaves, stems, or bracts. Although, these glands are present since the early stage of development on both leaves and

inflorescences, artemisinin increases at anthesis, suggesting that it accumulates as the glands reach physiological maturity, a stage which coincides with the end of cell expansion in floret development (Ferreira *et al*., 1995). As glands approach maturity, there appears to be a cellular discharge into the subcuticular space around the apical cells and the contents are spread over the epidermis when the glands burst. After anthesis, artemisinin decreases and so does the number of intact glands. The association of artemisinin with glandular trichomes sequestration explains why artemisinin was not detected in parts of the plant that do not bear glands, such as pollen or roots (Ferreira *et al*., 1995) or in a glandless biotype (Duke *et al*., 1994). Glandular trichomes are observed in leaves and stems of differentiated shoot cultures and artemisinin content of shoot cultures *in vitro* was similar to artemisinin content in vegetative clones grown in the greenhouse (Ferreira *et al.,* 1995).

2.11 Artemisinin Extraction

Artemisia annua is processed through solvent extraction, using hexane and petroleum ether (Vonwiller *et al*., 1993). Or using hydrocarbon extraction processes (El-Sohly *et al*., 1990). Other solvents being considered for extraction of artemisinin are supercritical carbon dioxide, ethanol, ionic liquids and hydro fluorocarbon HFC-134a and it has been observed that, extraction of artemisinin using these solvents has proven to be better interms of high process efficiency than the traditional hydrocarbons (Vonwiller *et al*., 1993). At present, few analytical procedures exist for the analysis of artemisinins and derivates. These are the Indirect Columetric Assay (ICA) (Michael, 2000) and the high performance liquid chromatography (HPLC) (Bin, 2006).

Singh *et al.* (2010) working on *A. scoparia* reported that aerial plant parts and harvested callus were dried and powdered and extracted by continuous percolation over a period of four to six hours using five to ten fold volume of the non-aqueous solvent ethanol.The extraction process was repeated three to five times to ensure maximum extraction of artemisinin from the herb. The resulting extract was concentrated to 1 to 5 % of the original volume by distillation under vacuum.

The excess of water (four times of the reduced volume of ethanol extract) was added to the concentrated to make it 80 % aqueous followed by partitioning of the contents between water and hexane. For partitioning, the aqueous content and hexane were used in a ratio of 1:1 or 2:1 v/v. Partitioning of aqueous content with hexane were repeated three to five times using the same solvent ratio in order to ensure maximum transfer of artemisinin to hexane fraction. The combined hexane fractions were pooled together before they were distilled under vacuum (to recover the solvent for using again) to obtain 1-5 % of its original volume. The concentrated liquid was a light to dark green oily liquid. Ethyl acetate (10-20% v/v) is added to it. To remove the green pigmentation this liquid was treated with 1-3 % w/v of any colour absorbing substance like activated charcoal (Singh *et al.,* 2010). The yellowish liquid obtained after removal of activated charcoal by filtration was subjected to the evaporative crystallization yielding substantially pure artemisinin (Singh *et al*., 2010). The purified crystals thus obtained were further analyzed for artemisinin by Thin Layer Chromatography (TLC) as follows:

Thin glass plates coated with silica gel ‘G’ were prepared and activated. The extract so obtained was dissolved in ethanol and applied separately 1 cm above the edge of the activated plates along with the standard reference compound of artemisinin. The glass plates were then developed in an organic solvent mixture of dichloromethane: ethyl acetate (De Vries*et al*., 1999). The plates were sprayed with a spray mixture consists of 1.5 ml anisaldehyde, 30 ml glacial acetic acid, 225 mL methanol and 1.5 mlL sulphuric acid. The plates were dried for 5 min at 110-120ºC and after cooling, sprayed again for at least 10 seconds and dried for 10 min at constant temperature (Singh *et al*., 2010).

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study Area

In vitro experiments were conducted in the Biotechnology Laboratory, Department of Plant Science, while the *in situ* experiments were conducted on the farm of Institute for Agricultural Research (IAR) during the 2012 rainy season and 2013 dry season in Ahmadu Bello University, Zaria, latitude 11 º 11 N and 07º38 E, altitude 670 m above sea level.

3.2 Experimental Materials

For both the *in vitro* and *in situ* germination experiments, seeds of *Artemisia* Chiyong variety were obtained from the *Artemisia* programme unit of IAR. Nordal and leaf primordia explants were excised using sharp knife from *Artemisia* plants obtained from the IAR garden.



Plate ii a Seeds obtained from Chiyong variety of *Artemisia annua*



Plate ii b. Stem cuttings with node explants excised from *A. annua*



Plate iii a. Leaves of from Chiyong variety of *A. annua*



Plate iii b. Leaf primordia of *A.annua* obtained from the shoot apical meristem

3.2.1 Protocol for *in vitro* regeneration

Full strength Murashige and Skoog (MS) mediaaugmented with varying concentrations of plant growth regulators (PGRs) were prepared. Full strength MS media (Appendix i) consisted of the actual concentrations of various components of macro and micro salts as recommended by Murashige and Skoog (1962).

3.2.2Surface sterilization

Outside the laminar flow hood, explants were washed with water and with 3 drops of Hederol wash soap solution and rinsed 5 times under running tap for 10 minutes.

In the laminar flow hood, the explants were further sterilized by soaking in 90 % ethanol for 5 minutes. The ethanol solution was decanted and the explants were rinsed in autoclaved distilledwater. It was followed by immersing in 1% Mercuric Chloride (HgCl) for 4 minutes. Explants were then rinsed 5 times in double distilled water before inoculation on the media (Hamish,1998, Razdan,2002)

3.2.3 Media preparation

For organogenesis using seed, nodal and leaf explants, full strength Murashige and Skoog (1962) media containing 30 g/l sucrose, 100 mg/l Myo-Inositol augmented with varying concentrations of plant growth regulators (PGRs) were prepared (Tables3.1). Hormone free MS media served as control. The pH of the medium was adjusted to 5.8±1 before the addition of 8g/l agar as gelling agent. Then, 50ml each of the media was dispensed into bama bottles and autoclaved at 121 °C at 15 lb pressure for 15 min. Fresh and clean seeds, leaf primordia and stem cuttings with nodes of 1-3cm long were inoculated on a Murashige & Skoog (1962) basal media supplemented with

varying concentrations of the plantgrowth regulators (PGRs) using the procedure of Hamish, (1998) and Razdan, (2002).

Table 3.1: Combination of PGRs used as supplements in MS media for the regeneration of *Artemisia* seed, leaf primordia and nodal explants

PGRs Concentration (mg/l)					
GA3 x BAP		**BAP x NAA**		**GA3 x NAA**	
GA3	BAP	BAP	NAA	GA3	NAA
0.5	0.5	0.5	0.5	0.5	0.5
1	0.5	0.5	1.0	1	0.5
1.5	0.5	0.5	1.5	1.5	0.5
2.0	0.5	0.5	0.2	2.0	0.5
2.5	0.5	0.5	2.5	2.5	0.5
3.0	0.5	0.5	3.0	3.0	0.5
0.0	0.0	0.0	0.0	0.0	0.0

Foot Note: GA_3= Gibberellic acid. BAP= Benzylaminopurine. NAA=Naphthaleneacetic acid

For callus initiation, also full strength Murashige and Skoog (1962) media augmented with varying concentrations of GA_3, NAA, BAP,IBA, 2,4-D and Kinetin were prepared (Table 3.2). Callus was induced byinoculating a 1cm long piece of leaf explant using a sterilized forceps onto a solid MS mediumin the culture room and maintained at 24 ± 1°C using 14/10 light/dark period, under a light intensity of 3000 lux provided by cool-white fluorescent lamps and 50 to 60% relative humidity. The callus formed was sub cultured 3 times at 4 weeks intervals by transferring to a fresh medium. Friable and embyogenic calli were selected for amplification and morphogenesis adapting the procedure of Hamish, (1998) and Razdan, (2002). Calli materials were also harvested and air dried for 2 weeks in preparation for artemisinin extraction.

Table 3.2: Combination of PGRs used as supplements in MS media for Callus initiation in *A. annua*

PGRs Concentration (mg/l)									
BAP x NAA		**BAP x 2,4-D**		**GA_3 x NAA**		**BAP x IBA**		**Kin x NAA**	
BAP	NAA	BAP	2,4-D	GA_3	NAA	BAP	IBA	Kin	NAA
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.5	1.0	0.5	1.0	1.0	0.5	0.5	1.0	0.5	1.0
0.5	1.5	0.5	1.5	1.5	0.5	0.5	1.5	0.5	1.5
0.5	2.0	0.5	2.0	2.0	0.5	0.5	2.0	0.5	2.0
0.5	2.5	0.5	2.5	2.5	0.5	0.5	2.5	0.5	2.5
0.5	3.0	0.5	3.0	3.0	0.5	0.5	3.0	0.5	3.0
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Foot Note: BAP= Benzylaminopurine. NAA=Naphthaleneacetic acid. 2,4-Dichlorophenoxy acetic acid. GA_3= Gibberellic acid.IBA=Indole-3 -butyric acid.Kin= Kinetin.

Shoot multiplication.

Multiple shoots were observed on the plantlets initiated from the nodal cuttings, callus and leaf primordia. They were sub-cultured 4weeks after into multiplication media augmented with varying concentrations of GA_3, BAP and NAA combined treatments and monitored for plant height, vigour and percentage regeneration for a period of eight weeks.

Rooting media

Established plantlets were transferred into rooting media 4 weeks after. Full strength MS media augmented with varying concentration of Indole acetic acid (IAA), Naphthalene acetic acid (NAA) and Indole butyric acid (IBA) were used (Table 3.3). After inoculation, rooted plantlets were transferred into plastic containers with cowdung and loamy soil in the ratio of1:3 and tight for hardening and acclimatization before they were carried to the greenhouse and monitored for survival rate for 3 weeks before they were transplanted in the field.

Table 3.3: Combination of PGRs used as supplements in MS media for root initiation in *A. annua*

PGRs Concentration (mg/l)					
IBA x IAA		**IBA x NAA**		**NAA x IAA**	
IBA	IAA	IBA	NAA	NAA	IAA
1.5	9.0	1.5	9.0	1.5	9.0
3.0	10.5	3.0	10.5	3.0	10.5
4.5	12.0	4.5	12.0	4.5	12.0
6.0	13.5	6.0	13.5	6.0	13.5
7.5	15.0	7.5	15.0	7.5	15.0
9.0	16.5	9.0	16.5	9.0	16.5
0.0	0.0	0.0	0.0	0.0	0.0

Foot Note:IBA=Indole-3 -butyric acid. IAA= Indole-3- acetic acid. NAA=Naphthaleneacetic acid.

The following data were collected in the laboratory:

i. Days to regeneration was determined by recording the number of days taken by seeds to germinate from the day of inoculation (Fatimah, 2013).

ii. % regeneration was calculated according to Wiese and Binning (1987) i.e. $\%r = \frac{Gs}{Sp} x100$ WhereGs = number of germinating seed and Sp = number of seeds planted.

iii. Explants vigour was determined based on morphological appearance and when it has 5-6 leaves according to Gibson, (1980). A scale of 1 - 5 was used, where 1 = Very high vigour and 5 = very low vigour.

iv. Shoot and root lengths were determined by spreading a thread against the length of a plantlet in glass bottle after which it was placed on a measuring tape to measure its height in centimeter (cm).

3.2.4 Experimental Design and Data Analysis

The 12 concentrations for each combination were laid in a complete randomized design (CRD) with 2 repetitions. Each treatment was placed in glass bottle and six explants were inoculated and monitored for regeneration. Data collected were analyzed using Generalized Linear Model of Statistical Analysis System (SAS) program 9.3.1 software (SAS Institute, 2013).

3.3 Effective treatment for *in situ* germination

This experiment was conducted to determine the most effective physical, chemical and hormone treatment for the germination of *Artemisia annua* using seed in the field.

3.3.1 Soil Analysis

Soil samples were taken at random on each experimental plot at depths of 0-15 cm, 15-30 and 30-40 cm. The soil samples from each depth per replication were mixed thoroughly to form a composite sample. The composite soil samples were air-dried and sieved through a 2mm mesh and subjected to physical and chemical analysis at the Soil science laboratory of IAR, Ahmadu Bello University, Samaru, Zaria.

3.3.2 Nursery

The *A. annua* seeds collected for the *in situ* germination experiment were subjected to the following treatments in the horticultural garden of Institute for Agricultural Research, ABU Zaria:

i. Chemical treatment.

Seeds were soaked into 10, 20 and 30% Sulphuric acid (H_2SO_4) for 1, 2 and 3 minutes each with the control not soaked in the acid. Thereafter, the seeds were rinsed several times in clean water and sown in polythene bags containing river sand for germination.

ii. Hormone treatment

The seeds were soaked in three different GA_3 concentrations (100mg/l, 300mg/l, and 500 mg/l) with the control not soaked in GA_3 for 6, 12 and 24 hours each.

iii. Warm water treatment.

Seeds were soaked in water and placed in water bath and incubated at 20, 40 and 60 ºC for 1, 3 and 5 minutes each.

iv. Cold water treatment.

Seeds were washed and soaked in cold water of room temperature (37 ^{o}C) for 2, 4 and 6 hrs before planting.

3.4 Sowing

About 0.03g of the *Artemisia*(100) seeds was sown by broadcasting in polythene bags containing river sand sterilized by steaming and monitored for germination(Ferreira *et al*., 1997). Transparent polythene material was used to cover the seeds. This is to help maintain adequate moisture, temperature and humidity levels in the soil,which are essential for *Artemisia* seeds germination (Fig. I).Two weeks after germination, the tender seedlings were decongested by transferring into new containers of a mixture of 50% each of river sand and compost to facilitate drainage. A seed was considered germinated when the tip of the radicle had grown free of the seed coat (Wiese and Binning, 1987; Auld *et al.,* 1988).The germination rate was calculated as described under the *in vitro* experiment.

3.4.1 Watering

Depending upon the humidity in the area, the soil was kept moist and damp by watering morning and evening for the *Artemisia* seeds to germinate.

3.4.2 Effect of organic and inorganic fertilizer on biomass production *in situ*

The experiment was laid in a 4^2 factorial design in RCBD with 3 replications. Treatments comprising of poultry manure of 2.9 % nitrogen content applied at the rate of 0, 2, 4, 6 and 8 ton/ha and NPK fertilizer (15-15-15) at the rate of 0, 25, 50 and 75 kg N/ha was applied.

3.4.3 Ridge Preparation

The ridges were prepared after ploughing and harrowing the sandy loam soil using a tractor.This provide a good drainage and moist but damp soil condition. Thirty six plots, each measuring 5.0 x 4.5m were marked out (Ferreira *et al*., 1997).

Transplanting

Sixty days after germination, the plants were hardened gradually by exposing them to sunlight while reducing watering, prior to transplanting. Twenty seedlings were transplanted per plot of 5.0 x 4.5 m. There were four rows per plot and inter and intra row spacing of 1.5 ×1.0m respectively. Ridges were labeled and irrigated with the same volume of water or rain fed up to harvesting.

Weeding

Weeds were controlled by hoeing at two weeks interval (WAP).

The following data were collected from *In situ* experiment

Seven days after transplanting, plant height, canopy spread and stem width were recorded in cm at two-week interval up to flowering. Data from samples of 6 plants per plot were taken at the various growth stages.

i. Days to germination was determined by counting the number of days to germinate per seed

ii. Germination percent was calculated as described for *in vitro* experiment.

iii. Seedling vigour was determined as for i*n vitro*

iv. Plant height was determined using measuring tape was used to measure the height of the plant from the level of the soil to the top of the shoot apex.

v. The plant canopy spread was determined by stretching measuring tape horizontally across the plant.

vi. Stem width was determined 5 cm above the ground using a thread.

vii. Fresh leaf and root weight were obtained by using an electronic weighing scale.

viii. Dry leaf weight was determined using electronic weighing balance after air drying under the shade for three weeks.

ix. Crude extract was obtained and prepared in the Natural products laboratory of Pharmaceutical Chemistry Department, Ahmadu Bello University, Zaria (Figure 1).

