Addis Ababa University
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Micropropagation of *Prunus africana* (Hook. f.) Kalkman from seedling

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By

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List of Abbreviations

BAP	6-Benzylaminopurine
FAO	Food and Agriculture Organization
FRC	Forestry Research Center
IAA	Indol-3-Acetic acid
IBA	Indol-3-Butyric acid
masl	meters above sea level
MS	Murashige and Skoog
±	Plus or minus

ABSTRACT

The aim of this work was to develop micropropagation protocol for the threatened, multipurpose Afromontane tree, *Prunus africana* (Hook. f.) Kalkman, starting from aseptically grown seedlings. Application of 12% $CaOCl_2$ on propagula for 15 minutes, following washing with the common laboratory detergent OMO and rinsing in 70% ethanol for 2 minutes resulted in seedlings that grew healthily and were free from microbial contaminants. Twenty to twenty five-day-old seedlings were used as explants. Among the tested concentrations of growth regulators, 2 mg/l BAP and 0.1 mg/l IBA in full strength MS medium yielded well initiated shoots. Activated charcoal (1g/l) was used for the initiation step, but not required in the subsequent transfers to multiplication media, as browning due to phenolic exudates was not a serious threat. Throughout all experiments, an initial pH value of 5.5 was maintained. Maximum shoot multiplication (4.6 shoots per explant) was obtained in response to 1 mg/l BAP without IBA. The sufficiently long (3-5 cm) and healthy microshoots that were transferred to half strength IBA free MS medium with 0.1% activated charcoal gave maximum rooting percentage (60) medium within 20 days. This was significantly higher from other compositions at $P \leq 0.05$. The plantlets each with average 11 roots were transferred to pots, with compost, red soil and sand in a 1: 2: 1 ratio. The pots were placed in a glasshouse for hardening, were covered with polythene plastic for one week, partially uncovered for the other one week, and then completely uncovered. All plantlets survived well.

Key words/ phrases:- Acclimatization, Culture initiation**,** Micropropagation, Propagules, *Prunus africana*

1. INTRODUCTION

Trees are general requisites if the world's cultures, economy and environments are to sustain themselves on the side of human wellbeing (Badeg Bishaw, 2001). Soil and water conservation, resource recycle, feed and shade for wild animals, climate temperance, vast array of food, fuel, medicine, construction and clothing materials are among the advantages of forests. Legesse Negash (2002) described their importance as "Forests determine the basic fabric of life on earth".

The last decades have shown extensive biodiversity turn down, above all in tropical forests. According to Laurance *et al.* (2000) cited in Farwig *et al.* (2008), deforestation, forest fragmentation and selective logging are among the major causes for this general decline. Ethiopia is one of the victims in this region that have lost their indigenous forests from vastness of mountainous regions to satisfy demands of the zooming population (Badeg Bishaw, 2001; Tileye Feyissa, 2006). The causes of environmental degradation in Ethiopia are many. One of these causes is deforestation of remnant old forests for household and commercial uses (Badeg Bishaw, 2001). The household uses of woods are primarily for cooking and construction while the commercial use of woods is for a cash source to many poor households.

This extensive loss of forest covers featured unsustainable and underproductive agriculture and massive environment deterioration (Legesse Negash, 1995). The consequences of natural forest destruction embrace biodiversity degradation, soil loss by erosion, silting of dams and lakes, worsening of climate and diminishing of

underground water reserves (Badeg Bishaw, 2001; Legesse Negash, 2002). The direct correlation of forest depletion and environmental deterioration alerts us about the need for proper forest use and replenishment in order to have sustainably progressive livelihood.

Ethiopia has been one of the centers of plant genetic diversity whose indigenous forests on watersheds and woodlands have been repositories of biodiversity (Legesse Negash, 2002). Due to wrong perception of forests as inexhaustible resources, unrealized exploitation and negligence of conservation activities led to forest fragmentation and complete destruction. Even today, the increased human population along with its increased demands puts pressure on natural regeneration of the remaining vestigial forests (FAO, 1997; Mersha Geberehiwot, 2007). Indigenous trees of Ethiopia that were once densely populated are now on the verge of extinction (Legesse Negash, 2002). It is not difficult to understand that the loss of indigenous species is irreparable damage for the ecosystem.

There have been several attempts initiated since several decades ago, when the problem was realized. Introduction of exotic tree, *Eucalyptus* species, at the end of 19th century was one of these attempts to satisfy the nation's need for fuel and construction (Badeg Bishaw, 2001). Since six decades ago, introduction of other fast growing exotic trees was also undertaken for reforestation purpose (Forestry Research Cervices, 1985 cited in Legesse Negash, 2002). But these practices featured controversies. For example, it is reported that introduction of the eucalyptus is undermining not only soil fertility but also the diversity of vegetation growing beneath them. Fast growing exotic trees that were promising in reforestation practices are being challenged by widespread dieback

(Legesse Negash, 2002). But, generalization about these controversies is still lacking, and requires further researches.

In any ways, indigenous trees are best candidates for afforestation or reforestation of degraded lands (Tileye Feyissa, 2006). This is because not only they are well adapted to the soil and climate in the country, but also they can grow in association with other species. *Prunus africana* (Hook. f.) Kalkman is one of the many indigenous trees of Ethiopia that should get due consideration in the reforestation program. It is known to grow in friendly way with other fauna and flora of the ecosystem. The medicinal value of the bark to treat several health problems and the subsequent commercial exploitation from many African countries, its use for construction, fuel and other purposes makes the tree extremely valuable. Unrealized exploitation for these uses and other factors threatened this tree species to the extent it is listed in Appendix II of CITES (Convention on International Trade in Endangered Species of wild fauna and flora) (CITES, 2008) and other conventions.

Only a few researchers from Ethiopia and the rest African countries have been engaged to save this extremely valuable tree from extinction. Neither of these attempts has taken plant tissue culture technique as a conservation measure. Tissue culture, however, has wider scope and potential both in commercial and conservation activities. This work demonstrated the amenability of *P. africana* to micropropagation.

2. LITERATURE REVIEW

2.1. Description of *Prunus africana*

P. africana (Hook. f.) Kalkman, synonymous with *Pygium africanum*, belongs to family Rosaceae and subfamily Prunoideae. It is known commonly as *Tikur Inchet* in Ethiopia. It is also named as African Cherry, African prune, bitter almond, iron-wood or red stinkwood (Page, 2003; Legesse Negash, 2004). *P. africana***,** wild relative of plume tree (Page, 2003), is the only one that is found in Africa among about 200 species of genus *Prunus* (Legesse Negash, 2002; Page, 2003).

It is an evergreen graceful tree that can grow upto 40 m in height (Hedberg, 1989) and 1 m in diameter (Dawson and Powell, 1999) upon maturity. Its bark is dark-brown to gray color and rugged. The leaves are elliptic-oblong, have 15 X 5 cm surface area, and shallowly crenate – serrate (Hedberg, 1989). They are shiny dark – green on the upper side and light green on the underside. Veins and midribs are conspicuous and prominent, especially on the underside of leaves (Legesse Negash, 2002).

The tree has raceme inflorescence composed of 10 to 30 small, white or greenish, hairy and fragrant flowers with male and female parts, creamy white petal, and Ovid ovary. Pollination is commonly effected by insects (Legesse Negash, 2002). Fruits develop four to six months past pollination. Fruits are drupe, spherical, bitter, 5 to 8 mm wide and 9 to 11 mm long, pinkish – brown, turning to dark – red or reddish – brown pulp when they get ripe (Legesse Negash, 2002).

The drupe fruits are monkey's favorite and hence, seed dispersal is by these animals (Farwig *et al*., 2006). Inside the fruit is bilaterally symmetrical and to some extent oval seed with leathery seed coat.

Germination is special (Fig. 1) in that the radicle comes out as a mucilaginous mass, gradually narrowing into a well defined embryonic root. The plumule appears to be enclosed within a natural split of the radicle, thus disentangling itself through split during germination (Legesse Negash, 2002).

Figure 1. The special germination of *P. Africana* (Photo taken in laboratory)

*2.*2 Ecology and distribution of *P. africana*

P. africana is a geographically widespread Afromontane tree growing in humid and semi humid highlands and midlands of sub- Saharan Africa (Angola, Cameroon, Democratic Republic of Congo, Ethiopia, Kenya, Malawi, Nigeria, Somalia, South Africa, Sudan, Swaziland, Tanzania, Uganda, Zimbabwe) and other outlying islands like Bioko, Grand Comore, Madagascar, Sao Tome (Dawson *et al.*, 2000).

It best grows in montane or riverine forests, forest edges and forest gaps, where annual rainfall ranges from 1000 to 2000 mm (Legesse Negash, 2002). It is known for high light requirement (Legesse Negash, 2002) and usually found in regions with altitudes ranging from 1700 to 2500 masl (Hedberg, 1989).

2.3 Economic and ecological importance of *P. africana*

P. africana is a multipurpose tree. The front line importance of this tree is through the medicinal value of its bark to treat several human diseases: The bark is so far applied to treat Prostate disorders (Benign Prostate hyperplasia and prostate gland hypertrophy), sexual dysfunction, chest pain, malaria, inflammation, fever, kidney disease, and stomachache (Legesse negash, 2002; Page, 2003) .

Benign Prostate Hyperplasia (BPH), enlargement of prostate gland which is about 33% prevalent amongst older men (50 or above) in UK, is characterized by urinary obstruction and irritation (Page, 2003). Prostate surgery and herbal treatments are among classical BPH treatments. The prostate surgery may cause series problems that range from urinary incontinence to impotence while synthetic drugs administered during the first stage of the disease are accompanied by side effects like nausea and fatigue (Legesse Negash, 2002; Page, 2003).

The bark of *P. africana* has valuable medicinal importance in the treatment of BPH without complications encountered in using classical treatments (Cunningham and Mbenkum, 1993). Europeans realized the medicinal importance of this tree species about two centuries ago after indigenous society of South Africa and Cameroon informed European soldiers about its relaxing effect on bladder pains. Traditionally, the

powder of the bark is added in water, and the resulting red liquid or suspension is used as remedy for the stomachache. Besides, leaves are boiled and inhaled for fever treatment or drunk as an infusion for improved appetite (Legesse Negash, 2002).

The bark extracts were patented in 1966, and the French entrepreneur, Debat, was the first to acquire the patent (Debat, 1966 cited in Cunningham and Mbenkum, 1993). Fifteen years ago, about 4, 000 tones of bark were exported from Africa to Belgium, France, and other pharmaceutical industries (Page, 2003). The global annual trade in *Prunus* bark is variously estimated to be worth between US$150 and US$220 million, and the price of a kilogram of the bark is 2 USD (Cunningham and Mbenkum 1993). In line with the pharmaceutical application and export income, collecting the bark by itself is a job opportunity for laborers recruited under the exporters.

To date, there is no any publication about Ethiopian genotypes of *P. africana* in relation to the bark chemistry and its medicinal value. But there are strong indicators about the suitability and potential use of Ethiopian provenances. One among the indicators is the close genetic similarity of ours to Kenyan (a genetic distance less than 0.1) (Dawson and Powell, 1999), which has been exported to France (Cunningham and Mbenkum, 1993). The major chemical constituents, quality standards and purity assays of Ethiopian provenances are not either researched or published though clear and easy protocols are accessible.

The other importance of the tree is through its excellent timber for heavy construction works, household furniture, flooring, turnery, moldings, poles and mortars. It is

generally among highly preferred because of its durability followed by wood resistance to rot, termites and woodborers. (Legesse Negash, 2002; Kakudidi, 2007).

Beyond these uses, domestic fuel, erosion control and soil fertility improvement are citable (Tesfaye Wubet *et al*., 2003). Legesse Negash (2002) further out lined that this indigenous tree is used in apiculture due to high quantity of pollen and nectar. The graceful and evergreen tree has also aesthetic value to the ecosystem. Worth mentioning, *P. africana* is an important part of the montane ecosystem that fills gap in the integrity of the forest and provides food resources as well as shelter for rare birds (Cunningham & Mbenkum, 1993).

2.4 Problems associated with *P. africana*

This species was once densely distributed in Ethiopia, Eastern Zimbabwe, Mozambique, Kenya, Uganda, Democratic Republic of Congo, Cameron, Guinea, and Madagascar. (Legesse Negash, 2002). Unrealized exploitation for commercial use by pharmaceutical companies in Spain, Italy, Germany and France put pressure on the natural stand. This and use of the tree for construction and timber is significantly accounted for fragmentation and clearance of *Prunus* population in Cameroon (Kakudidi, 2007; Cunningham and Mbenkum, 1993). The tree is also threatened in Madagascar, Kenya and Equatorial Guinea (Dawson *et al*., 2000).

As a result of over-exploitation, trade in *P. africana* products is regulated under Appendix II of the Convention on International Trade in Endangered Species of wild fauna and flora (CITES, 2008).

Although there is no information about the extent of bark export, Ethiopia has now only fragmented and sparsely distributed stands in Gojam, Gondar, Shewa, Arsi, Bale, Harerge, Wollega, Ilubabor, Kefa and Sidamo regions (Legesse Negash, 2002). The species is suffering from overexploitation to satisfy the increasing demand associated with the growing population, leading to devastating consequences for the forest ecosystem (Tesfaye Wubet *et al.*, 2003). One can predict what would be the fate of the remaining populations if the poor Ethiopian society realize the cash value and start exploiting them.

Complimentary to human's impact; there are also some biological and ecological factors that have contribution for the diminished and fragmented populations of *P. africana* not only in Ethiopia but also in the other countries (Dawson *et al.,* 2000). Some of these problems are well researched and became grounds for conservation practices. Some others, on the other hand, are controversial and not yet clearly understood.

2.5. Research and development on *P. africana*

The research interest of European pharmaceutical companies is mainly on how to increase the product standard and quantity, while that of ours focuses on how to ensure sustainability of the species.

Genetic variation among eight countries provenances was studied using RAPD (Random Amplified Polymorphic DNA) by Dawson and Powell (1999). The research was conducted with the intent of having clear understanding on the patterns of variation

within and among populations of tropical *Prunus*, which is in-turn essential for devising optimum genetic management strategies for conservation and sustainable utilization. According to this diversity analysis, the *Prunus* in Ethiopian (particularly in *Lepsi- Arsi*) is closely related to that in Kenya (genetic distance less than 0.1) (Dawson and Powell, 1999), which implies the potential use of Ethiopian provenance in medicinal application.

Seed of *P. africana* is intermediate in nature, which limits *ex situ* seed storage. The best conditions for seed storage were obtained when seed from mature (purple) fruit was harvested directly from trees and depulped immediately after collection, followed by storage, without drying, at 5° Celsius. However, even under these conditions, germination was only 35% after 12 months of storage (Sacandé *et al*., 2004). Long term seed storage of *P. africana* as a means of *ex situ* conservation is therefore not advisable, although short-term storage across planting seasons is possible.

Few research activities have been also conducted on the reproductive biology of *P. africana* and revealed that the species is predominantly outcrossing; and pollination and seed dispersal in a given population is effected over a relatively long period of time (Were *et al.,* 2001). These observations combined with the frequent low density, patchy and unusual size class distribution of *P. africana* in forest (Ewusi *et al*., 1992; Ewusi *et al*., 1997), increase concerns regarding effective population sizes of exploited natural stands and their long term reproductive viability for in situ conservation.

It is also found that there are some ecological and/or biological factors that contribute at least in part for the species to be imperiled. According to one research in Uganda (Paul

et al., 2004), *P. africana* seedlings suffer from greatest mortality rate till the edge of one year. This research further showed that about 60 % of seedlings die in shrub dominated site and 45% in forest not due to edaphic and physiographic factors. It is rather due to light inaccessibility for this light loving species in forest and smothering effect of shrubs through network of branches (Paul *et al.*, 2004).

Farwig *et al*. (2006) tried to demonstrate that local disturbance of forests is advantageous for the dispersal of *P. africana*, as its attractive fruits become more available for frugivers, and this in-turn helps the regeneration and community wide species diversity. On the other hand, Farwig *et al.* (2008) indicated the probable decrease in seed dispersal service, pollination success and seedling establishment due to decline of pollinators and seed dispersers. The latter work ended up its conclusion by recommending further investigation on whether this threatened tree species get regeneration advantage by the local disturbance (Farwig *et al.,* 2008). Successful plantations and enrichment plantings have been limited to Kenya where over a period of 90 years, *P. africana* has been planted by the Forest Department for Timber. However, their planting material is often of unknown origin and of narrow genetic base. They have, therefore, a limited utility for conservation purposes (Eyog *et al*., 2001).