3.5 Effective protocol and effect of organic and inorganic fertilizer on *in vitro* and *in situ* artmisinin production

Shoot cultures regenerated *in vitro* from leaf primordia, nodal cuttings and calli were harvested and placed on filter papers to remove excess water, air dried and then weighted and prepared for artemisinin extraction.

Three plants from each plot of the field experiment were harvested before flowering and at full bloom. The sampled plants were uprooted and leaves were cut with a sharp knife and air dried to obtain the biomass used for artemisinin extraction.

3.5.1 Sample preparation

Harvested leaf from both *in vitro* and *in situ* experiments and the calli materials were soaked in ethanol and extracted by maceration adapting the method of Singh *et al.,* (2010).

3.5.2 Determination of Artemisinin

For the artemisinin extraction, 50 g of *Artemisia* leaf sample was weighted and soaked in 500ml concentrated ethanol.It was allowed to stay for 7 days and was kept open to concentrate to 5% of initial volume by allowing the solvent to evaporate. Then 250ml distilled water was added to dilute the substrate.Partitioning was achieved by adding n-hexane ranging from 600ml and shaken gently using separation funnel. In order to ensure maximum artemisinin extraction, the process was repeated 3-5 times and depending on the artemisinin content of a sample, 4-6 hours were spent using five to ten fold volume of the ethanol (Singh *et al.*, 2010)

Light to dark green colour oily liquid was observed.The distilled water was then removed and the n-hexane was decanted. It was allowed again to concentrate to original volume and 30% ethyl acetate was added to dissolve the concentrated substrate. Thereafter, 1.5g of activated charcoal was added to decolorize the compound and was filtered using a filter paper in a funnel. A light yellow liquid was obtained as Artemisinin whish was allowed to crystallize.The isolated compound was weighed to quantify the Artemisinin and transferred into tubes for storageuntil they are needed (Singh *et al.*, 2010).

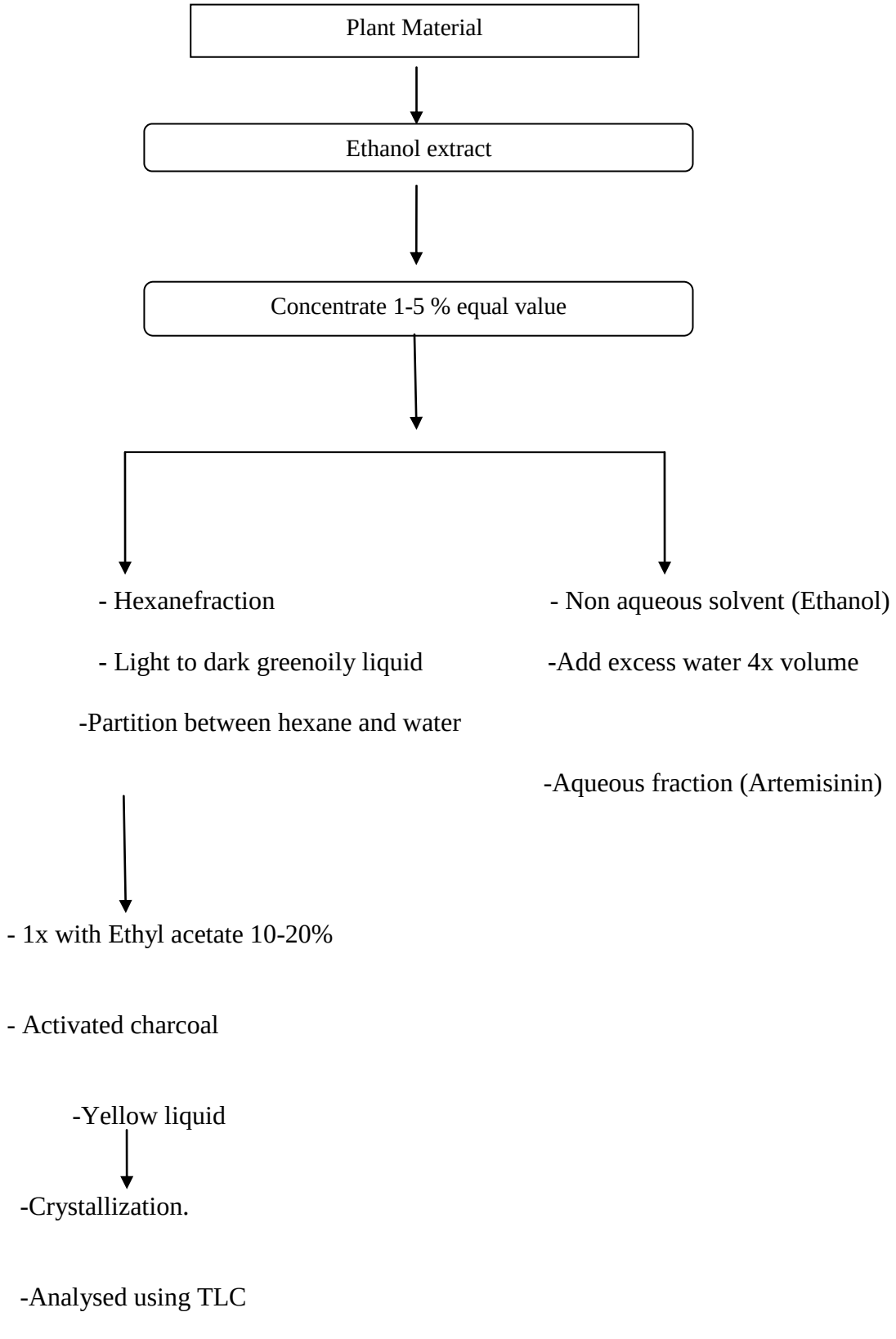


Fig. 3.1 Schematic procedure for Artemisinin extraction.

Source: (Singh*et al.,* 2010)

3.5.3 Statistical Analysis

The data was analyzed using Analysis of Variance (ANOVA), SAS (2013) statistical package. Least significant difference (LSD) was used to compare treatment means ($P < 0.05$) when F was significant.

CHAPTER FOUR

4 RESULTS

4.1 Effective protocol for*in vitro* regeneration

Results of Analysis of Variance (ANOVA) indicated significant difference among the treatments compared with the control which did not show any response ($P<0.05$).The addition of plant growth regulators had significant effect on the *in vitro* seed germination of *A. annua* compared with the hormone free MS mediaas the control which did not show any response (Plate i). Green, aromatic and deeply dissected leaf materials were regenerated using leaf primordia excised from 70 days old *A. annua* inoculated on a full strength MS media supplemented with varying concentrations of some plant growth regulators (Plate ii). Shoots were induced from the nodal cuttings on MS media supplemented with varying concentration of some plant growth regulators (PGRs) that significantly influenced the in vitro cultures of *A. annua* in comparison with the half strength and hormone free MS media which did not show any response (Plate iii).

4.1.1 Effects of GA_3 and BAP

However, concentration 1.5mg/l of $GA_3$and 0.5 mg/lBAPrecorded the shortest days to germination. Highest regeneration percentage was observed at 2.0 mg/l of $GA_3$and 0.5 mg/l of BAP. A concentration of 2.5 mg/l of GA_3 and 0.5 mg/lof BAP had the best vigor.Highest shoot height was observed at 2.0 mg/l concentration of GA_3 and 0.5 mg/l of BAP(Table4.1).

GA_3 produced a significant effect on the seed, leaf and nodal explants with respect to vigour, shoot length, days to regeneration and percentage regeneration except on the leaf's percentage regeneration. Also, BAP had significant effect on all the parameters studied on all the explants except on seed's

percentage regeneration. Similarly, a significant combined effects of GA_3 x BAP was observed on all the explants except on node's days to regeneration (Table 4.1)

4.1.2 Effects of BAP and NAA

Best vigour was observed with 0.5 mg/l of BAP and 0.5 mg/l of NAA. Highest shoot height was recorded at 0.5 mg/l of BAP and 0.5 mg/l of NAA. However, lowest days to regeneration were observed with equal concentrations of 0.5mg/l of both BAP and NAA. Similarly, highest percentage regeneration was observed at equal concentrations of 0.5 mg/l of both BAP and NAA (Table 4.2).

BAP had significant effects on the vigour and shoot length of the seed, leaf and nodal explants with no significant difference on seed's days to regeneration. However, NAA produced significant effects on the seed, leaf and node with respect to vigour and shoot height but with no significant difference on days to regeneration and percentage regeneration on seed, leaf and node explants (Table 4.2).

A significant combined effectof BAP and NAA was recorded on the vigour and shoot height of the seed, leaf and nodal explants, but no significant difference was observed with respect to days to regeneration and percentage regeneration on the seed explant. There was significant difference on percentage regeneration in leaf and nodal explant. (Table 4.2)

4.1.3 Effects of GA_3 and NAA

Concentrations of 1.0 mg/l of GA_3 and 0.5 mg/l produced the highest vigour. Also, a concentration of 1.5 mg/l of GA_3 and 0.5mg/l of NAA had the best shoot height. However, 0.5mg/l of GA_3 and 0.5mg/l of NAA had the lowest days to regeneration (Table 4.3).

A highly significant effect was produced by GA_3 on the vigour and shoot height of the seed, leaf and nodal explants with no significant effects on days to regeneration and percentage regeneration. On the

other hand, NAA produced a significant effect on leaf explant with respect to all the traits studied with no significant effect on days to regeneration and percentage regeneration on seed and nodal explants (Table 4.3).

A significant combined effect of GA_3 and NAA was observed on seed, leaf and nodal explants with respect to vigour and shoot height and significant effect was recorded on days to regeneration and percentage regeneration (Table 4.3).

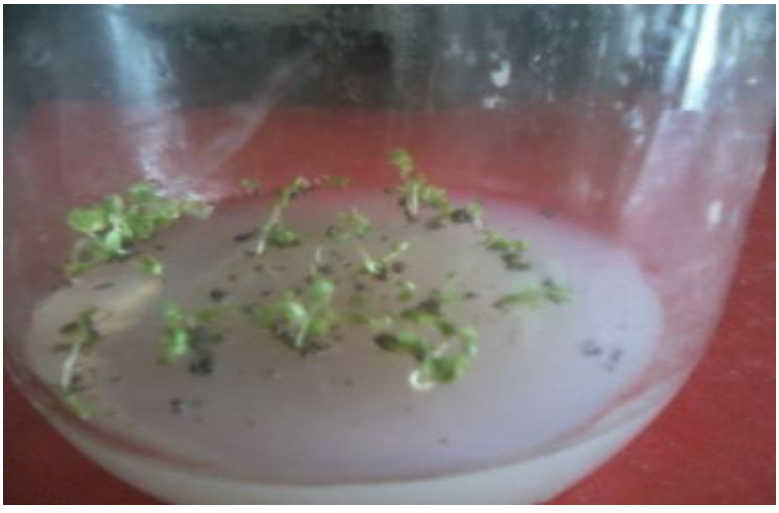


Plate iv a. Three days old plantlets germinated *in vitro* from *A. anua* seed



b.

b. One week old *in vitro* germinated *A. annua* from seed.

Table 4.1 Combined effects of GA_3 and BAP on the *in vitro* regeneration of *A. annua* using seed, leaf and nodal explants.

PGRs	Vigour	Shoot height (cm)	Days to regeneration	Regeneration (%)
GA_3				
0.5	1.39 bc	1.37 bc	5.72 a	8.38 ab
1	1.46 abc	1.90 a	3.61 ab	11.45 ab
1.5	1.09 c	1.06 a	1.63 bc	12.55 ab
2	1.53 abc	1.94 a	5.83 a	18.98 a
2.5	1.89 a	1.57 ab	3.89 ab	9.87 ab
3	1.53 ab	1.33 bc	3.72 ab	6.83 ab
Control	NR	NR	NR	NR
SE±	0.17	0.14	1.23	4.86
BAP				
0.5	1.94 a	1.97 b	5.28 a	14.57a
SE±	0.11	0.93	0.81	3.18
Explant x PGRs				
Seed x GA_3	1.5**	2.11**	9.16*	17.67*
Leaf x GA_3	1.58**	1.33**	7*	21.43^{NS}
Node x GA_3	1.49**	1.76**	2.5NS	27.17*
SE±	0.29	0.25	2.13	8.41
Seed x BAP	1.65**	1.82**	6.93**	12.45^{Ns}
Leaf x BAP	2.25**	3,08**	7.36**	28.4**
Node x BAP	1.52**	1.77**	3.14^{NS}	22.07*
SE±	0.19	0.16	1.39	5.51
Seed x GA_3 x BA	3**	2.12**	17.5**	38.3*
Leaf x GA_3 x BA	3**	2**	16**	46.6*
Node x GA_3 x BA	2.23**	3.21**	6^{NS}	31.5**
SE±	0.49	0.43	3.69	14.57

Foot note: *, ** Significant at 5% and 1% respectively. NS= Not significant. GA_3 = Gibberellic acid. BAP= Benzylaminopurine.**NR=** No response.

Table 4.2 Combined effects of BAP and NAA on the *in vitro* regeneration of *A. annua* using seed, leaf and nodal explants.

PGRs	Vigour	Shoot height (cm)	Days to regeneration	Regeneration (%)
BAP				
0.5	1.36 a	1.14 ab	2.67 a	5.50 a
SE±	0.08	0.13	1.03	2.38
NAA				
0.5	1.66 a	1.52 a	3.07 a	6.18 a
1	0.85 b	0.66 b	2.19 a	1.57 b
1.5	NR	NR	NR	NR
2	NR	NR	NR	NR
2.5	NR	NR	NR	NR
3	NR	NR	NR	NR
Control	NR	NR	NR	NR
SE±	0.51	0.09	0.68	1.56
Explant x PGRs				
Seed x BAP	0.86**	0.73 **	2.28 ^{NS}	0.78 ^{NS}
Leaf x BAP	2.44 **	2.28 **	4.00 *	14.69 **
Node x BAP	1.67 **	1.52 **	2.92 *	4.99 ^{NS}
SE±	0.09	0.14	1.17	2.7
Seed x NAA	1.02 **	0.88 *	2.00 Ns	1.33 S
Leaf x NAA	2.10 **	1.49 **	4.7 *	9.93 ^{NS}
Node x NAA	1.16 **	1.93 **	3.33 ^{NS}	6.6 ^{NS}
SE±	0.14	0.22	1.79	4.13
Seed x BAP x NA	3.08 **	2.67 **	6.00 ^{NS}	4.00 ^{NS}
Leaf x BAP x NA	2.79 **	2.59 **	2.00 ^{NS}	26.5 **
Node x BAP xNA	2.95 **	3.55 **	3.00 ^{NS}	18.25 *
SE±	0.23	0.38	3.1	7.15

Foot note:*, ** Significant at 5% and 1% respectively. NS= Not significant. NAA = Naphthaleneacetic acid. BAP= Benzylaminopurine. NR= No response.

Table 4.3 Combined effects of GA_3 and NAA on the *in vitro* regeneration of *A. annua* using seed, leaf and nodal explants.

PGRs	Vigour	Shoot height (cm)	Days to regeneration	Regeneration (%)
GA_3				
0.5	1.00 b	0.84 b	1.67 a	8.24 a
1	1.83 a	0.65 bc	2.33 a	2.92 ab
1.5	1.75 a	1.32 a	1.94 a	3.15 ab
2	0.96 bb	0.55 cd	1.83 a	2.86 ab
2.5	0.00 c	0.33 d	1.94 a	1.28 b
3	0.00 c	0.65 cb	2.23 a	2.2 ab
Control	0.00 c	0.00 e	0.00 a	0.00 b
SE±	0.11	0.08	1.08	2.21
NAA				
0.5	1.58 a	1.37 a	2.93 a	5.57 a
SE±	0.07	0.05	0.71	1.44
Explant x PGRs				
Seed x GA_3	0.59 **	0.52 **	1.26 ^{NS}	0.99 ^{NS}
Leaf x GA_3	0.66 **	0.76 **	2.24 *	5.81 *
Node x GA_3	0.79 **	0.58 **	1.64 *	2.05 ^{NS}
SE±	0.04	0.05	0.71	1.44
Seed x NAA	1.59 **	0.77 **	2.14 ^{NS}	2.29 ^{NS}
Leaf x NAA	1.97 **	2.29 **	3.71 *	11.52 **
Node x NAA	1.59 **	1.04 **	2.93 ^{NS}	4.5 ^{NS}
SE±	0.07	0.08	1.23	2.50 ^{NS}
Seed x GA_3 x NAA	3.50 **	3.15 **	6.00 ^{NS}	7.50 ^{NS}
Leaf x GA_3 x NAA	2.22 **	2.59 **	2.00 ^{NS}	25.00 *
Node x GA_3 xNAA	2.00 **	1.60 **	7.00 ^{NS}	8.35 ^{NS}
SE±	0.19	0.23	3.24	6.62

Foot note:*, ** Significant at 5% and 1% respectively. NS= Not significant. GA_3= Gibberellic acid. NAA = Naphthaleneacetic acid. NR= No response



Plate v a. Four weeks old *in vitro* regenerated leaf biomass from leaf primordium of *A. annua*



b. Six weeks old *in vitro* regenerated leaf biomass from leaf primordium of *A. annua*

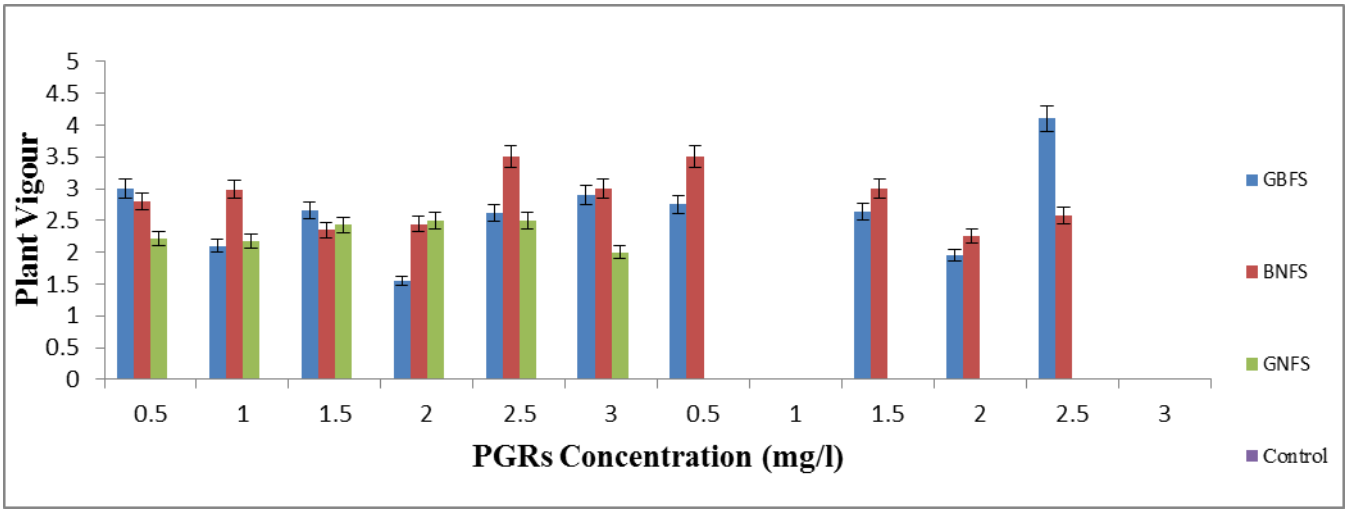


Figure 2 Effects of varying PGRs concentration on plant vigour of *A. annua*

Foot Note: GBFS = GA_3 x BAP in full strength MS media.**BNFS** = BAP x NAA in full strength MS media

GNFS = GA_3 x NAA in full strength MS media.

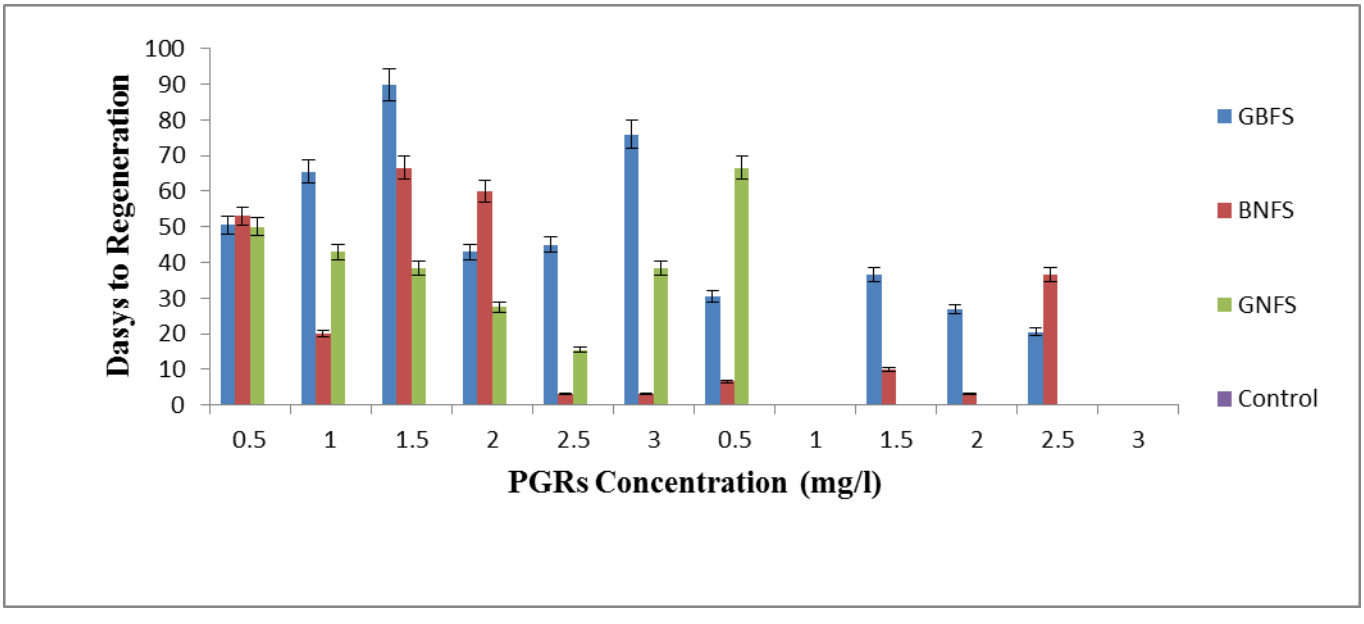


Figure 3.Effects of varying PGRs concentration on days to regeneration of *A. annua*

Foot Note: GBFS = GA_3 x BAP in full strength MS media.**BNFS** = BAP x NAA in full strength MS media.

GNFS = GA_3x NAA in full strength MS media.

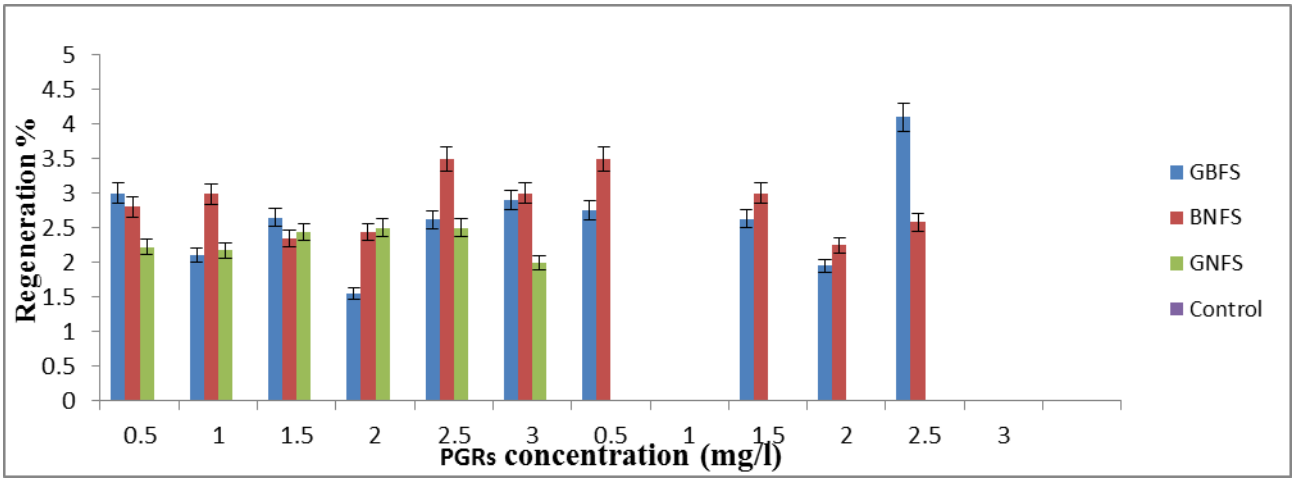


Figure 4 Effects of varying PGRs concentration on regeneration percent of *A. annua*

Foot Note: GBFS = GA_3 x BAP in full strength MS media. **BNFS** = BAP x NAA in full strength MS media. **GNFS** = GA_3 x NAA in full strength MS media.

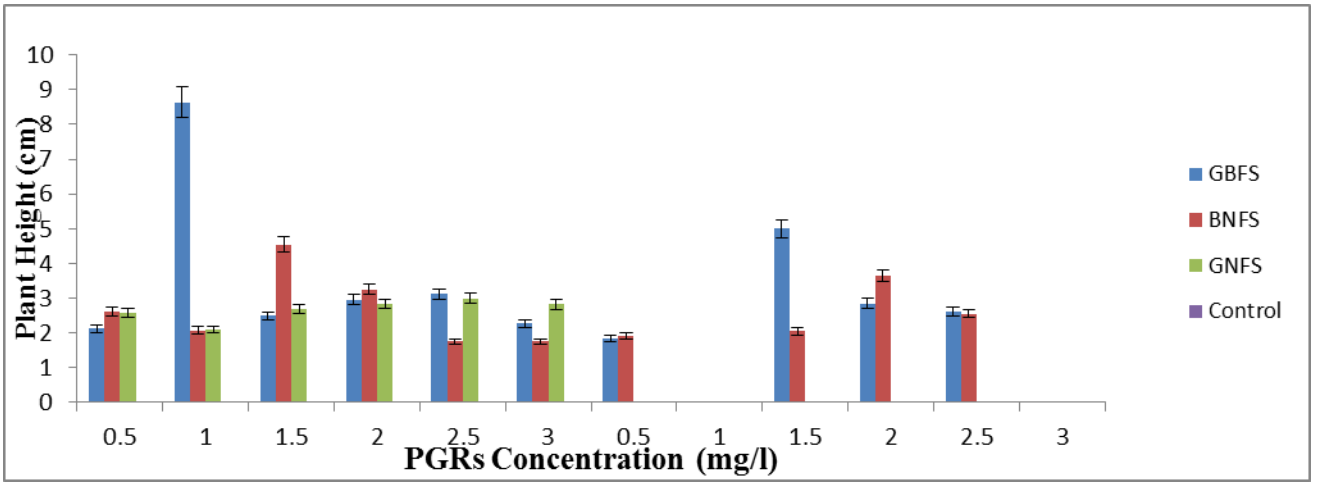


Figure 5 Effect of varying PGRs concentration on plant height of *A. annua*

Foot Note: GBFS = GA_3 x BAP in full strength MS media. **BNFS** = BAP x NAA in full strength MS media. **GNFS** = GA_3x NAA in full strength MS media.



Plate vi. *In vitro* regeneration of *A. annua* plantlets from nodal explant

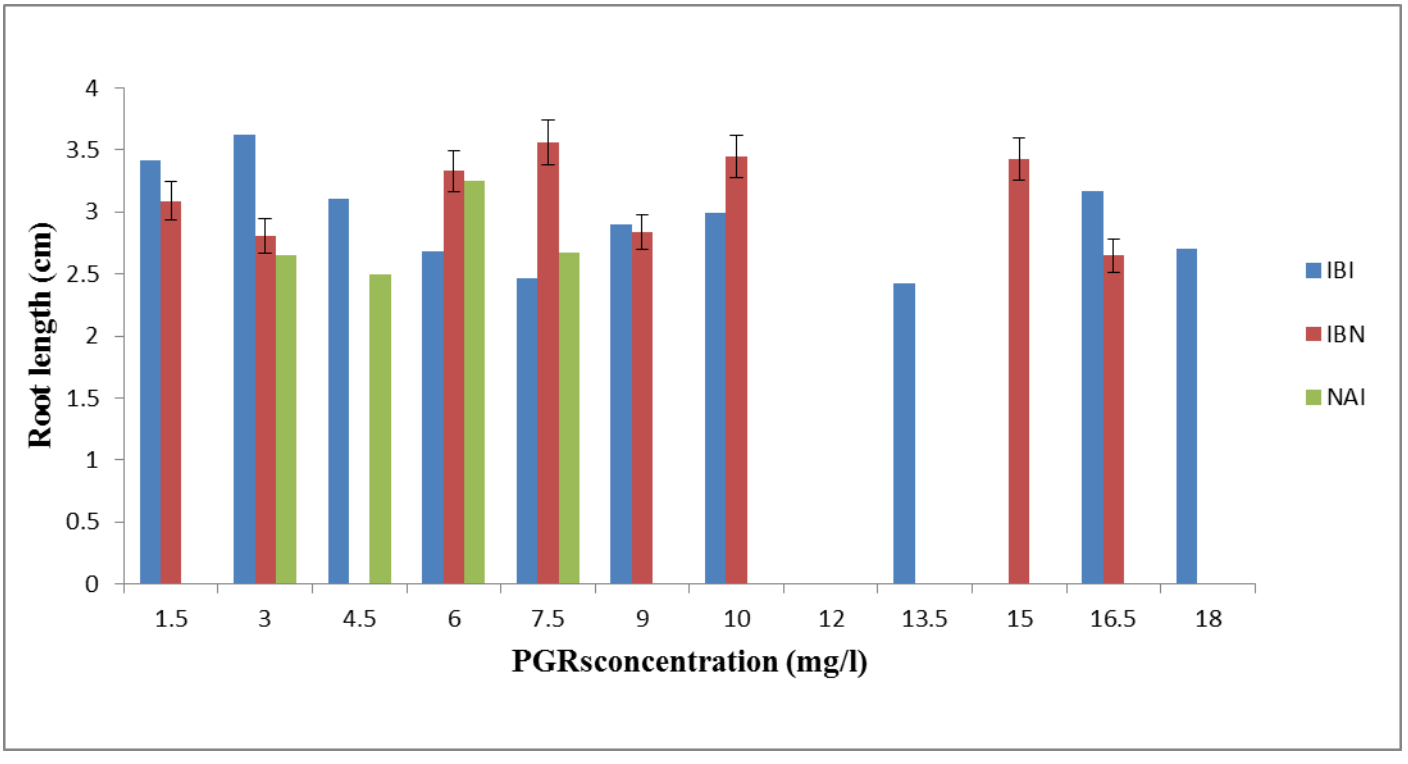


Figure 6. Effects of varying PGRs concentration on plant root length of *A. annua*

Foot Note: IBI = IBA x IAA (Indolebutyric acid & Indole acetic acid, IBN= IBA x NAA (Indolebutyric acid& Nephthyle acetic acid. NAI= NAA x IAA (Nephthyle acetic acid & Indole acetic acid.