The precautionary and alleviation practice in Ethiopia is underway and Legesse Negash (2002) notified the importance of researches on this and other endangered indigenous trees before the ugly face of extinction occurs whenever the low income Ethiopian realize the commercial value of this tree and start unrealized logging of the natural stand.

Rapid propagation methods for this threatened species are researched in Ethiopia (Legesse Negash, 2004). Mean germination in different conditions: seed coat removed or intact seed, with or with out germination promoting hormone (GA_3), in glasshouse or nursery, and seed gathering conditions (from beneath trees with soil and litter). To mention some of the improvements obtained from these trials, 98.6% mean germination 35 days after sawing on seedbed setup in glasshouse from red soil, compost and horse dung in equal proportion; 91% mean germination within 21 days by removing seed coat, disinfecting and sowing on filter paper in Petri plates; and propagation of *P. africana* by means of wildlings with limited application is also recommended.

2.6 Plant tissue culture techniques

Micropropagation is the one aspect of plant tissue culture that allows the production of large numbers of plants from small pieces of the stock plant in relatively short periods of time. It is a technique for rapid multiplication of plants (Singh, 2003). It is an aseptic, sophisticated laboratory procedure that requires unique facilities and special skills (Hartmann *et al*., 2004).

Micropropagation confers distinct advantages not possible with conventional propagation techniques. It is possible to multiply a single explant into several thousands in less than one year. Actively dividing cultures are continuous sources of plantlets without seasonal interruption. It has a great commercial potential due to the speed of propagation, its high quality and the ability to produce disease – free plants (Hartmann *et al*., 2004; Tileye Feyissa, 2006).

Using methods of micropropagation, the nurseryman can rapidly introduce plants of desired trait in sufficient quantities. It is likely that micropropagation in the forestry sector will become commercially more important when compared to vegetative propagation through cuttings, because the higher multiplication rates through micropropagation offer a quicker capture of genetic gains obtained in clonal forestry strategies (Ruane and Zimmermann, 2001).

The importance of *in vitro* methods for the propagation of plants in botanic gardens is also widely recognized (Fay, 1992). The use of these techniques has allowed the propagation of many species which prove problematic with conventional horticultural methodology.

Hartmann *et al***.** (2004) classified the developmental stages of micropropagation into culture initiation, multiplication, root formation and acclimatization. The first activity in culture initiation is to select and take explant. The explant may be taken from shoot tip, leaf, and lateral bud, stem or root tissue. Seeds are preferred to vegetative materials as a source of explant for its ability of maintaining wider genetic base of the plant, and its tolerance to the toxic effect of disinfectants (Fay, 1992).

Explant disinfection is complete elimination of bacteria, fungus and algae by appropriate chemicals so that the culture to be established is free from contaminants (Fay, 1992; Hartmann *et al*., 2004). Disinfection is effected by chemicals which are toxic for the contaminants but relatively non toxic to the plant. The commonly used disinfectants are calcium hypochlorite, sodium hypochlorite and mercury chloride. Usually, the treatments are done following rinsing the explant with 70% ethanol.

Optimizing disinfection involves determining the type of chemical used, effective concentration and time of exposure (Fay, 1992).

The disinfected explant should be transferred to a defined culture medium plus specified combinations of growth regulators. There are several types of culture medium with more or less similar composition, but the most widely used medium is Murashige and Skoog (1962) medium. The culture medium contains major minerals (macronutrients) that are indispensable from plant growth, minor minerals (micronutrient) required for the normal physiological functioning, energy source, organic supplements like Myo-inositol and thiamine (vitamin B1), support system (agar or gelrite), and antioxidant, for example, activated charcoal (Fay, 1992).

Among the five classes of growth regulators, the three are mainly used in plant tissue culture: cytokinins, auxins and gibberellins. Cytokinins are normally used to induce shoot proliferation by breaking dormancy in lateral buds (suppressing apical dominance). Auxins are used in optimum combination with cytokinin to induce callus, or used alone to induce root. Gibberellins are commonly used in vegetative propagation where shoot elongation following proliferation proves difficult to achieve. In addition, they can be used to promote seed germination (Fay, 1992; Hartmann *et al.*, 2004).

Factors that affect plant's response to tissue culture include media composition, growth regulator combinations, genotype, explant type, organic component, and developmental stage of the explants both at the time of culture initiation and subsequent culture processes (Hartmann *et al*., 2004).

2.7 Application of tissue culture for *P. africana*

Initial application of micropropagation was restricted to fruit crops such as strawberry and raspberry, and its scope extended to hardwoods as methods were developed later (Ahmed *et al*., 2001). The number of woody plant species which have been clonally propagated through tissue culture is increasing at a rapid rate, and in fact, most commercially important ornamental species and hard woods have been studied (Chalupa, 1987). Perhaps the best publicized of the early research on woody plants involved apples, Douglas fir, and rhododendrons (Aboel-Nil, 1987).

The micropropagation of *Prunus* species like *P.avium* (L.) Moench and *P. padus* (L.) are well studied (Chalupa, 1987) and their results create a good insight about amenability of *P. africana* for tissue culture. So far, there was no any attempt to mitigate the extinction risk of *P. africana* by tissue culture approaches. It might be partly due to the notion that it simply germinates and grows in conventional propagation. Researches, on the other hand, indicated that the seed has availability problem, its recalcitrance poses storage problem (Sacandé *et al*., 2004; Legesse Negash, 2002); and there are seedling survival problems (Paul *et al*., 2004).

Micropropagation and other *in vitro* techniques are important to reduce the extinction rate of such plants (George and Sherrington, 1984 cited in Fay, 1992). The conclusion of electronic forum by FAO in 2000 indicates that tissue culture techniques are suitable for special situations; such as the conservation and management of *P. africana (*Ruane and Zimmermann, 2001).

Micropropagation is also promising to mitigate the extinction problem of the tree by giving alternative population for commercial or household consumption, saving the natural stand from further damage. If further investigations on Ethiopian provenances reveal superior genotype for the medicinal and the subsequent commercial value, Ethiopia can be benefited from *Prunus* export as other African countries do. If this becomes feasible, micropropagation will provide clonally propagated populations for the commercial purpose. The same application can be extended to the timber production which in turn saves the natural *Prunus* stand in undisturbed ecosystem.

3. OBJECTIVES

3.1 General Objective

The general objective of this research was to develop micropropagation protocol for *P. africana* from seedling.

3.2 Specific Objectives

The specific objectives are:

- to optimize germination in aseptic condition
- to optimize combinations of growth regulators for culture initiation and shoot multiplication
- to examine the rooting response of shoots at different IBA concentrations
- to harden the plantlets in glasshouse and observe the survival

4. MATERIALS AND METHODS

All laboratory experiments were conducted in Plant Physiology and Plant Tissue Culture Laboratory of Biology department, Addis Ababa University. The greenhouse trial was also cariied out in the greenhouse of AAU, Science Faculty.

4.1 Media

4.1.1 Stock solution preparation

Murashige and Skoog medium was entirely used in this experiment. Media were prepared in stock solutions i.e., macronutrient, micronutrient and organic supplements. Recommended amounts of components to, in weight per liter (Appendix 1) were measured; dissolved in double distilled water; poured into labeled plastic bottles; and stored at - 5 ºC until used.

The stock solutions of growth regulators were prepared in 1 mg/ml concentration in such a way that 50 mg crystal is dissolved in 50 ml total volume. Indole-3-butryric acid (IBA) was the auxin used in the experiment, and 50 mg crystal was first dissolved by three to four drops of 1M NaOH. The completely dissolved solution was poured into labeled volumetric flask, filled with double distilled water to the volume mark, and stirred with magnetic stirrer. The cytokinin, 6-Benzyl aminopurine (BAP), was prepared in a similar way to that of auxin. The IBA stock solution was poured into 5 ml test tubes while the BAP is placed in a bottle. The growth regulators were stored at +4 ºC for immediate use and at –5ºC for short time storage (a week).

4.1.2 Culture media composition and condition

The culture media for shoot initiation and multiplication consisted of MS basal medium in a full strength i.e., containing macronutrient, micronutrient, vitamins and 3% (w/v) sucrose with or without combinations of growth regulators. But for rooting, half strength MS medium was used. The pH of all media used was adjusted to 5.5 using 0.1 M HCl or 0.1 M NaOH. Agar (0.7%) and activated charcoal (0.1%) was also added in culture initiation media.

The media in flasks were covered with aluminum foil and heated on the hot plate in the presence of magnetic stirrer. For seed germination, nutrient free agar medium and growth regulator free MS medium with out activated charcoal were autoclaved at 121^{o}C for 15 minutes. The media were then taken out, poured into plates, and allowed to cool down in the laminar air flow cabinet. For culture initiation, multiplication and rooting, 50 mm diameter Baby food jars filled with 40 ml of the media, or Magenta G_7 culture vessels filled with 60 ml of the media were autoclaved at 121 ^{o}C for 15 minutes and allowed to cool in laminar air flow cabinet.

4.2 Establishment of aseptic seedling

The seeds of *P. africana*, collected in the middle of May, 2007 from *Menagesha* (20 km west of Addis Ababa) were kindly provided by Forestry Research Center (FRC) of Ethiopia in June, 2008. As the report of FRC, the seeds had 75 % moisture content, 93 % purity, and 60 % mean germination at the time of collection. The packs of seeds were stored both in FRC and Plant physiology and Tissue Culture Laboratory at +4 ^{0}C until used.

The following treatments were carried out to get seedlings free from contaminants relatively in a short period of time. Decontamination was applied on whole seeds and seeds with out seed coat (propagula). Removal of seed coat was done based on work of Legesse Negash (2004. This was done by cutting the coat using scissors carefully without damaging the embryo and the cotyledon. Whole seeds were also used incase the naked embryo or cotyledon might be uncontrollably damaged by toxic effect of disinfectants. Both whole seeds and propagula were washed with OMO, and cleaned by running tap water. The seeds were then placed in 70% ethanol for two minutes, and directly subjected to chemical treatment as described below.

The whole seeds were treaded with 5% sodium hypochlorite (NaOCl) or 10% calcium hypochlorite ($CaOCl_2$) for 15 minutes, rinsed four times in sterile double distilled water, and sawn on growth regulator free MS media in Petri plates. The Petri plates were then sealed by tape, labeled, and placed on growth chamber at 25 $\pm$ 2 ^{0}C, at 12 hrs photoperiod. NaOH was used only at 5% because this concentration was available and dilution was difficult or impossible because of its chemical nature. Fifty seeds treated with 5% NaOCl were decoated in aseptic condition before sawing. For all treatments, ten seeds per plate and 5 plates for each treatment were used. The propagula were sterilized by 5% NaOCl, 10%, 12% or 15% $CaOCl_2$ each for 15 minutes following the OMO and ethanol common pretreatments, sown on growth regulator free MS basal medium, sealed and placed on the similar growth chamber. Disinfection was repeated en masse using the best optimized procedure (washing propagula with OMO, placing in 70% ethanol for 2' , treating with 12% $CaOCl_2$ for 15'), and disinfected propagula were sown on nutrient free agar medium to be economic.

4.3 Culture initiation

The shoot tips of 20 to 25 day old seedlings, grown free from contaminants, were used as explants. The ability of explants to be well initiated was compared among ten combinations of BAP and IBA (Table 1) and the control (without growth regulators) . Activated charcoal, agar, MS basal medium compositions and growth conditions were the same for all treatments. Eight jars, each with four explants, were used for each treatment. They were properly sealed, labeled, and placed on the growth chamber with average photoperiod of 12 hrs using cool white fluorescent light (40 $\mu molm^{-2}s^{-1}$) at a temperature of 25 $\pm$ 2 ºC. Every change in growth was observed and recorded.

4.4 Shoot multiplication

Before the shoot multiplication experiment was carried out, the need of activated charcoal was assessed. For this, healthy and young shoots that induced microleaves in culture initiation were transferred to multiplication media containing 0.1, 1.0 and 5.0 mg/l BAP in combination with 0.1 and 1 mg/l IBA without activated charcoal. Fifteen well initiated shoots were used per treatment, five explants in each jar. The jars were labeled, sealed and placed randomly on the growth chamber with the similar photoperiod and temperature as that of initiation. The browning on the media and response of explants were observed.

Based on the above need assessment, activated charcoal was not used in the multiplication medium. The brown distal ends of en masse initiated shoots were cut off and shoot tips were transferred to the 17 shoot multiplication media compositions (Table 1). Thirty shoots were taken for each treatment in 6 jars, five shots per explant.

The cultures were labeled, sealed and randomly placed on the growth chamber of the predefined photoperiod and temperature. Growth regulators free MS basal medium was used as a control to test the response of explants to shoot multiplication.

Subculturing was done once by separating the microshoots and transferring to fresh media of the same compositions as the previous. The multiplied shoots were subcultured one times. Shooting experiment was repeated twice.

4.5 Rooting and acclimatization

About 5 cm long micro shoots were transferred to half strength MS medium with 0.0, 0.1 and 1.0 mg/l IBA either in combination with 0 and 1 g/l activated charcoal (Table 1). The media pH and other growth condition were as stated above.

After twenty days, plantlets with well developed shoots and roots were washed gently under running tap water and transferred to pots containing compost, red soil and sand with a ratio of 1:2:1. Pots with transferred plantlets were covered with thin transparent polythene plastic to ensure high humidity. Then, they were transferred to green house of Plant Physiology (AAU) and watered every one or two days. Polythene plastics were removed partially after one week and completely after two weeks.

Table 1. Combinations of growth regulators for culture initiation, multiplication, and rooting media

Culture initiation Media			**Multiplication media**			**Rooting media**		
Treat't	BAP	IBA	Treat't	BAP	IBA	Treat't	IBA	AC(g/l)
1	0.1	0.1	**1**	0.1	0.00	**1**	0.0	0
2	0.1	1.0	**2**	0.1	0.01	**2**	0.0	1.0
3	1.0	0.1	**3**	0.1	0.05	**3**	0.1	0
4	1.0	1.0	**4**	0.1	0.10	**4**	0.1	1.0
5	2.0	0.1	**5**	1.0	0.00	**5**	0.1	0
6	2.0	1.0	**6**	1.0	0.01	**6**	0.1	1.0
7	5.0	0.1	**7**	1.0	0.05			
8	5.0	1.0	**8**	1.0	0.10			
9	7.0	0.1	**9**	5.0	0.00			
10	7.0	1.0	**10**	5.0	0.01			
Control	0.0	0.0	**11**	5.0	0.05			
		2.0	**12**	5.0	0.10			
			13	10.0	0.00			
			14	10.0	0.01			
			15	10.0	0.05			
			16	10.0	0.10			
			Control					

Concentrations of BAP and IBA are in mg/l
AC (activated charcoal) is added in shoot initiation and rooting media

4.6 Data analysis

Percentage of germination, average number of shoots per explants, percentage of root formation and average number of roots per plantlet were calculated. Statistical data analysis was done by using SPSS 13 software. Data were subjected to analysis of variance (ANOVA) to detect the significance of differences among treatments at or below 0.05 probability level. Where appropriate, the means of different treatments were displayed and their homogeneity was analyzed using Duncan homogeneity test at 5% confidence interval.

5. RESULTS

5.1 Germination in aseptic condition

Whole seeds under all treatments were unable to germinate within these days, and were gradually contaminated by fungi and bacteria. Propagula disinfected by 5% NaOCl for 15 minutes failed to give germination (Fig. 2 D), but no germination. An attempt to remove seed coat after disinfecting with 5% NaOCl showed repeated bacterial and fungal contamination. Application of 10% $CaOCl_2$ for 15 minutes gave pure seedlings in a small size experiments (when the seeds in a disinfecting jar are less than 200). The same result could not be obtained when tried to repeat in large scale (300, 500), but rather fungal and bacterial contaminations (Fig. 2 C).