4.1.4 Callus induction:

One week after inoculation, explants swelled before callus initiation commenced from the cut edges. Callus appeared yellowish, compact, friable and nodular (Plate.iv a). Series of changes occurred after callus initiation ranging from metabolic and morphological. Callus proliferation occurred after callus tissue was sub cultured.Callus turned greenish after transferred into the multiplication media and morphogenesis by way of shoot formation also commenced. Colour, friability and quantity of callus obtained from different treatments varied. Browning of some calli was also observed after subculture (Plate.iv b&c). Numerical assessment (1-5) was used for grading the vigor with 1 being the best.



Plate vii a. Friable and embryonic callus initiated from *A. annua* leaf explant.



b. Amplified callus of *A. annua* after subcultured

Results of Analysis of Variance had indicated that a low BAP concentration (0.5 mg/l) and a high concentration of NAA (3.0 mg/l) as well as equal concentration of BAP (1.0 mg/l) and IBA (1.0 mg/l) had the least days to initiation (Fig 7)

For regeneration percent, equal low concentrations of BAP (0.5 mg/l) and NAA (0.5 mg/l) had the highest performance. It was followed by GA3 (1.0 mg/l) and NAA (1.0 mg/l) and low concentrations (0.5 mg/l) of BAP and (1.0 mg/l) IBA (Fig.8)

However, no significant difference was observed between treatments with respect to vigor (Fig.9) Equal low concentrations of BAP (0.5 mg/l) and (0.5 mg/l) NAA and BAP(1.0 mg/l) combined with IBA(1.5 mg/l) had the highest height.(Fig.10).

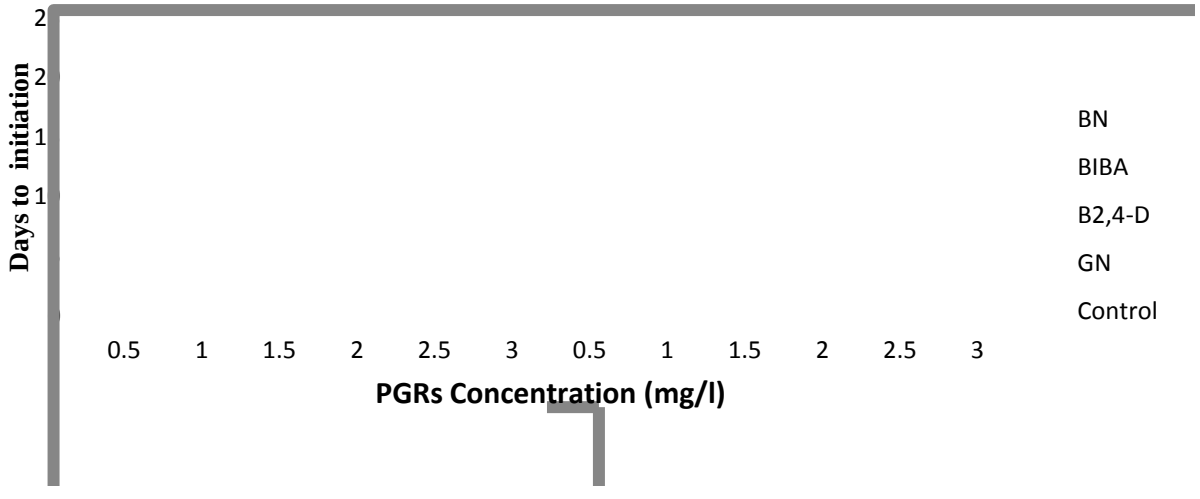


Figure 7. Effects of varying PGRs concentration on days to callus initiation in *A. annua*

Foot Note: BN=Benzyle amino purine x Nephthyle Acetic Acid.BIBA= Benzyle amino purine Indolebutyric acid. B2,4-D= Benzyle aminoPurine x 2,4-Dichlrophynoxyacetic acid. GN= Giberellic acid x Nephthyle Acetic Acid

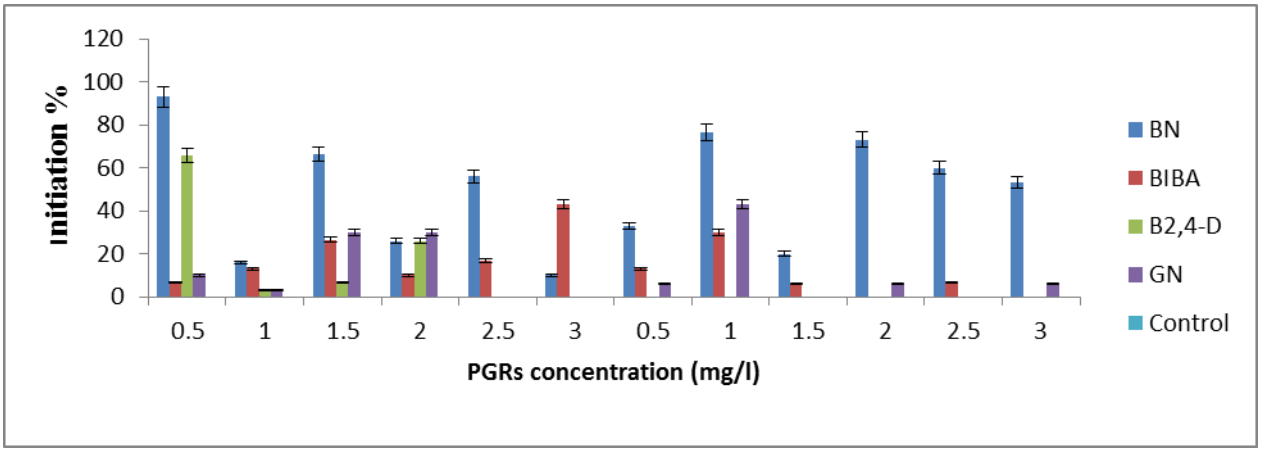


Figure 8. Effects of varying PGRs concentration on callus regeneration percent of *A. annua*

Foot Note: BN=Benzyle amino purine x Nephthyle Acetic Acid.BIBA= Benzyle amino purine x Indolebutyric acid, 2,4-D= Benzyle aminoPurine x 2,4-Dichlrophynoxyacetic acid. GN= Giberellic acid x Nephthyle Acetic Acid

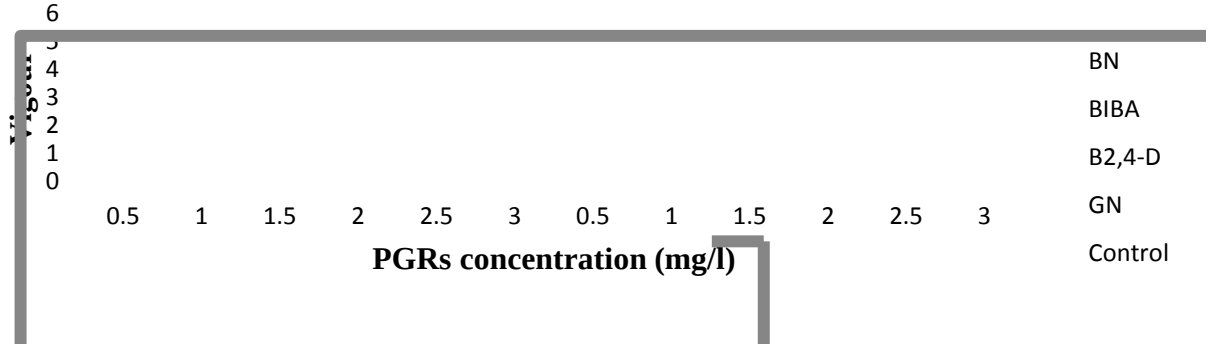


Figure 9. Effects of varying PGRs concentration on callus vigour of *A. annua*

Foot Note: BN=Benzyle amino purine x Nephthyle Acetic Acid.BIBA= Benzyle amino purine x Indolebutyric acid, 2,4-D= Benzyle aminoPurine x 2,4-Dichlrophynoxyacetic acid. GN= Giberellic acid x Nephthyle Acetic Acid.

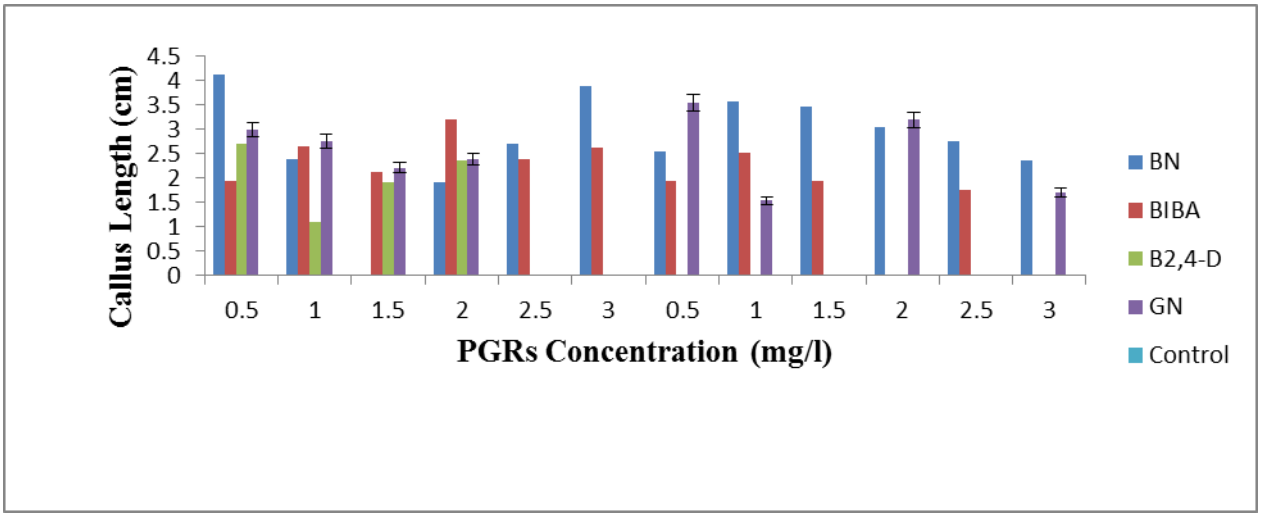


Figure 10. Effects of PGRs concentration on callus diameter of *A. annua*

Foot Note: BN=Benzyle amino purine x Nephthyle Acetic Acid.BIBA= Benzyle amino purine x Indolebutyric acid . B2,4-D= Benzyle aminoPurine x 2,4-Dichlrophynoxyacetic acid. GN= Giberellic acid x Nephthyle Acetic Acid

4.2 Effective treatment for*in situ* germination

Results of soil analysis indicated that, the soil sample was a sandy loam soil which is ideal for the propagation of *A. annua*. The soil was also found to contain 3.8 % carbon (C), 4.25 % Phosphorous (P) and 0.050 % Nitrogen (N) elements. Similarly, the poultry manure was found to have 2.90% Nitrogen elements.

Hypogeal type of germination was observed to occur 2-3 days after sowing (Plate vi). Leaves were found to be aromatic, deeply dissected and range from 3.0 to 8.5cm in length (Plate vii). Results obtained from this study showed no significant difference between the seasons with respect to germination, shoot and root lengths. However, warm water treatment at 60^0C for 2minutes during the rainy season trial had the least days to germination (Table 4.4). Similarly, warm water treatments in both rain and irrigation trials at 40^0C for 3minutes and 60^0C for 3 minutes produced the highest vigour (Tables4.5 &4.6). Highest germination percent (96%) was observed during the rainy season trial with 10% Sulphuric acid treatment (Table 4.4). Fourteen days after germination,the young seedlings were transfrered to the nursery stage(Plate vii).



Plate viii. *Artemisia annua* seeds sown and covered with transparent polythene.



Plate ix. *Artemisia* seedlings at Pre- nursery stage.

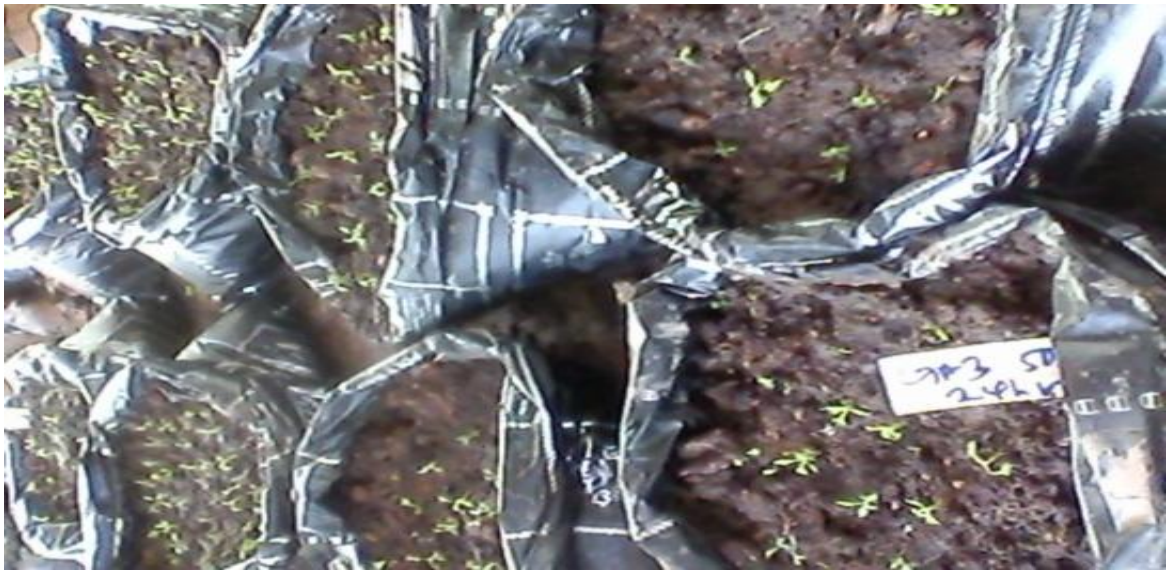


Plate x a. Seedlings of *A. annua* thinned to enhace growth.



b. *Artemisia* seedlings at the Nursery.