Propagula started germinating 13 days after sawing. After 25 days, 70% mean germination was obtained from the seeds treated by 12% and 15% $CaOCl_2$ for 15 minutes (Fig. 2 A & B).

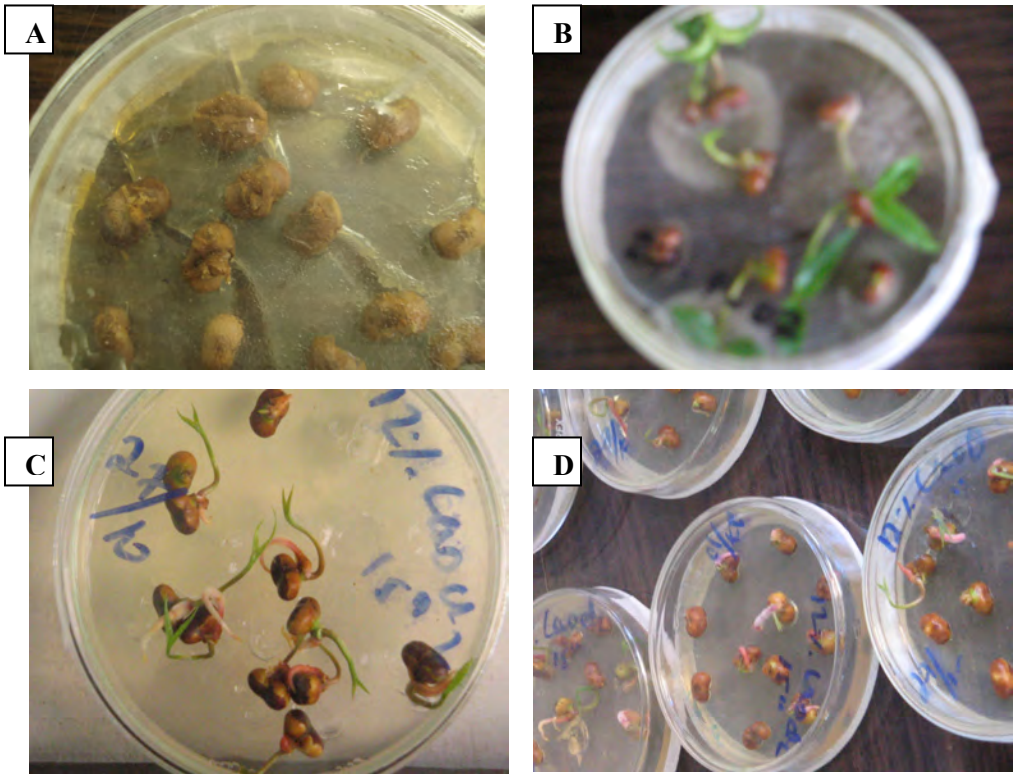

Figure 2. Germination of variously treated seeds after 20 days **A**) Propagula treated by 5% NaOCl for 15' **B)** Propagula treated with 10% $CaOCl_2$ for 15' when number of seeds treated in a jar exceed 200 **C**) Propagula treated with 12% $CaOCl_2$ for 15' **D)** En masse germination of propagula by 12% $CaOCl_2$ for 15'

5.2 Culture initiation

Healthy and young shoots with average 1.97 ± 0.71 microleaves per explant (Table 2) were obtained from cultures initiated on MS basal medium with 2 mg/l BAP and 0.1 mg/l IBA plus 1 g/l activated charcoal (Fig. 3 A).

Table1. Culture initiation in several combinations of BAP and IBA

Shoot initiation media			**Observation**		
Treat't	BAP	IBA	The previous leaves	Microleaves per explant	Rooting (%)
1	0.1	0.1	Matured, dry	1.0	40%
2	0.1	1.0	Brittle, small	1.1	40%
3	1.0	0.1	Mature, dry	-	16%
4	1.0	1.0	Brittle, pale, dying	-	9%
5	2.0	0.1	Healthy and young	1.97	-
6	2.0	1.0	Brittle, thick ,small	1.0	-
7	5.0	0.1	Either died / matured	-	13%
8	5.0	1.0	Small and callus like	-	16%
9	7.0	0.1	Complete death	-	-
10	7.0	1.0	Retarded growth	1.0	-
control	0	0	matured	1.0	20%

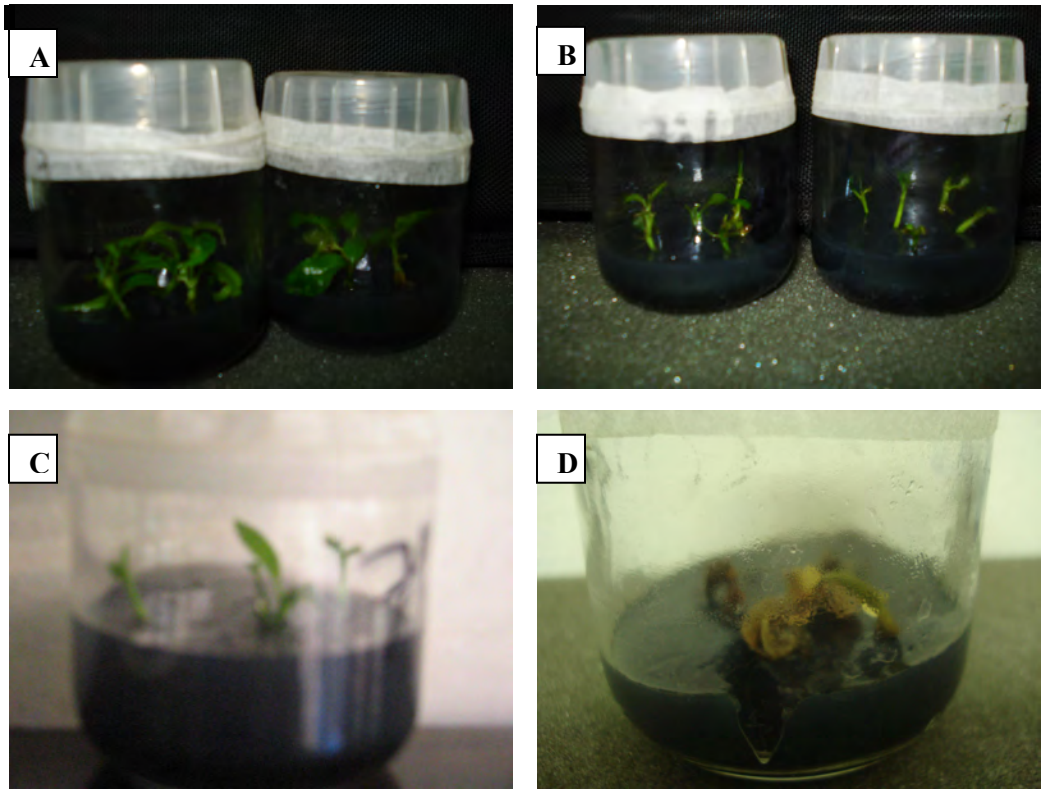

Figure 3. Culture initiation in different growth regulator combinations **A)** 2mg/l BAP and 0.1 mg/l IBA **B)** 7 mg/l BAP and 1mg/l IBA **C**) 0.1 mg/l BAP and 0.1 mg/l IBA and **D**) 7mg/l BAP and 0.1 mg/l IBA

The remaining media either lead to certain unwanted features like rooting, leaf maturity or retarded growth and death. Nine to forty spontaneous rooting percentages were obtained in response to media of several BAP and IBA combinations.

5.3 Shoot multiplication

Mild browning shown on the shoot bases did not introduced to the shoot multiplication medium. Browning at second, third and fourth weeks were minimal. It was not also serious enough to stop multiplication (Fig. 4).

Figure 4. Minimal browning observed in activated charcoal-free multiplication medium after four weeks

Responses of shoots to the different combinations of growth regulators were observed starting from a week after culturing, and the data taken at the fourth week was statistically analyzed (Appendix 2). The multiplication medium with 1 mg/l BAP devoid of IBA gave the best multiplication, 4.6 ± 0.29 shoots per explant after three to four weeks of the first culture (Fig. 6). Four weeks after subculturing, shoots gave higher number of shoots (4.8), but not significantly different at $P \leq 0.05$ when compared to the initial culture of the same combination.