Table 4.4 Germination and seedling growth of *A. annua* in respect of GA_3, Cold water, Sulphuric acid and Warm water treatments during raining season

Treatment	Days to Germination	Vigour	Germination (%)	Shoot Length(cm)	Root Length(cm)
$GA_3$100mg/l					
6hrs	3.67 de	2.00 d	73.67 c	1.98 e	0.48 d
12hrs	2.67 e	1.33 e	63.50 d	2.48 c	0.53 cd
24hrs	3.33 de	1.33 e	81.00 b	2.67 b	0.48 d
300mg/l					
6hrs	3.67 de	2.33 d	57.50 e	2.62 b	0.53 cd
12hrs	3.67 de	3.33 bc	49.67 f	2.51b	0.58 c
24hrs	3.67 de	4.33 a	26.83 h	2.11 c	0.58 c
500mg/l					
6hrs	5.00 c	3.33 bc	37.83 g	2.57 b	0.58 c
12hrs	5.00 c	3.33 bc	48.83f	2.43 c	0.60 ab
24hrs	0.00 f	0.00 f	0.00 i	0.00 f	0.60 ab
Cold water					
2hours	2.33 e	4.33 a	58.83 e	2.32 c	0.55 c
4hours	4.00 d	3.67 b	45.33 f	2.99 a	0.60 ab
6 hours	3.33 de	4.33 a	42.37 f	2.31 c	0.61 ab
Sulphuric acid					
10%/ 1min.	4.67 d	1.67 e	95.50 a	2.36 c	0.64 a
2mins	4.00 d	2.33 d	70.17 c	2.22 cd	0.61 ab
3mins	2.33 e	3.00 bc	51.00 e	2.74 b	0.62 ab
20%/ 1min.	3.67 de	2.33 d	58.33 e	2.46 b	0.61 ab
2mins	8.67 a	3.33 bc	50.67 de	2.83 a	0.62 ab
3mins	6.00 b	4.00 ab	25.67 g	2.25 cd	0.61 ab
30% 1min.	6.33 b	4.33 a	21.00 g	2.57 b	0.56 c
2mins	2.33 e	1.67 ef	65.67d	2.05 d	0.55 c
3mins	5.33 c	3.33 bc	41.17 f	2.20 cd	0.55 c
Warm water					
20^0c/1min.	8.00 ab	4.33 a	21.67 gh	2.87 a	0.52 cd
2mins.	2.67 e	2.33 d	55.83 d	2.41 cd	0.56 c
3mins.	7.33 a	3.67ab	30.83 g	2.86 a	0.58 c
40^0c/1min.	4.33 d	3.67ab	35.50 g	2.52 b	0.60 ab
2mins.	2.33 e	2.33 d	60.83 d	2.65 a	0.55 cd
3mins.	3.00 d	1.33 e	66.17 d	2.46 c	0.60 ab
60^0c/1min.	2.67 e	1.67 e	65.17 d	2.59 b	0.61 ab
2mins.	2.00 e	2.00 de	66.67 d	2.62 a	0.68 a
3mins.	2.67 e	1.33 e	72.67 c	2.54 b	0.65 a
Control	3.67 de	2.33 d	75.50 c	2.53 b	0.45 d
SE±					

Means within a column followed by the same letter are not significantly different using LSD at 0.05% .

Table 4.5 Germination and seedling growth of *A. annua* in respect of GA_3, Cold water,Sulphuric Acid and Warm water treatments during the Irrigation trial.

Treatment	Days to Germinatio	Vigour	Germination (%)	Shoot Length(cm)	Root Length(cm)
GA_3 100mg/l					
6hrs	3.33 g	2.33 d	61.17 de	1.90c	0.38 b
12hrs	3.00 g	2.33 d	78.50 c	1.64f	0.41 b
24hrs	4.67 f	3.00 c	60.50 de	2.10 ab	0.48 ab
300mg/l					
6hrs	6.00 d	4.33 a	52.00 f	0.56 a	0.50 c
12hrs	6.33 d	4.33 a	46.17 h	1.78 d	0.48 b
24hrs	5.33 e	3.33c	50.83 fg	1.67 e	0.51 b
500mg/l					
6hrs	5.00 e	2.67cd	55.00 f	1.93 c	0.51 b
12hrs	7.00 c	2.67cd	84.67 b	1.67 e	0.53 b
24hrs	6.00 d	1.67 f	27.17 g	1.71 d	0.52 b
Cold water					
2 hours	4.33 f	2.00 e	75.67 c	2.21 ab	0.55 ab
4 hours	3.33 g	2.33 d	90.83 a	1.97 d	0.55 b
6 hours	5.33 e	2.67cd	60.83 de	1.86 c	0.56 b
Sulphuric acid					
10%/ 1 min.	4.67 f	2.33 c	84.00 b	1.79 d	0.58 b
2mins	7.00 c	2.67cd	80.83 bc	2.00 ab	0.58 b
3mins	2.67 h	2.33 c`	61.17 de	1.91c	0.58 b
20%/ 1min.	4.33 f	4.33 a	50.00 fg	1.83 c	0.59 b
2mins	2.67 h	3.67bc	31.33 i	2.26 ab	0.57 ab
3mins	13.00 a	3.67 bc	30.83 i	1.98 c	0.58 b
30%/ 1min.	13.00 a	3.33c	35.33 i	1.79 d	0.55 b
2mins	3.33 g	4.00 ab	51.17 fg	1.94 c	0.55 b
3mins	7.33 c	4.33 a	46.17 h	1.86 c	0.55 a
Warm water					
20^0c/1min.	13.00 a	4.33 a	32.17 i	2.87 a	0.52 a
2mins.	3.67 fg	4.00 ab	54.50 f	2.16 ab	0.56 ab
3mins.	10.67 b	3.33c	55.67 f	2.11 ab	0.58 ab
40^0c/1min.	7.33 c	4.00 b	38.83 i	1.82 c	0.60 b
2mins.	3.33 g	3.33c	58.50 de	1.98 c	0.56 b
3mins.	4.33 f	2.67cd	60.67 de	2.04 ab	0.60 b
60^0c/1min.	3.33 g	2.67cd	68.33 d	1.97 c	0.61 b
2mins.	2.33 h	1.67 f	71.50 cd	2.16 ab	0.68 ab
3mins.	2.67 h	1.00 g	72.50 cd	2.04 ab	0.65 b
Control	4.00 f	3.00c	50.83 fg	1.85c	0.46 b
SE±					

Means within a column followed by the same letter are not significantly different using LSD at 0.05% .

4.3 Effect of Organic and Inorganic Fertilizers on biomass production *in situ*

Results of combined analysis of the effect of organic (Poultry manure) and Inorganic manure (NPK 15:15:15) on the growth and artemisinin content of *A. annua* indicated significant difference between treatments ($P<0.05$) on some of the parameters studied compared with the control except on plant height, number of branches, leaf fresh weight, leaf dry weight, whole fresh weight before flowering and leaf dry weight after flowering during the irrigation trial.

6t/ha of poultry manure during the raining season had the highest plant height (Table 4.7) followed by 4t/ha of poultry manure during the irrigation trial(Table 4.6). Similarly, rain season trial had the highest number of branches with 50kg/ha of NPK followed by same quantity of NPK during the irrigation trial (Tables 4.6 and 4.7).

Rain trial had the best leaf fresh weight after flowering with 6t/ha of poultry manure followed by irrigation trial with 4t/ha of poultry manure (Plate viii). The best leaf dry weight before flowering was observed with 25kg/ha during the rain trial. It was followed by the same quantity of NPK during the irrigation trial. Similarly, rain had the highest leaf fresh weight before flowering with 2t/ha of poultry manure followed by the same quantity during the irrigation trial. Also, the rain season had the best result with respect to leaf dry weight after flowering with 8t/ha of poultry manure followed by the same quantity during the irrigation trial (Tables 4.6 & 4.7).

No significant difference was observed between the two seasons with respect to canopy spread. However, rain season gave the best result with 100kg/ha of NPK followed by irrigation trial with 25kg/ha of NPK (Tables 4.6 7 4.7)

25 kg/h of NPK produced the highest whole plant fresh weight before flowering during the raining season trial (Plate ix) followed by 6t/ha of poultry manure. The lowest whole plant fresh weight was observed with 2t/ha of poultry manure during the irrigation trial (Table 4.6)



Plate xi. a. Three weeks old *Artemisia* plants



b. *Artemisia* plants at the pre flowering stage



Plate xii. ***In situ Artemisia*** **plants at the flowering stage.**

Table 4.6 Effects of organic and inorganic manure on the growth of *A. annua* during the irrigation.

	Growth parameters								
Treatment	**PH (cm)**	**NB**	**CS (cm|)**	**LFWBF (g)**	**WFWBF (g)**	**LDWBF (g)**	**LDWAF (g)**	**LFWAF (g)**	**WFWAF (g)**
NPK.									
25kg/ha	42.26a	25.67a	39.62a	63.72a	208.97a	36.97a	15.13a	25.30bcd	265.92ab
50kg/ha	49.05a	26.00a	32.24ab	42.67a	262.67a	26.87a	14.57a	16.38bcd	363.80a
75kg/ha	42.31a	24.17a	25.61b	33.98a	187.55a	29.77a	15.07a	19.73bcd	199.65b
100kg/ha	46.45a	23.83a	30.49b	39.65a	246.93a	32.75a	13.83a	31.02ab	162.90b
Poultry manure.									
2t/ha	47.23a	24.00a	28.25b	63.77a	250.70a	30.40a	13.72a	18.42bcd	256.77ab
4 t/ha	50.00a	24.00a	29.57b	46.13a	214.87a	25.50a	18.70a	44.68a	181.00b
6 t/ha	46.89a	25.67a	27.58b	47.87a	248.40a	26.10a	17.48a	12.30d	177.55b
8 t/ha	46.22a	24.83a	30.13b	53.08a	107.53a	18.37b	19.28a	26.88bc	234.63b
Control.	41.86a	26.67a	25.28b	44.07a	259.95a	27.13a	21.43a	21.783bcd	248.13ab
SE±									

Means with same letters are not significantly different using LSD at 0.05%.

Foot note: PH= Plant height. NB= No. of Branches. CS= Canopy spread. LFWBF= leaf fresh weight before flowering. WFWBF = Whole fresh weight before flowering. LDWBF= Leaf dry weight before flowering. LDWAF= leaf dry weight after flowering. LFWAF= Leaf whole fresh weight after flowering. WFWAF= Whole fresh weight after flowering.

Table 4.7 Effects of organic and inorganic manure on the growth of *A. annua* during the rain season.

	Growth parameters								
Treatment	**PH (cm)**	**NB**	**CS (cm)**	**LFWBF (g)**	**WFWBF (g)**	**LDWBF (g)**	**LDWAF (g)**	**LFWAF (g)**	**WFWAF (g)**
NPK									
25kg/ha	71.17c	39.33ab	40.00b	125.80a	758.20a	36.97a	16.32a	174.88ab	4455.00a
50kg/ha	80.50ab	41.50a	43.00ab	122.46a	705.10a	26.87a	15.67a	179.93ab	3892.50a
75kg/ha	77.00ac	35.50b	44.83ab	120.38a	606.70a	29.77a	16.20a	200.60ab	3384.20a
100kg/h**a**	74.00c	38.167al	46.33a	188.67a	571.30a	32.75a	14.75a	261.60a	4039.50a
Poultry Manure									
2 t/ha	73.67c	39.178ab	44.50ab	179.87a	502.50a	30.40a	14.75a	231.83ab	4189.20a
4 t/ha	76.00c	27.33ab	44.83ab	149.54b	598.10a	25.50a	19.68a	209.35ab	4333.80a
6 t/ha	84.5a	35.00b	44.50ab	145.67b	672.10a	26.10a	17.78a	258.58a	4150.20a
8 t/ha	76.92c	36.83ab	45.17ab	147.55b	649.40a	18.37b	20.38a	152.77b	3619.40a
Control	97.167ab	38.500ab	46.00a	139.40b	673.30a	27.13a	25.55a	202.20ab	4457.60a
SE±									

Means with same letters are not significantly differentusing LSD at 0.05%.

Foot note: PH= Plant height. NB= No. of Branches. CS= Canopy spread. LFWBF= leaf fresh weight before flowering. WFWBF = Whole fresh weight before flowering. LDWBF= Leaf dry weight before flowering. LDWAF= leaf dry weight after flowering. LFWAF= Leaf whole fresh weight after flowering. WFWAF= Whole fresh weight after flowering.

4.4 Protocol for Artemisinn content *in vitro*

No significant difference was observed between treatments with respect to aretmisininextracted from leaf material produced *in vitro* from leaf primordia ($P<0.05$). However, a combined treatment of 1.0mg/l GA_3 and 0.5 mg/l BAP had the highest artemisinin content 0.97 %.

A significant difference was observed between treatments with respect to artemisinin extraction using foliage materials generated from nodal cuttings. Equal concentration of 0.5 mg/l BAP and 0.5 mg/l NAA treatments had the highest content of 0.87 % followed by 2.5 mg/l of $GA_3$and 0.5mg/l of NAA with 0.83 %.

However, the extract obtained from calli material was colourless rather than yellowish. So there were indications of artemisinin in callus material that was allowed to turn green. However, no significant difference was observed between the different treatments and only traces of artemisinin were observed.

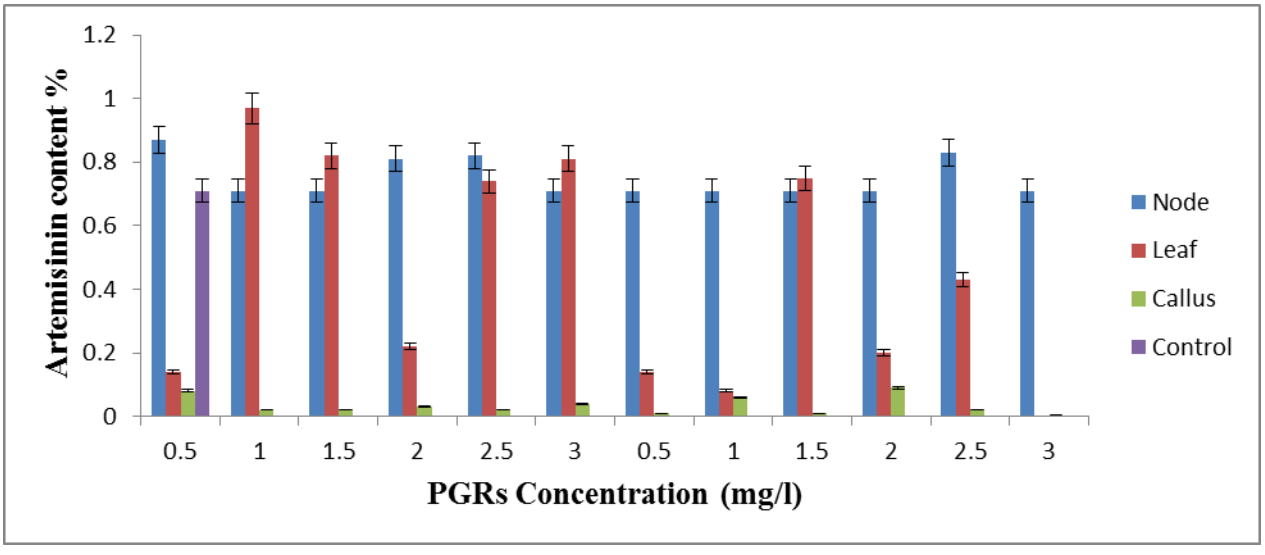


Figure 11. Effectof PGRs concentration on artemisinin content of leaf material regenerated *in vitro* from node, leaf primordia and callus of *A. annua*.

4.4.1 Effect of organic and inorganic fertilizer on artemisinin production i*n situ*

Results of Analysis of Variance indicated significant difference between treatments with respect to artemisinin content compared with the control ($P<0.05$). 6t/ha of poultry manure had the highest artemisinin content of 2.1 %followed by 75kg/ha on NPK with 1.5%. Lowest artemisinin content of 0.4 % was recorded with the control.

No significant difference was observed between the rain and irrigation trials.However, Rain season trial produced the highest artemisinin quantity of 1.1%followed by the irrigation trial with 0.9%. Similarly, no significant difference was observed between periods of harvesting leaf material for artemisinin extraction. Leaf material harvested before flowering had 1.0%artemisinin content while those harvested after flowering produced 0. 9% artemisinin content (Table 4.8).