Within four weeks, the bases of shoots produced small, fade or dark brown calli in 0.1 mg/l BAP combined with 0.01, 0.05 and 0.1 mg/l IBA (Fig. 5 C). Neither of these

shoots differed significantly from control in shoot ($P \leq 0.05$). One mg/l BAP in combination with 0.01, 0.05 and 0.1 mg/l IBA also gave calli at the shoot base, and multiplication was significantly lower than multiplication in 1 mg/l BAP without auxi ($P \leq 0.05$). The mean number of shoots produced per explant in these combinations was not significantly different among each other but significantly higher as compared to the control.

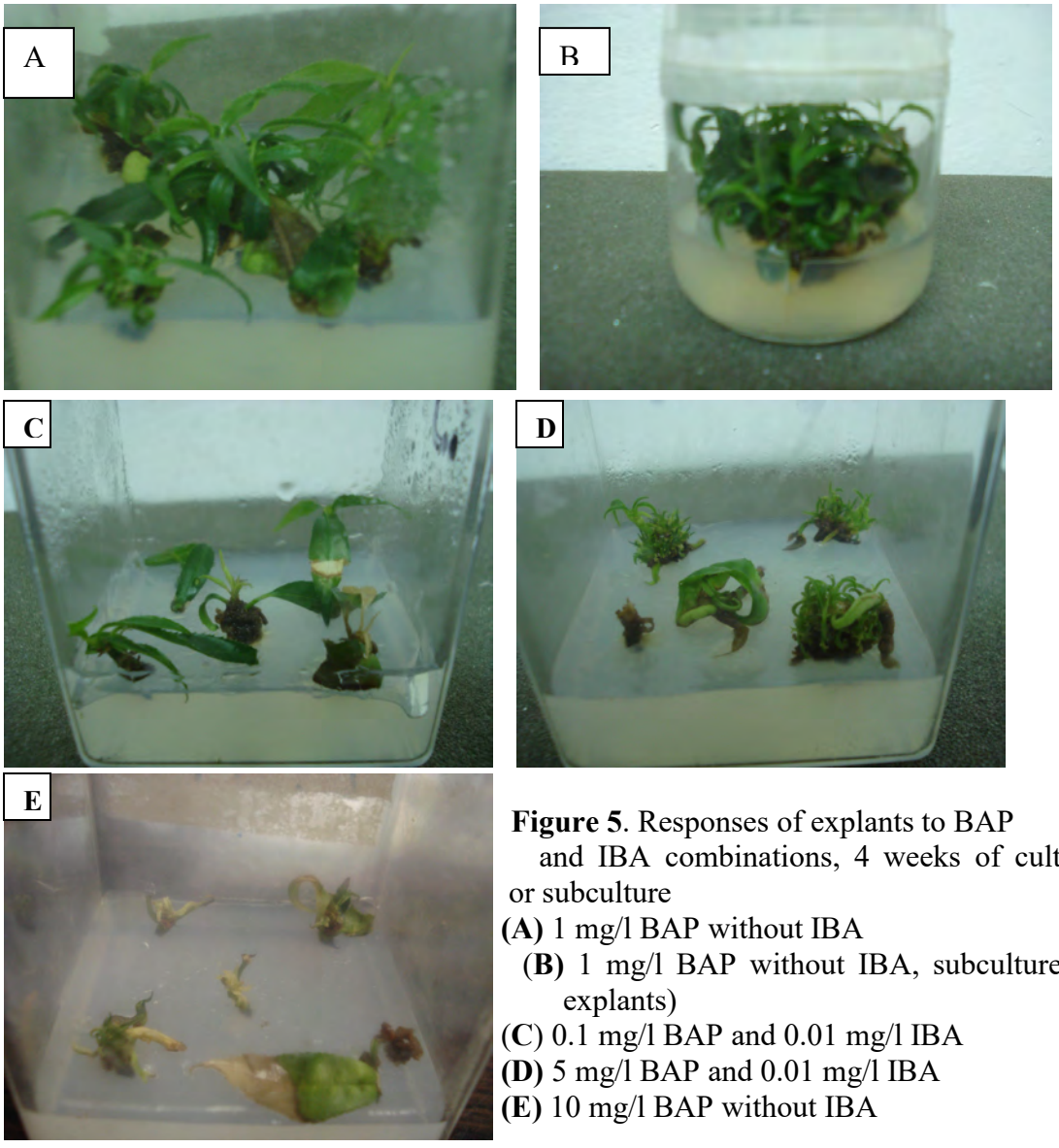

Figure 5. Responses of explants to BAP and IBA combinations, 4 weeks of culture or subculture
(A) 1 mg/l BAP without IBA
(B) 1 mg/l BAP without IBA, subculture (3 explants)
(C) 0.1 mg/l BAP and 0.01 mg/l IBA
(D) 5 mg/l BAP and 0.01 mg/l IBA
(E) 10 mg/l BAP without IBA

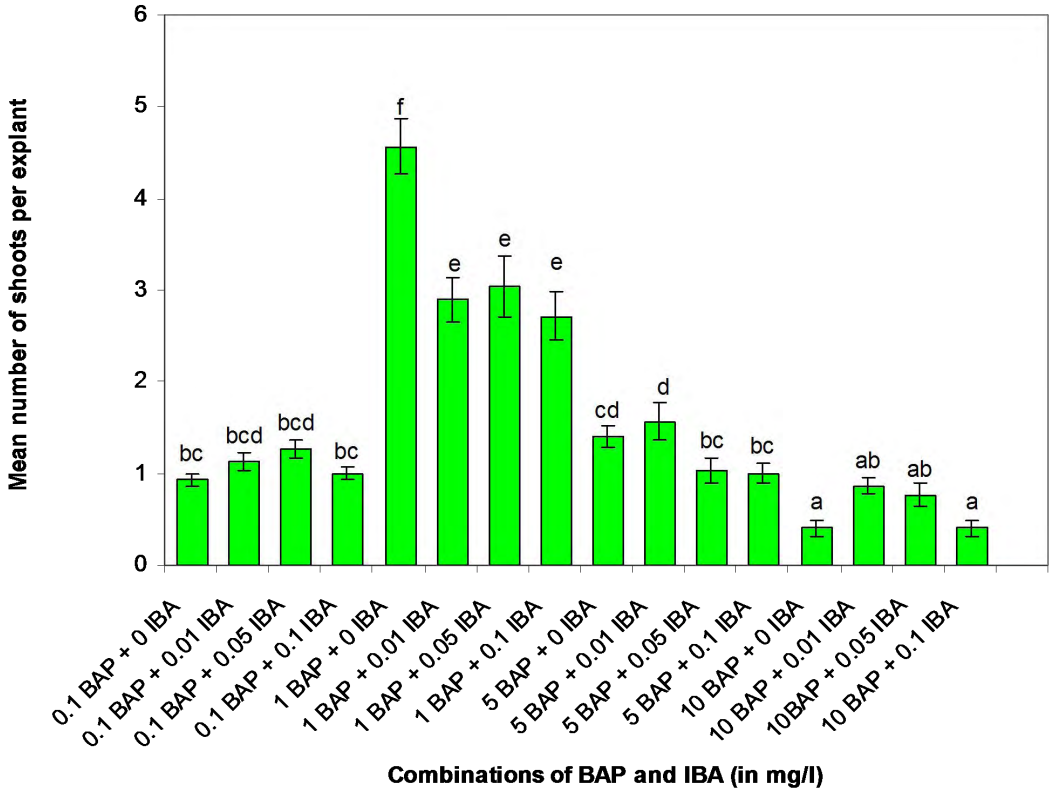

Figure 6. Effect of different concentrations and combinations of growth regulators on shoot multiplications

The medium with 5 mg/l BAP without IBA or with only 0.01mg/l IBA produced callus at the bases of the shoot which is subsequently regenerated to multiple dwarf shoots (Fig. 5 D). On the other hand, 10 mg/l BAP combinations resulted in death of shoots (Fig. 5 E) with 0 and 0.1 mg/l IBA. The multiplication of survived shoots was not significantly different from that of control ($P < 0.05$).