Table 4.8 Combined Effects of Poultry manure, NPK and Season on artemisinin content of *A. annua* produced *in situ*

Season	Artemisinin content
Rain	1.04 a
Irrigation	1.15 a
SE±	0.15
Season x Poultry Manure	0.39 ^{NS}
Season x NPK	0.36 ^{NS}
SE±	0.76
Poultry Manure	
2t/h	0.96 b
4t/h	1.09 ab
6t/h	1.75 a
8t/h	0.68 b
Control	0.99 b
SE±	0.24
NPK	
25kg/h	1.44 a
50kg/h	0.93 a
75kg/h	0.95 a
100kg/h	1.02 a
Control	1.14 a
SE±	0.24

4.4.2 Relative effectiveness of *in vitro* and *in situ* artemisinin production.

Results of analysis of variance showed significance difference with respect to artemisinin content an *Artemisia annua*($P<0.05$). Lef material regenerated *in vitro* from both leaf primordial and nodal explants produced artemisinin content of 0.97 % and 0.82 % respectively 9Fig. 12). These are low quantities compared with the artemisinin obtained from in situ leaf materials where poultry manure level of 6 t/ha produced the highest artemisinin content of 2.1 % and it was followed by level of 75 kg/ha of NPK which produced 1.5 % of artemisinin (Fig. 12). Callus materials that were allowed to commence differenciation and turned green showed evidence of artemisinin of 0.14 % (Fig. 12)

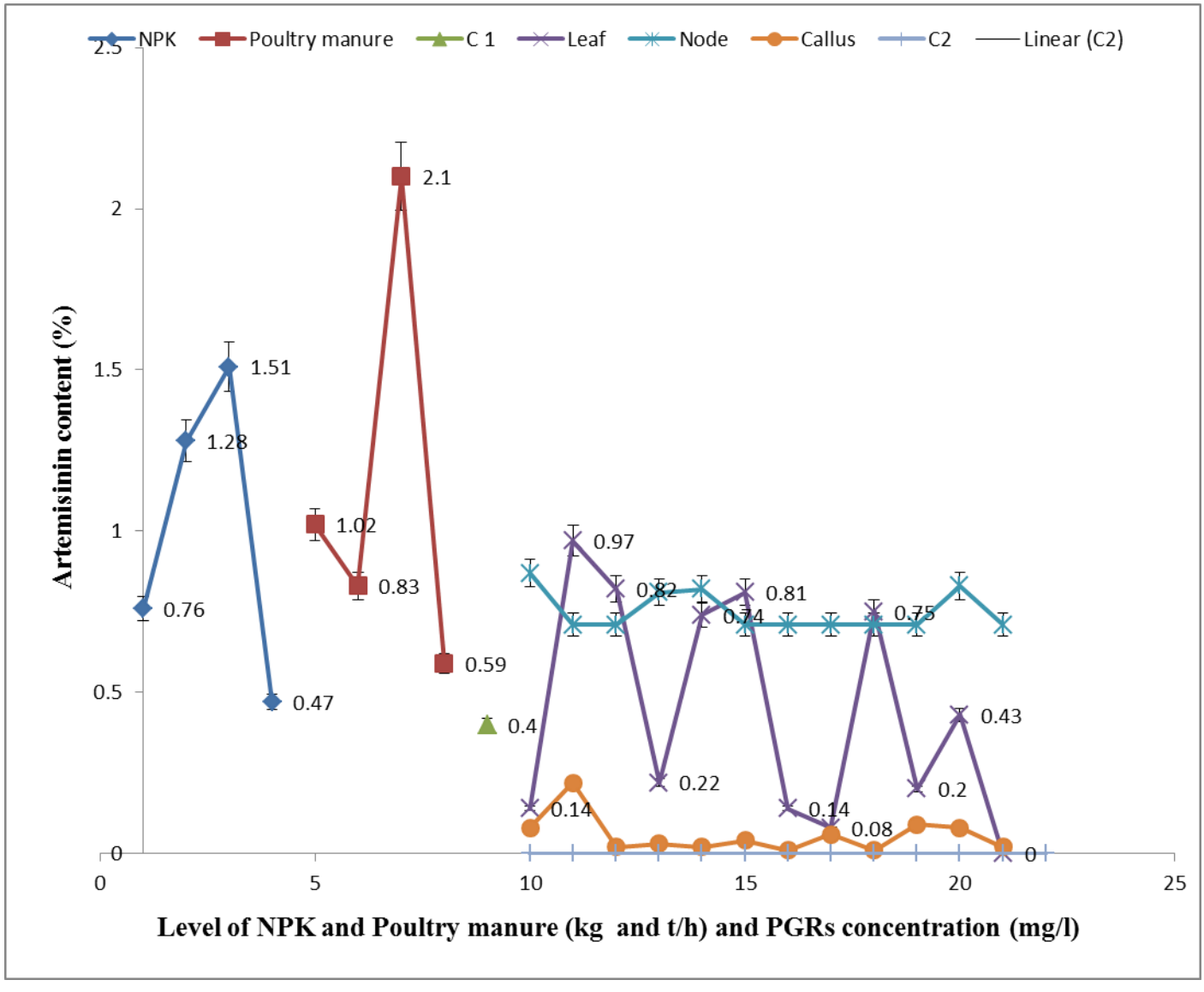


Figure 12. Effectiveness of *In vitro* and *in situ* techniques in the production of artemisinin in *A. annua*.



Plate xiii. a. Seperation of Artemisinin using n-hexane in a seperation funnel.



b. Artemisinin allowed to crystalized after extraction.

CHAPTER FIVE

5 DISCUSSION

5.1 Protocol for*in vitro* regeneration

This experiment was based on the principle of manipulation of the MS media and the hormonal concentrations (Murashige, 1990; Uranbey *et al*., 2005; Bewley and Black (1994).The germinating seeds of*A. annua* exhibited ahypogeal type of germination by having thecotyledon remaining below the media surface. A seed was considered germinated when the tip of the radicle had grown free of the seed coat emerging through the outer covering (Wiese and Binning, 1987; Auld *et al*., 1988). Exposure of the shoot tip to light enabled it to photosynthesize thereby straightening the epicotyls. (Moore, 1979; Osborne *et al.,* 1985).

Chemical dormancy reported due to phenolic secretion on the seed of *A. annua* seeds was overcome by the use of GA_3 (Bewley and Black, 1994, Nicolas, 2003, Farouk *et al*., 2008*)*. Similarly, the cytokinin (BAP) had positively influenced the physiological process of the seed germination as reported by Thomas, 1989, Syed, 2001) it also enhances lateral bud growth (Tamas, 1987). All seeds responded to treatments when kept under the light. This is contrary to the findings of Jamaleddine *et al.* (2011) who reported that seeds of *A. annua* germinated after exposure to dark. Germination of seeds of *A. annua*, commenced 3 days after inoculation, and 50 to 60 % of the seeds regenerated to plantlets. This is contrary to the findings of Jamaleddine *et al.* (2011) and Mannan *et al.*(2012) who observed that, plant growth regulators do not reduce the number of days to germination in the *ln vitro* cultures of *A. annua* and *A.absinthium* compared to 6 - 7 days obtainable when grown under field conditions. Also, no increase in the germination percentage was observed. This is not in conformity with the results obtained by Nikolić *et al*.

(2006) working on seeds of *Lotus coniculatus* who reported that different hormones and their optimum concentrations stimulated germination percent at least two fold. Multiple shoots were recorded from this experiment as also reported by Jamaleddine *et al.* (2011). Similarly, the addition of GA_3, auxin and cytokinin had significantly shortened the number of days to germination, vigour and shoot length. Mark (2003) had also suggested that the hormones GA_3, BAP, and NAA stimulate growth in*A.annua.*

The concentration of growth substances in an *in vitro* media has been manipulated by several workers to induce shoot regeneration in cultures (Razdan, 2002) Leaves were found to be aromatic, deeply dissected and range from 3.0 to 8.5cm in length (Ferreira and Janick, 2009). Similarly, explants isolated from younger parts of a plant were reported to be more regenerative than those from older regions and that regeneration potential of a tissue culture diminishes with maturation (Razdan, 2002). Apical dominance has been demonstrated to be under control of various growth regulators, therefore early regeneration within 3 days achieved at a balance between GA_3 and NAA, revealed strong evidence that Gibberellic acid and Auxins balance is important in organ differentiation and its further development. It is also an indication of the role of GA_3 in stimulating early growth and development of plants by promoting cell division in epical meristem and cambium tissues (Taylor *et al.,* 1997). Mark (2003) had also suggested that the hormones GA_3, BAP, and NAA would stimulate growth of *A. annua.*

The best vigour at GA_3& BAP combination is a reflection of the function of GA_3 in causing the breakdown of stored starch and proteins into sugars and amino acids useful for seedlings growth (Graham *et al.,* 2006; Taylor *et al.,* 1997) as well as that of BAP in enhancing lateral bud growth

by promoting cell division in shoot meristems, influencing the development of Vascular tissues, promoting the development of shoots from undifferentiated tissues of cultured tissues (Graham, *et al*., 2006).

Highest regeneration percent produced by the combination of GA_3 and BAP can also be attributed to the fact that, shoot grown on culture media containing cytokinins develops precociously proliferating to form clusters of secondary and tertiary shoots. Likewise, GA_3 has been used in the shoot proliferation media to improve shoot elongation, rate of multiplication, growth and quality of shoots (Razdan, 2002).

The *in vitro* cultures were kept under inflorescent light, it is therefore evident that the photosynthetic pigment in cultured tissues absorbed light thereby inducing the morphogenesis of these tissues as it was reported by Kato (1978) that bud induction in excised leaf segments of lily *Heloniopsis orientalis* was to a certain extent controlled by the photosynthetic system in its cells hence controlling the organogenic differentiation and growth of shoots in cultures.

Cytokinins such as BAP were observed to influence a diversity of responses when applied to plant tissues, or organs. Consequently, it has been used for the induction of organogenesis in several plants (Baskaran and Jayabalan, 2005).

It was also reported to influence a multitude of morphological and physiological processes, including cell division and elongation, and the morphological appearance of cultured tissues. (Syed, 2001; Thomas, 1989)

The best vigor obtained at high GA_3 and a low BAP concentration is an indication of dual functions of BAP in enhancing lateral bud growth (Wickson,1958) by promoting cell division in shoot meristems, influencing the development of Vascular tissues, promoting the development of shoots from undifferentiated tissues of cultured tissues (Graham*et al*., 2006) one hand and the role of GA_3 in causing the breakdown of stored starch and proteins into sugars and amino acids useful for seedlings growth (Graham *et al.* , 2006; Taylor*et al.,* 1997)

However, the early shooting (3days) and high percentage regeneration (93%) observed may be attributed to the combined effect of GA_3 and BAP in stimulating early growth and development of plants. by promoting cell division in epical meristem and cambium tissues. (Taylor *et al.*, 1997).

According to Sayed (2001), the mechanism of action of GA_3 is not clear, however it act with other hormones (e.g. BAP) to synergistically bring about extensive growth as it stimulated internode growth of dwarf maize, pea and bush bean to attain normal height. Similarly, both hormones were reported to greatly increase growth by promoting cell division and enlargement hence facilitating shoot growth of normal plants (Mark, 2003).

In this study, low BAP and high GA_3 were seen to produce high multiplication rate. This is contrary to the findings of many workers who observed shoot induction and multiplication of *A. annua* by using high concentration of BAP (Mackay and Kitto, 1988; Geng *et al*., 2001; Aslam *et al*., 2006). Similarly, the effect of BAP on stimulating shoot formation was reported for other species from the genus *Artemisia* (Holobiuc and Blindu, 2007; Zia *et al*., 2007).Insignificant rooting (45%) obtained with IBA, IAA and NAA was contrary to the findings of Nam-Cheol

et al. (1992). who reported satisfactory rooting percentage using the same hormone types working on *A annua*.

Callus production is a process of dedifferentiation of organized tissue as indicated by changes in morphology and metabolic activity. Callus can be initiated from explants of almost any part ofthe original plant such as leaves, roots, and stems. Callus can be induced either by injury orby the addition of growth regulators – usually a proper ratio of auxin and cytokinin (Sugimoto *et al*., 2011). When a surface is cut, the cells at the wound site undergo division. With the firstdivision being synchronous after 4-6 weeks of culture and continuous cell division, the explants tissue may have produced double its original weight in callus tissue and after initiation is the maintenance or proliferation of the callus material (Hamish and Sue, 1989,Razdan, 2002).

This provides a source of uniform cells for a variety of purposes such as regeneration. Juvenile leaves contain higher population of actively dividing cells therefore more responsive to callus initiation. The parent plant must be healthy and actively growing therefore, not about toenter period of dormancy (Razdan, 2002, Hamish and Sue, 1989).

Swelling of the explants was observed one week after inoculation. However, callus initiation was observed after the second week. Yellowish, compact and nodular callus at cut edge of the explants may be due to the wound caused during the process of cutting which resulted in a synchronous cell division. This is considered as a process of dedifferentiation of organized tissue and its similar to the work of Hamish and Sue (1989). An embryonic callus possesses high regenerative capacity compared to those derived from mature organs such as leaf, stem and root. This is true of conifers, cereals etc. The most serious draw back in the use of callus cultures for

shoot multiplication is the genetic instability of their cells due to which initial plant regeneration capacity of the tissue may decline with passage of time (Razdan, 2002) Manipulation of the media components may reverse de-differentiated process and the tissue re-differentiated back to an organized form (Hamish and Sue, 1989). Control of differentiation was found to be dependent upon the presence of Auxins and cytokinin. The physiological state of the tissue provided actively dividing cells and has an influence on the plant's response to the tissue culture program (Hamish and Sue, 1989, Brisibe *et al.,* 2008). Muhammad *et al.* 2007 reported that, Cytokinins and Auxins were found to be effective in callusculture of *A. annua*.

Callus initiation is probably dependent upon genotype, medium and the explant used (Paniegoand Giulietti, 1994) and it is accompanied by series of changes in both the appearance and metabolism of the cells (Hamish and Sue, 1989). Variations in colour and friability may be gene dependent. However, browning of callus after sub culture may be due to phenolic secretion which may be overcome by the addition of activated charcoal (Razdan, 2002, Hamish and Sue, 1989).

Skoog and Miller (1957) demonstrated that, root and shoot formation from undifferentiated callusof tobacco was dependent upon the balance of auxin and cytokinins. However, initiated calli undergo differentiation when they were transferred to bright light (Razdan, 2003).This probably triggers chlorophyll formation, hence commencement of photosynthesis. This eventually led to organogenesis.

5.2 Effective treatment for *In situ* germination

The early germination observed in three days may be as a result of covering the seeds after sowing with transparent polythene bag which help in maintaining adequate moisture, warm and humidity levels in the soil, which are essential for germination of *Artemisia* seed. Dormancy of some seeds was reported to be overcome when soil temperatures are too warm (Nicolas, 2003). Similarly, germination of *A. annua* seeds was enhanced by the weakening of the seed coat as a result of soaking in warm water. This provided an optimum temperature suitable for influencing the rate of enzyme –controlled reactions that triggered germination (Taylor, 1997; Style, 2008). The chemical dormancy reported in *Artemisia* seeds due to the accumulation of phenolic compounds strengthened the plant cell walls by polymerizing into lignin. This physiologically contributes to the maintenance of dormancy by impeding water and gas to and from the embryo, chemically by inhibiting germination and mechanically by restricting the growth of the embryo (Mott and Groves, 1981; Farouk *et al*., 2008; Style, 2008) This eventuallyrestricted water uptake by the impermeable outer part of the epidermal layer of malphigian cells, therefore restraining the expansion of the radicle and manifestation of germination (Bewley and Black, 1994).

The aromatic, deeply dissected leaves ranging from 3.0 to 8.5cm in length observed, is similar to the findings of Ferreira and Janick (2009). The germinating seeds of*A. annua* exhibited ahypogeal type of germination by having theCotyledon remaining below the soil surface. A seed was considered germinated when the tip of the radicle had grown free of the seed coat emerging through the outer covering (Wiese and Binning, 1987; Auld *et al*., 1988). Exposure of the shoot tip to light enabled it to photosynthesize thereby straightening the epicotyls. (Moore, 1979; Osborne*et al.*,1985).

All treated seeds were covered with transparent polythene thereby exposing them to light which is an important regulatory environmental signal that triggers germination and they responded favorably (Modares and Hashemi, 2003). This is contrary to the findings of Jamaleddine *et al*. 2011 who reported that seeds of *A. annua* germinated after exposure to dark.