5.4 Rooting

Well developed shoots that were transferred to rooting media showed visually detectable variation in rooting potential with different IBA concentrations. The half strength MS medium without IBA and with 1% activated charcoal gave 60% rooting

(Fig. 7 and 8). The average root number per explant was 6 after two weeks and 11 after 3 weeks. The same growth regulator combination with out activated charcoal gave 20% rooting and 2 roots per explant. The 0.1 mg/l IBA gave 20% rooting, and no root was obtained by 1 mg/l IBA. Instead, the bases of shoots increased in diameter (callus), the second being wider than the first, and leaves became gradually pale.

Figure 7. Rooting in a half strength MS medium with out IBA (left and right), root hairs (center)

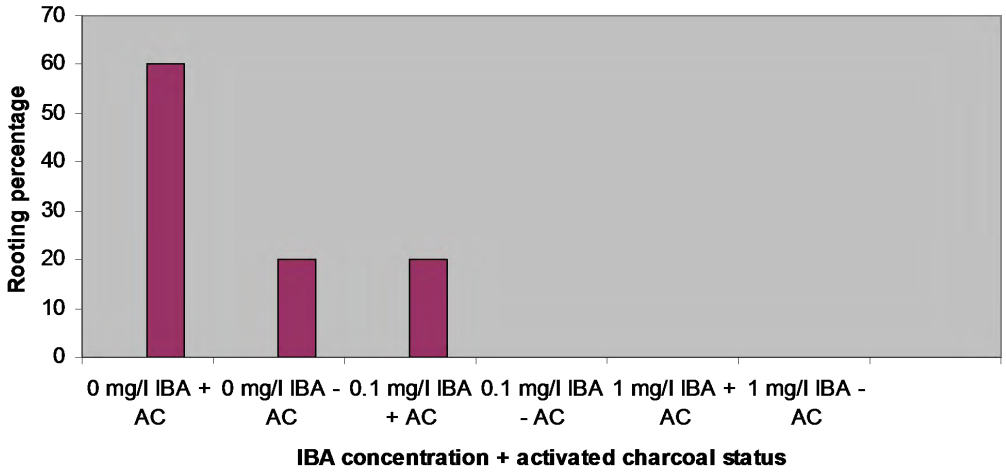

Figure 8 Effect of IBA on rooting of shoots

5.5 Acclimatization

After 3 weeks, all the plantlets transferred to the glasshouse were grown healthy, and survived (Fig. 9). Comparison to the wildings was possible, as the time was cotemporary to the *in vivo* germinating. They had normal leaf development and did not show any detectable differences in morphology.

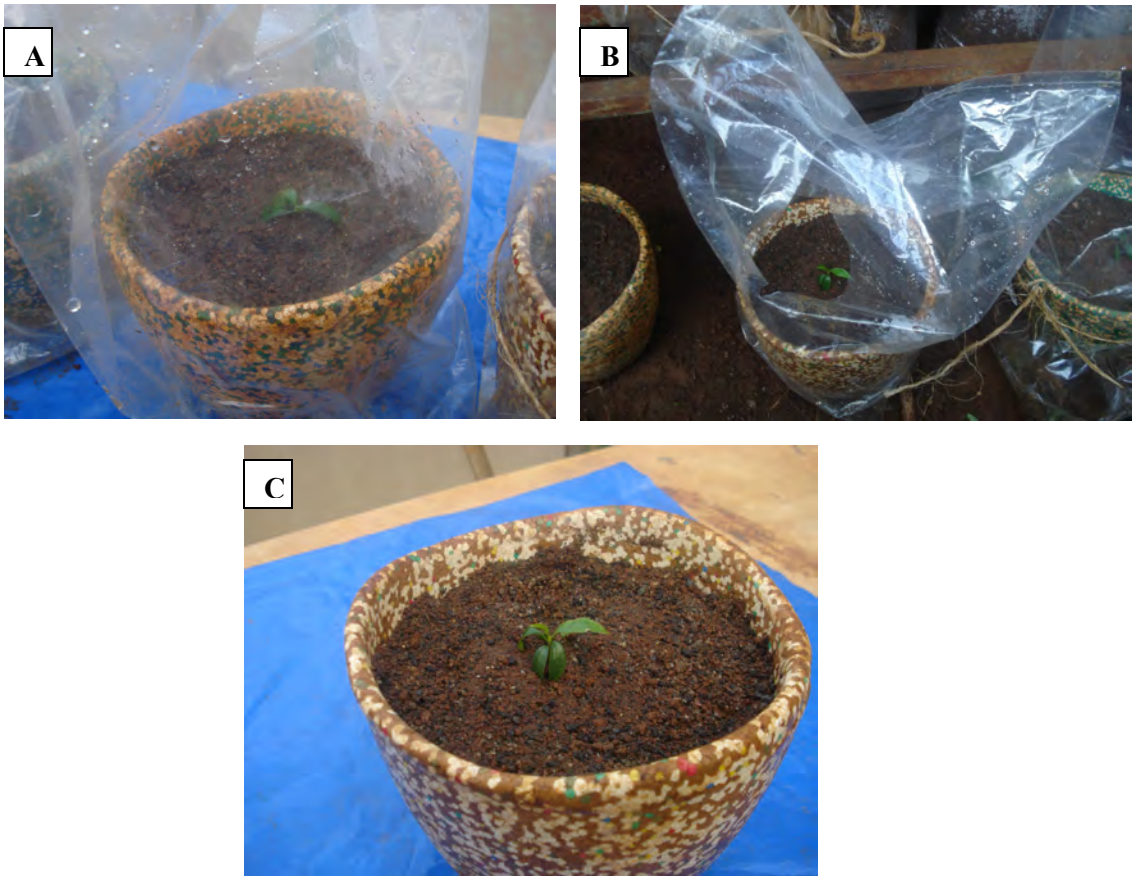

Figure 9. Micropropagated plantlets of *P. africana* acclimatized in a glasshouse (A) after one week of transfer into soil mix. (B) After two weeks (C) after three weeks

6. DISCUSSIONS

6.1 Germination in aseptic conditions

Seed pretreatments like washing by laboratory detergent and placing in 70% ethanol for 1 or 2 minutes are commonly used and recommended by Young (1919).

The result of disinfection is also consistent with the recommended concentration and exposure time of calcium hypochlorite. Disinfecting with 10% $CaOCl_2$ was found effective only for small scale trial but not for en masse sterilization. This may be due to higher contamination load and other volume related factors when the number of seeds is high. Sodium hypochlorite failed to enable germination at the applied concentration. This concentration might have killed the exposed embryo and/or cotyledon due to the corrosive effect of sodium hypochlorite (Ditommaso and Nurse, 2004).

Legesse Negash (2004) reported that 91% germination was obtained and start of germination time was shortened to 21 days by removing the seed coat. The germination percentage (70) in this experiment was not as high as the original work (91%). One of the factors that contribute for the difference is the intermediate nature of the seed, that is, its germination declines along time of storage (Legesse Negash, 2002, 2004). According to Dawson *et al.* (2004), only 1/3 of seeds can germinate after 12 months storage under conventional storage conditions. The seeds used in this experiment had been stored for about five months (until they were used in September, 2007). The germination decline over time has limiting power on seed based conventional propagation, and this underlines the importance of *in vitro* propagation if it is possible

to optimize protocols and get maximum number of plantlets within a short period of time.

The other factor that may, at least in part, contribute to the difference is the effect of the disinfectant that may damage on the exposed embryo and cotyledon. Calcium hypochlorite is among known disinfectants for their germination retarding and inhibiting effect on seeds of forest trees (Spaeth and Afanasiev, 1939). Provenance difference in germination vigor may also have its own contribution.

6.2 Shoot initiation

The best shoot initiation at the 2 mg/l BAP plus 0.1 mg/l IBA and 0.1% AC was due to the cumulative effect of several factors. The incorporated activated charcoal adsorbed the growth inhibitory phenolic compounds that are released from explants, which is common in tree species (Hartmann *et al.*, 2004). In this study, browning or severe blackening, which is the serious problem in culture establishment, is avoided. The activated charcoal, however, reduces or prevents shoot multiplication since it adsorbs growth regulators and lowers pH of the medium. Series of subculturing were not required to avoid the browning and other constraints. This might be because of the explant juvenility, at which the extent of browning is commonly low.