Germination of seeds of *A. annua*, commenced 2-3 days after sowing and 96 % of the seeds responded to treatment. This is contrary to the findings of Mannan *et al*. (2012) and other workers who observed that seeds of *A. annua* and *A. absinthium* germinated in 6 - 7 days as obtained when grown under field conditions. The insignificant height observed with the *Artemisia* young seedlings may be attributed to lack of ample reserved nutrients such as Carbohydrate, lipid and protein to enable the seedlings achieve critical size advantage (Michael, 2000).

Application of 50kg/ha of NPK had the highest number of branches. This is similar to the findings of Ming (1994) who observed substantial number of branches with application of average level of compound fertilizer. However, this is contrary to the findings of Arul (2002) and Rao *et al*. (1985) who recorded highest number of branches with poultry manure.

The highest leaf fresh weight and leaf dry weight observed with 6 t/ha of poultry manure corresponds with the findings of MajbuRahman *et al*. (2003) who attributed that to the photosynthetic efficiency of poultry manure on plant which yields high number of leaves and stems and increased vegetative growth. Similarly, Arul (2002), Khalid and Shafei (2005) observed that, application of poultry manure increases nitrogen level of a soil, which in turn increased production of leaves resulting in increased fresh herbage yield. The use of organic

fertilizers plays an essential role in plant growth and development, biosynthesis of the organic substance at all levels and growth yield characters such as biomass yield as reported by Marculescu *et al*. (2002).

This also agrees with the findings of Dixit (1997) and Mathias (1997) who reported that, poultry manure increased plant shoots. This was attributed to probably low soil nutrients status by Yeboah (2010) Similar results were also reported by Agyenim (1999)working on maize and found that the total weight of dry matter was higher in plants treated with poultry manure.

6 t/ha of poultry manure gave the highest plant height this contradicts what was reported by Martinez and Staba (1988) that N-fertilization stimulates the vegetative development of the plant, the greater the application of N, the greater the height of the plant. This may also be attributed to adequate supply of nutrients that influenced cell division and cell enlargement resulting in better plant height as suggested by Martinez and Staba (1988) and Gandh (1996).

This suggests that application of poultry manure at a rate of 6 t/ha may be considered as the optimum rate above which A*rtemisia* does not respond. Application of 8 t/ha of Poultry manure may be considered as excessive and is not efficiently utilized by plants. Consequently, high quantities of dissolved salt accumulate in the soil making nutrients unavailable (Hileman, 1971).

Raining season had the highest plant height, root and leaf weights compared with the irrigation trial this might not be unconnected with the fact that water constitutes 80-95 % of the mass of growing plant tissues and plays a crucial role for plant growth by acting as base material for all metabolic activities and helps to keep the plant erect by maintaining plant's turgidity (Taiz *et al.*, 1998; Araya, 2007).

Water is a constituent of protoplasm, maintaining cell turgidity for structure and growth. Growth occurs as water enters the cell in response to an osmotic driving force, and the pressure of the intracellular water inflates the cellulosic wall which bounds the cell. The primary process of volume expansion is driven by water uptake. The structural rigidity of the soft plant tissues is also conferred by water. This can be seen in the wilting of leaves of plants subjected to sudden drought. (Araya, 2007; Holbrook, 2010).

The Canopy spreadresults corroborate with the findings of Ferraira *et al.* (1995) who reported that organic amendments promoted vigorous growth and development, strong root system and stem development of *Artemisia*. Poultry manure contains appreciable amount of nitrogen that results in the production of larger stem width and longer internodes (Bandel *et al.*, 1972; Kallah and Adamu, 1998).

Extensive work on the production of artemisinin from *A. annua* has been done (Acton and Klayman, 1985; El-Sohly, 1990; Ferreira and Janick, 1996; Ferreira and Simon, 1995). Several studies on the artemisinin content of *Artemisia* species revealed that only *Artemisia apiacea* and *A. annua* contain artemisinin (Liershc *et al*., 1986; McIntosh and Olliaro 2004). The quantities obtained from these species were very low and varied according to seasons, growth conditions, as well as geographical locations (Abdin *et al*., 2003). According to Laughlin (2002), different quantities of artemisinin were reported in different plant parts, stages of vegetative growth and origin of the plant. For example, *A. annua* from Italy was reported to contain only 0.04 % to 0.05 % artemisinin dry weight.Trichomes are considered as appendages that emerged from the epidermal cells of leaves, stems, and flowers. They occur in a variety of shapes and sizes such as

unicellular or multicellular, branched or unbranched, and glandular or nonglandular. The glandular trichomes play vital role in the synthesis of artemisinin (Duke *et al.,* 1994; Ferreira and Janick, 1996, Bui *et al*., 2011)

Glandular secretory trichomes GSTs are located mainly on the leaves and flowers of *A. annua* (Ferreira and Janick, 1996, Olsson *et al*., 2009). Since trichomes are the sites of synthesis of artemisinin, it should be expected that more trichomes would result in more artemisinin produced, and indeed, there is a strong correlation between artemisinin concentration and trichome density (Graham *et al.,* 2006; Kapoor *et al*., 2007; Arsenault *et al.*, 2010)

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 *Artemisia annua* through the application of fertilizers. The pathway of artemisinin synthesis and the mechanism for its accumulation in leaves and inflorescences most probably determined the final artemisinin content in *Artemisia annua* and the effect of other environmental factors such as nutrient availability might be incidental; a situation observed by Chalapathi *et al*., (2004).

Artemisinin has also been reported from tissue culture of *A. annua* (Fulzele *et al.*, 1991; Jaziri, 1995; Martinez and Staba, 1988). Plants respond to their environment by altering shape, pigment content, and their secondary metabolites. Some environmental factors also alter aretmisinin production. Lulu *et al.* (2008) showed that, chilling can affect both artemisinin content and transcript levels of *ADS* and *CYP* in *in vitro* cultures. Efforts to enhance artemisinin content through adjustment the nutrients, hormones, growth condition, and stresses were also

reported (Misava, 1985; Stafford *et al*., 1986; Ramachandra and Ravishankar, 2002; Wang and Tan, 2002., Liu, 2003).

According to Ferreira *et al*. (1997) and Bhakuni *et al*. (2001) there is relatively low level of artemisinin in *A. annua* (0.01-0.8 %). Therefore, several efforts have been made to enhance artemisinin production with the view 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).

Using samples from leaf primordial and nodal cuttings to have the highest artemisinin content from a combined treatment of 3.0 mg/l GA3 and 1.0 mg/l BAP and equal combination of 0.5 mg/l BAP and 0.5 mg/l NAA is similar to the findings of Maes *et al*. (2010) who reported significant increase in the density of trichomes in response to 6-benzylaminopurine (BAP) and jasmonic acid (JA). Similarly, Mannan *et al*. (2008) and Banyai *et al*. (2011) observed enhancement of artemisinin content and leaf biomass in *A. annua* by exogenous GA_3 treatment. The increased artemisinin content was associated with an increased synthesis of key enzymes in the artemisinin biosynthesis pathway due to the GA_3 application. Exogenous GA_3 application continuously enhanced the artemisinin content from the vegetative stage to the flower initiation in both plant lines and gave a significantly higher leaf biomass. Consequently, the artemisinin yield in GA_3-treated plants was much higher than in the control plants.

In another development, Maes *et al*. (2010) sprayed two *A. annua* cultivars with 50 mg BAP and 100 mg GA_3 and saw that both phytohormones stimulated filamentous trichome development,

but only BAP stimulated glandular trichome development in both cultivars. Although the two cultivars showed an increase in glandular trichomes, the low-artemisinin-producing cultivar showed a greater increase than the high-artemisinin-producing cultivar (Maes *et al*., 2010). Also, compared to the control, only GA_3 increased the trichome size of the low-artemisinin-producing but not the high-artemisinin-producing cultivar. This suggested that trichomes in the high-artemisinin cultivar were already replete with artemisinin. Gibberellic acid (GA_3), abscisic acid (ABA), and cytokinins are phytohormones known to affect artemisinin production. GA_3 and BAP are also known to stimulate trichome development (Maes *et al*., 2010; Mannan *et al*., 2008). When the *A. tumefaciens* cytokinin biosynthetic gene, isopentenyl transferase was transferred and constitutively expressed in *A. annua*, the *in vivo* content of cytokinins increased along with chlorophyll and artemisinin (Sa *et al*., 2001) suggesting that biosynthesis of sartemisinin is regulated by cytokinins. This is not surprising since trichome development is known to be stimulated by BAP and the growth of the *A. annua* plants as well as the variations in artemisinin contents were attributed to environmental factors including hormones than to genetic factors. (Bui *et al*., 2011) The yield of artemisinin was higher (0.015 %) in leaf parts in comparison to its traces of 0.001 % in callus cultures (Singh*et al*., 2010). Exposure of plantlets to light might also be contributed to the level of artemisinin content. Because light was reported to exert stimulatory or inhibitory influence on the accumulation of secondary metabolites such as artemisinin in plant tissues and it is involved in the regulation of the secretion mechanism (Liu *et al*., 2002).

With the exception of Jha *et al*. (1988) most authors (He *et al*., 1983; Nair *et al*., 1986; Tawfiq *et al*., 1989; Fulzele *et al*., 1991; Kim *et al*., 1992; Woerdenbag *et al*., 1993; Brown, 1994; Paniego

and Giuliette, 1994; Ferreira and Janick, 1996) either reported no artemisinin or only trace amounts produced from callus cell.

Leaf materials obtained from this work were soaked directly into solvents without being crushed. This is in compliance with the fact that, Artemisinin and its precursor have been shown to be localized 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 *et al*., 1990).

Lommen *et al.* (2006) suggested that, upon maturation, trichomes collapse and release artemisinin, which may inhibit the artemisinin biosynthetic pathway. Artemisinin biosynthetic pathway is regulated through feedback inhibition and that ruptured trichomes may reduce production (Arsenault *et al.,* 2010).

Despite the fact that, the roots produce neither artemisinin nor its precursors, they play a critical role in artemisinin biosynthesis and possibly in the perception of signals that affect its synthesis. How the roots affect artemisinin production is still unclear. Clearly more research is needed to elucidate how roots increase artemisinin in the shoots of *A. annua*. (Khanhyan *et al.,* 2011). *In vitro* cultured shoots of *A. annua* that develop roots produce more artemisinin than shoots without roots Ferreira and Janick, 1996).

Production of highest artemisinin content by 6 t/ha of poultry manure is in accordance with the results obtained by Marculescu *et al*. (2002) who observed that, the use of organic manure plays

an essential role in plants growth and increased biosynthesis of secondary metabolites. This may be due to the influence of poultry manure in promoting vegetative growth hence increased herbage production and subsequently high crude extract yield (Singh, 2001; Singh *et al*., 2004). However, Singh (2001) observed that crude extract contents were not influenced by the application of fertilizers. This is contrary with a report by Delabays *et al*., (2001) that, artemisinin content is influenced by environmental factors such as nitrogen availability.

Lowest artemisinin content recorded with the control is an indication of its agronomic limitation since leaf biomass is an important attribute in *Artemisia* which influences artemisinin content (Yeboh, 2010). This probably suggests that artemisinin content was influenced by soil fertility. This is in consistence with the findings of Cox (1992) who reported that artemisinin content is influenced by genetic factors and not soil condition.

A. annua is classified as a short-day plant. Environmental variations, such as light, temperature, water and salt, were reported to affect the artemisinin yield (Weathers *et al.,* 1994). 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 *et al*., 1990; Laughlin, 1995). The artemisinin content of *A. annua* was reported by Delabays *et al.* (2001) to vary with season. Drought was also observed to affects artemisinin content in *A annua* (Marchese *et al.,* 2010). For example pot-grown plants deprived of water for 38 - 62 hours showed increased aretmisinin levels in leaves. Only the shorter duration treatment was effective as it did not reduce leaf biomass.

No significant difference was observed between periods of harvesting leaf material for artemisinin extraction. However, leaf material harvested before flowering had the highest

artemisinin content compared to those harvested after flowering. This confirms the fact that more trichomes are found on younger leaves therefore produce more artemisinin than older leaves (Ferreira and Janick, 1996). However, in mature plants, senescent leaves were found to produce nearly half of the total artemisinin in the plant (Lommen *et al.,* 2007, Woerdenbag *et al*., 1994) compared yield response of artemisia to different harvesting stages and noted that harvesting at pre-flowering stages promotes the production of high artemisinin contents. Laughlin *et al*. (2002) also confirmed that the optimum time to harvest artemisia is just before flowering; this is also important because of the allergic reaction of the pollen. It was further observed that time of harvesting at different ecological zones for high artemisinin content remains a technological problem. Several investigators, (Laughlin, 1994, Woerdenbag*et al*., 1994, Laughlin *et al*., 2002) have reported similar results of higher artemisinin content at pre-flowering. The slight decline in artemisinin content at full bloom is probably due to the partition of artemisinin or its precursors into flowering tops or due to its catabolism as suggested by (Laughlin *et al*., 2002).

However, 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 *et al*.,1990;Parakasa,1997;Sukhmal *et al*., 2001) reported that, organic and inorganic fertilizers increased leaf biomass production and subsequently led to an increase in artemisinin yield. This explains why evaluation of leaf biomass accumulation or production by *Artemisiaannua* was more important, since it is a precursor for higher artemisinin yield (EABL, 2005).This is contrary to an observation that artemisinin reaches its peak during flowering. The association of peak artemisinin with flowering is related to the abundance of glandular trichomes in the inflorescence particularly florets and receptacle (Ferreira *et al*., 1995). Morales *et al*. (1993) also confirmed that peak artemisinin was achieved during full bloom in a number of

greenhouses and field trials. This is confirmed by Ferreira *et al.* (1995) who found that artemisinin reaches its peak during full flowering in a Chinese clone for both greenhouse and field conditions. EABL (2005) also reports that, when half to three quarters of the plants shows signs of bud initiation, artemisinin content will be at maximum.

According to Ferreira *et al*. 1995, Ma *et al*. 2008, Arsenault *et al.* 2010, artemisinin content was found to be high either before or after full flower, suggesting that the flowering process may be involved in regulation of artemisinin biosynthesis. This appears unlikely as two studies that induced premature flowering using the early flowering gene CONSTANS (Wang *et al.,* 2004) or the flowering promoting factor1 (Wang *et al*., 2007) showed no significant increase in aretmisinin levels compared to wild type controls. This suggested that the high level of artemisinin found before or after the flowering stage is not directly related to the flowering period. As *A. annua* plants develop from vegetative to reproductive growth, artemisinin levels increase (Ferreira *et al*., 1995; Lommen *et al.*, 2006; Ma *et al.,* 2008*;* Arsenault *et al*., 2010).

Artemisinin content in *A. annua* propagated *in situ*, was reported by several workers to be highest at thepre-flowering period (Acton *et al*., 1985; Liersch *et al*., 1986; Woerdenbag *et al*., 1991; El-Sohly 1990; Woerdenbag *et al*., 1994), other workers reported it reaching its peak during the flowering stage (Singh *et al*., 1988; Pras *et al*., 1991; Morales *et al*., 1993; Ferreira *et al*., 1995; Laughlin 1995). However, Ferreira *et al*. (1995) observed artemisinin reaching its peak during the full flowering for both greenhouse and field conditions.

In vitro production of artemisinin was reported using differentiated shoot cultures of*A. annua* (Martinez and Staba 1988; Fulzele *et al*., 1991; Whipkey *et al*., 1992; Ferreira and Janick

(1996b), Brown (1994) obtained 0.0038 % of artemisinin callus cultures of *A. annua* bearing shoots. Nair *et al*. (1986) and Jha *et al*. (1988) reported trace amounts of artemisinin in *in vitro* root culture, however, Weathers *et al.* (1994) reported 0.4 % level of artemisinin in hairy root cultures of *A. annua* transformed with *Agrobacterium rhyzogenes*. Similarly, He *et al*. (1983); Nair *et al*. (1986); Tawfiq *et al*. (1989); Fulzele *et al*. (1991); Kim *et al*. (1992) ; Woerdenbag *et al*. (1993); Brown (1994); Paniego and Giuliette (1994); Ferreira and Janick (1996b), either reported no artemisinin or only trace amounts produced from callus. The highest level of artemisinindry weight obtained fromshoot of *A. annua* inoculated in a hormone-free medium was 0.287 % (Ferreira and Janick, 1996).

5.3 Summary

The addition of plant growth regulators had significant effect on the *in vitro* seed germination of *A. annua* compared with the hormone free MS mediaas the control which did not show any response. Green, aromatic and deeply dissected leaf materials were regenerated using leaf primordia excised from 70 days old *A. annua* inoculated on a full strength MS media supplemented with varying concentrations of someplant growth regulators. Shoots were induced from the nodal cuttings on MS media supplemented with varying concentration of some plant growth regulators (PGRs) that significantly influenced the in vitro cultures of*A. annua* in comparison with the half strength and hormone free MS media which did not show any response.

In vitro experiment with GA_3 and BAP, concentrations 1.5 mg/l of $GA_3$and 0.5 mg/l BAP recorded the shortest days to *in vitro* germination. Highest regeneration percentage was observed at 2.0 mg/l of GA_3 and 0.5 mg/l of BAP. A concentration of 2.0 mg/l of GA_3 and 0.5 mg/l of BAP had the best vigor.Highest shoot height was observed at 2.0 mg/l concentration of

GA_3 and 0.5 mg/l of BAP. GA_3 produced a significant effect on the seed, leaf and nodal explants with respect to vigour, shoot length, days to regeneration and percentage regeneration except on the leaf's percentage regeneration. Also, BAP had significant effect on all the parameters studied on all the explants except on seed's percentage regeneration.

Using BAP and NAA, best vigour was observed with 0.5 mg/l of BAP and 0.5 mg/l of NAA. Highest shoot height was recorded at 0.5 mg/l of BAP and 0.5 mg/l of NAA. However, lowest days to regeneration were observed with equal concentrations of 0.5mg/l of both BAP and NAA. Similarly, highest percentage regeneration was observed at equal concentrations of 0.5 mg/l of both BAP and NAA.

However, with $GA_3$and NAA plant growth regulators, concentrations of 1.0 mg/l of GA_3 and 0.5 mg/l produced the highest vigour. Also, a concentration of 1.5 mg/l of GA_3 and 0.5 mg/l of NAA had the best shoot height. However, 0.5mg/l of GA_3 and 0.5mg/l of NAA had the lowest days to regeneration

In situ germination experiment results indicated no significant difference between the seasons with respect to germination, shoot and root lengths ($P \geq 0.05$). Warm water treatments in both rain and irrigation trials at 40 ^{0}C for 3minutes and 60 ^{0}C for 3minutes produced the best vigor. Highest germination percent of 96%was observed during the rainy season trial with 10 % Sulphuric acid treatment.

Application of 6 t/ha of poultry manure during the raining season had the highest plant height followed by 4 t/ha of poultry manure during the irrigation trial. Similarly, rain season trial had the highest number of branches with 50 kg/ha of NPK followed by same quantity of NPK during

the irrigation trial. Similarly, rain trial had the best leaf fresh weight after flowering with 6 t/ha of poultry manure followed by irrigation trial with 4 t/ha of poultry manure. The best leaf dry weight before flowering was observed with 25 kg/ha during the rain trial. It was followed by the same quantity of NPK during the irrigation trial. Similarly, rain had the highest leaf fresh weight before flowering with 2 t/ha of poultry manure followed by the same quantity during the irrigation trial.

Also, the rain season had the best result with respect to leaf dry weight after flowering with 8t/ha of poultry manure followed by the same quantity during the irrigation trial.Treatment 6 t/ha had the highest artemisinin content of 2.1 % followed by treatment 75 kg/ha with 1.51 %. Lowest artemisinin content of 0.4 % was recorded with the control.

Combined treatment of 1.0 mg/l GA_3 and 0.5 mg/l BAP produced the highest artemisinin content of 0.97 % from *in vitro* leaves harvested from leaf primordia. It was followed by 0.5 mg/l of GA_3 and 0.5 mg/l of BAP which produced 0.87 % artemisinin from leaf material harvested *in vitro*from nodal explants.

5.4 Conclusion

Treating *Artemisia* using the plant growth regulators hadsignificant effect on its *in vitro* regeneration. It has also reduced the effect of the phenolic secretions reported on the seeds thereby enhancing its germination. These are promising and viable approaches to faster *in vitro* regeneration of *Artemisia* plant hence supply of the raw materials needed for the production of anti malaria drugs for the fight against malaria fever.

The use of Murashige and Skoog (MS) media in combination of the following varying concentrations of 0.5 mg/l, 1.5 mg/l and 2.0 mg/l of GA_3, BAP and NAA is an effective protocol for the *in vitro* regeneration of *Artemisia annua* using seed, leaf primordia and nodal explants. Soaking *Artemisia* seeds in 10 % Sulphuric acid for 1 minute was found to be the best treatment with early *in situ* germination and high germination percent of *A. annua*.

Artemisia and artemisinin production is possible in Nigeria. Leaf biomass harvested from the application of 6 t/h of poultry manure and 75 kg/h of NPK during the *insitu* experiment produced the highest artemisinin content. However, 1.0 mg/l of GA_3 and 0.5 mg/l of BAP is an effective protocol for *in vitro* artemisinin production. *In situ* regenerated plantlets produced the highest quantity of Artemisinin than those produced *in vitro.*

5.5 Recommendations

The following are made as recommendations.

i. The identified concentrations of plant growth regulators and quantities of poultry and NPK are recommended for *in vitro* and *in situ* biomass and artemisinin production in *A. annua*
ii. Covering *A. annua* seeds with a transparent polythene material under moist condition is a simple and affordable technique especially to local farmers. It gives a good result in the germination and seedling production of the plant compared to not covering.
iii. Planting in March can be tried for the *in situ* germination of *A. annua*. However, more work need to be done to ascertain more suitable planting dates. This is to facilitate timely transplanting of the seedlings on the field at the on set of rainfall.

iv. *Artemisia* and artemisinin production is possible in Nigeria. Therefore, there is the need for research on advanced methodsand techniques forconfirmation of theidentity and purity of artemisinin obtained from both in *vitro* and *in situ* regenerated leaf materials.

v. Vegetative propagation using stem and root cuttings may also be a viable means to large scale propagation.

vi. More research efforts are needed for economic exploitation of *Artemisia annua*and its resources.

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APPENDICES

Appendix i. Murashige and Skoog (MS) Plant Tissue Culture Media Composition

1. Macro salts		
Ammonium nitrate	NH_4NO_3	1,650 mg/l
Calcium chloride	$CaCl_2 \cdot 2H_2O$	440 mg/l
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	370 mg/l
Potassium phosphate	KH_2PO_4	170 mg/l
Potassium nitrate	KNO_3	1,900 mg/l
2. Micro salts		
Boric acid	H_3BO_3	6.2 mg/l
Cobalt chloride	$CoC_{l2} \cdot 6H_2O$	0.025 mg/l
Cupric sulphate	$CuSO_4 \cdot 5H_2O$	0.025 mg/l
Ferrous sulphate	$FeSO_4 \cdot 7H_2O$	27.8 mg/l
Manganese sulphate	$MnSO_4 \cdot 4H_2O$	22.3 mg/l
Potassium iodide	KI	0.83 mg/l
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/l
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	8.6 mg/l
$Na_2EDTA\ 2H_2O$		37.2 mg/l
3. Vitamins		
i-Inositol		100 mg/l
Niacin		0.5 mg/l
Pyridoxine HCL		0.5 mg/l
Thiamine · HCl		0.1 mg/l
Glycine		2 mg/l

Appendix ii. Mean square for effects of GA_3 and BAP on the *in vitro g*ermination and Seedling growth of *A. annua*using seed explants in MS media.

Source	DF	Vigour	Shoot height (cm)	Days to regenereatio	Regeneration (%)
GA_3	6	3.93**	4.12**	80.30**	274.61^{NS}
BAP	2	13.23**	12.35**	231.17**	651.15^{NS}
GA_3 x BAP	12	2.21**	1.35**	39.53*	205.87^{NS}
SE±		0.47	0.2	2.63	11.64

Foot note: *, ** Significant at 5% and 1% respectively

GA3= Giberellic acid. BAP= Benzyle purineaminoacid. NAA= Nephthyle acetic acid

Appendix iii. Mean square for effects of NAA and BAP on the *in vitro g*ermination and Seedling growth of *A. annua* using seed explants in MS media.

Source	DF	Vigour	Shoot height (cm)	Days to germination	Regeneration (%)
NAA	6	1.47**	1.06**	11.05^{NS}	1.51^{NS}
BAP	2	3.52**	2.54**	24.38^{NS}	2.63^{NS}
NAA x BAP	12	1.47**	1.06**	11.05^{NS}	1.51^{NS}
SE±		0.15	0.07	2.49	0.9

Foot note:*, ** Significant at 5% and 1% respectively

NAA= Nephthyle acetic acid. BAP= Benzyle purineaminoacid.

Appendix iv. Mean square for effects of GA_3 and NAA on the *in vitro* germination and Seedling growth of *A. annua* using seed explants in MS media.

Source	DF	Vigour	Shoot height (cm)	Days to germination	Regeneration (%)
GA_3	6	3.39**	2.66**	16.21^{NS}	15.20^{NS}
NAA	2	3.86**	2.79**	17.60^{NS}	19.23^{NS}
GA_3 x NAA	12	2.79**	2.25**	15.26^{NS}	9.41^{NS}
SE±		0.15	0.22	2.81	2.46

Foot note: *, ** Significant at 5% and 1% respectively. GA_3= Gibberellic acid. NAA= Nephthyle acetic acid.

Appendix v. Mean square for effects of GA_3 and BAP on the *in vitro* regeneration of *A. annua* using leaf primordia explants in MS media.

Source	DF	Vigour	Shoot height (cm)	Days to regeneration	Regeneration (%)
GA_3	6	3.55*	5.65**	32.33^{NS}	248.72^{NS}
BAP	2	25.07**	33.68**	199.45*	2916.56**
GA_3 x BAP	12	1.24^{NS}	5.18**	25.70^{NS}	134.35^{NS}
SE±		0.57	0.59	4.87	14.95

Foot note: *, ** Significant at 5% and 1% respectively

GA3= Giberellic acid. BAP= Benzyle purineaminoacid. NAA= Nephthyle acetic acid.

Appendix vi. Mean square for effects of NAA and BAP on the *in vitro* regeneration of *A. annua* using leaf primordia explants in MS media.

Source	DF	Vigour	Shootheight (cm)	Days to regeneration	Regeneration (%)
NAA	6	2.79**	4.62**	12.23^{NS}	133.96^{NS}
BAP	2	22.08**	19.03**	106.84**	824.26*
NAA x BAP	12	2.19**	1.47^{NS}	20.63^{NS}	186.35^{NS}
SE±		0.29	0.62	3.65	10.83

Foot note: *, ** Significant at 5% and 1% respectively. GA3= Giberellic acid. BAP= Benzyle purineaminoacid. NAA= Nephthyle acetic acid.

Appendix vii. Mean square for effects of $GA_3$and NAA on the *in vitro* regeneration of *A. annua* using leaf primordia explants in MS Full strength media.

Source	DF	Vigour	Shoot height (cm)	Days to germination	Regeneration (%)
GA_3	6	0.53**	0.74**	33.27^{NS}	261.81^{NS}
NAA	2	18.10**	24.58**	54.38^{NS}	464.69^{NS}
GA_3 x NAA	12	0.53**	0.74**	24.27^{NS}	126.66^{NS}
SE±		0.19	0.29	3.87	10.03

Foot note: *, ** Significant at 5% and 1% respectively. GA3= Giberellic acid. NAA= Nephthyle acetic acid.

Appendix viii. Mean square for effects of GA_3 and BAP on the *in vitro* regeneration of *A. annua* using nodal explants in MS media

Source	DF	Vigour	Shoot height (cm)	Days to regeneration	Regeneration (%)
GA_3	6	2.31^{NS}	37.8**	4.41*	560.7^{NS}
BAP	2	15.6**	16.6**	35.17*	1731.9^{NS}
GA_3 x BAP	12	2.01^{NS}	2.29**	8.22^{NS}	291.03^{NS}
SE±		0.42	0.42	2.21	16.06

Foot note: *, ** Significant at 5% and 1% respectively. GA3= Giberellic acid. BAP= Benzyle purineaminoacid. NAA= Nephthyle acetic acid.

Appendix ix. Mean square for effects of NAA and BAP on the *in vitro* regeneration o *A. annua* using nodal explants in MS media.

Source	DF	Vigour	Shoot height (cm)	Days to regeneration	Regeneration (%)
NAA	6	2.93**	2.61**	9.54^{NS}	31.19^{NS}
BAP	2	9.82**	8.42**	30.74^{NS}	102.12^{NS}
NAA x BAP	12	3.60**	1.86**	15.24^{NS}	30.12^{NS}
SE±		0.25	0.24	2.75	4.34

Foot note: *, ** Significant at 5% and 1% respectively. GA3= Giberellic acid. BAP= Benzyle purineaminoacid. NAA= Nephthyle acetic acid.

Appendix x. Mean square for effects of GA_3 and NAA on the *in vitro* regeneration of *A. annua* using nodal explants in MS media.

Source	DF	Vigour	Shoot height	Days to regeneration	% Regeneration
GA_3	6	3.95**	2.31**	27.02^{NS}	31.01^{NS}
NAA	6	8.84**	3.10**	31.36^{NS}	72.60^{NS}
GA_3 x NAA	12	2.08**	1.22**	12.11^{NS}	24.94^{NS}
SE±		0.22	0.2	3.04	3.97

Foot note: *, ** Significant at 5% and 1% respectively. NS = Not significant. GA_3= Gibberellic acid.

NAA = Naphthaleneacetic acidNR= No response

Appendix xi. Mean square for effects of NPK and Poultry Manure on the Growth of *A. annua* under rain and irrigation season.

Source.	DF	PH (cm)	NB	CS	LFWB	LDWB	WFWB	LDWA	LFWA	WFWA
Treatment	8	166.0*	17.3	21.2	1418.2	1993.8**	36111.59	2638.44	4316.7	423647
Season	1	2974.1	4880.3	154.8	3997.4	228086.6	4673178*	538397*	1019523*	441209769.2**
Trt *Seasc	1	90.7	14	154.8	44.9	685.32	8630.13	3318.89	3985.41	414798.6
Error	40	41.5	13.45	17.8	887.9	582.56	25159.5	1958.8	2813.72	1322164.9
Grand Mean		75.5	31.26	43.7	54.29	70.12	440.49	85.91	110.81	2040.07

*, ** Significant at 5% and 1% respectively

Appendix xii. Mean square for effects of Poultry manure and NPK on Artemisinin content in *A. annua*

Source	DF	Artemisinin content
Season	1	0.48 ^{NS}
Poultry Manure	4	4.70 *
NPK	4	1.34 ^{NS}
Season x Poultry Manure	4	5.32 *
Season x NPK	4	1.58 ^{NS}
Season x Poultry x Manure x NPK	32	1.69 ^{NS}
SE±		0.76

Foot note: *, ** Significant at 5% and 1% respectively. NS = Not significant.