6.3 Shoot multiplication

The shoot multiplication did not require application of IBA to give maximum shoot multiplication. According to Hartman *et al.* (2004), auxin is either used in small concentration, or it is not completely added in shoot multiplication medium. The other reason may be due to the physiology of the explant (actively growing seedling) where

endogenous IAA is reported to be high (Savidge *et al*., 1982). The seedlings are therefore expected to produce more endogenous auxins by this stage. The probably produced endogenous auxin and the carry over effect of 0.1 mg/l IBA used in culture initiation medium may be sufficient to give the maximum shoot multiplication in combination with 1 mg/l BAP. The average number of shoots per explant was also consistent with results from micropropagation of other *Prunus* species like *P. padus* and *P. avium* (Chalupa, 1987).

The induction of small calli at bases the shoots with low concentrations of IBA in combination with upto 5 mg/l BAP may be due to the level of endogenous auxin that could not be balanced with the incorporated BAP to give multiple healthy shoots. The calli that was induced from media with 5 mg/l BAP plus 0.05 mg/l IBA gave dwarf shoots because of suppressing effect of BAP on apical growth. But the 0.1 mg/l auxin was found toxic in combination with extremely low or high level of BAP. Welander and Snygg (1987) and George (1993) reported that endogenous and exogenous auxins have synergetic effect to the optimum amount and become supra-optimal beyond certain level.

There was no browning in the multiplication media once the phenolic compounds released from the explants, the initiated shoots do not require use of activated charcoal in multiplication stage (Tileye Feyissa, 2005). The result from the need assessment of activated charcoal in this study was also consistent with this generalization in that activated charcoal free media with several combinations of growth regulators were not browned after one month. The shoot multiplication and viability were not affected in relation to absence of activated charcoal.

6.4 Rooting

MS basal medium with half strength is the recommended rooting medium in most micropropagation techniques (Chalupa, 1987; Hartman *et al*., 2004). Similarily, Fotopoulos and Sotiropoulos (2005) reported effect of rooting of hybrids between *Prunus persica* and *P. amygdalus*. George (1993) and Reddy *et al.*(2001) on the other hand reported that using activated charcoal enhances rooting. The present work also showed similar result in that the rooting percentage and number of roots per explant are similar to micropropagation works on most of hard woods in general and *Prunus* in particular (Chalupa, 1987).

However, maximum rooting without IBA is somewhat unusual in hard woods tissue culture. The 9 to 40% spontaneous rooting obtained in the presence of BAP (in multiplication media), and maximum shoot multiplication without IBA are supportive to each other and to the result of rooting trial. They require minimum or no applied auxin probably because of endogenous auxins production in a sufficient amount (habituation) (George, 1993). Research on apple revealed that IBA may not be required for rooting if the endogenous auxin is sufficient to do so (Welander and Snygg, 1987). Worth mentioning, Chalupa (1987) indicated that microshhots of hardwoods can also give root without auxin treatment *in vivo*.

6.5 Acclimatization

The higher glasshouse survival rate was similar with that of *P. avium* and *P. padus* (Chalupa, 1987). During acclimatization, broadening of leaves and height increment of plantlets were consistent with that of Kadleček (1997) cited in Pospíšilová (1999). The high stomata closure in response to abiotic factor as inferred from *P. cerasifera* (Ehrh. L.) (Zacchini and Morini, 1998), and *P. serotina* (Ehrk. L.) (Drew *et al*., 1992) indicates that the *Prunus* species have no problem in relation to water loss.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Based on the results of this study, the following conclusions are drawn

- To get contamination-free seedlings, washing the propagula by OMO, placing in 70% ethanol for 2 minutes, and then applying 12 % $CaOCl_2$ for 15 minutes is effective
- Two mg/l BAP, 0.1 mg/l IBA and 1 g/l activated charcoal in a full strength MS medium at 5.5 pH gives best shoot initiation.
- One mg/l BAP without IBA and activated charcoal is the best growth regulator for multiplication in the same medium composition
- Half strength MS medium with 0.1% activated charcoal, with no applied auxin, gives better rooting.

7.2 Recommendation

There are various activities that have to be done in order to propagate *P. africana* by tissue culture techniques, and to asses its applicability in conservation practices. Therefore, future works should include:

- Evaluation of multiplication rate at different subcultures
- Trial and application of other commonly recommended techniques for further improvement in rooting success, and for enhanced field establishment
- Characterization of Ethiopian *Prunus* provenances in relation to their and commercial value, and their response to micropropagation
- Study on the genetic stability of micropropagated shoots after the several subcultures by applying molecular techniques

8. REFERENCES

Aboel – Nil, M. (1987). Tissue culture of Douglas-fir and Western North American conifers. **In**: *Cell and Tissue Culture in Forestry*, Volume 3, pp. 80 -101 (Bonga, J.M. and Durzan, DJ. eds). Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Ahmed Z., Akhter, M., Haque, S., Banu, H., Rahman, M. and Faruquzzaman, A. (2001). Novel Micropropagation. *Online J. Biol. Sci*. **1**(11): 1106- 1111.

Badeg Bishaw (2001). Deforestation and Land Degradation in the Ethiopian Highlands: A Strategy for physical Recovery. *Northeast African Studies* **8**: 17-25.

Balakrishnamoorthy, G., Jothi, L.J. and Murugesan, R. (2003). Effect of media and auxins of *in vitro* rooting in gerbera. *Madras Agri. J.* **90** (7-9): 511-514.

Chalupa, V. (1987). European hard woods, **In**: *Cell and Tissue Culture in Forestry*, Volume 3, pp. 80 - 101 (Bonga, J.M. and Durzan, D.J. eds). Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (2008 appendices). http://www.cites.org/eng/app/E-Jul01.pdf

Cunningham, A. B. and Mbenkum, F.T. (1993). Sustainability of harvesting *Prunus africana* bark in Cameroon – A medicinal plant in international trade. People and Plant working paper. May 1993, 32 PP.

Dawson, I.K., and Powell, W. (1999). Genetic variation in the Afromontane tree, *Prunus africana*, an endangered medicinal species. *Molecular Ecology* **8**: 151 – 156.

Dawson, I.K., Were, J. and Lengkeek, A. (2000). Conservation of Prunus africana, an over-exploited African medicinal tree. *For. Genetic resources* No. 28. Rome, Italy, 62 pp.

Drew, A.P., Kavanagh, K.L. and Maynard, C.A. (2006). Acclimatizing micropropagated black cherry by comparison with half-sib seedlings**.** *Physiol. Plant.* **86**: 459 – 464.

Ditommaso, A. and Nurse, R.E. (2004). Impact of sodium hypochlorite concentration and exposure period on germination and radicle elongation of three annual weed species. *Seed Science and Technology* **32**(2): 377-391.

Ewusi, B. N., Asanga, C.A., Ebai, E. S. and Nkongo, J.B. (1992). An evaluation of the quantity and distribution of *Pygeum africanum* on the slopes of Mount Cameroon. Report for Plantecam-Medicam, Douala, Cameroon.

Ewusi, B.N., Tako, C.T., Nyambi, J. and Acworth, J. (1997). Bark extraction: the current situation of the sustainable cropping of *Prunus africana* on Mount Cameroon. **In***: A strategy for the conservation of Prunus africana on Mount Cameroon*. Technical papers and workshop proceedings, pp. 39-54, (Davies, G. ed). Limbe Botanic Garden, Limbe.

Eyog, M., Kigomo, B. and Boffa, J.M. (2001). Recent research and development in forest genetic resources. Proceedings of the international workshop on the conservation and sustainable use of forest genetic resources in Eastern and Southern Africa, 6 - 11 December 1999, Nairobi, Kenya, pp 121 - 127.

FAO (1997). Sate of the World's Forests. Words and publications, Oxford, UK. http://www.fao.org/docrep/W4345E/W4345E00.htm

Farwig N., Böhning-Gaese, K. and Bleher, B. (2006). Enhanced seed dispersal of *Prunus africana* in fragmented and disturbed forests? *Oecologia* **147**(2): 238-252.

Farwig, N., Bleher, B., Von der Gonna, S. and Bohning – Gaese, K. (2008). Does forest fragmentation and selective logging affect seed predators and seed predation rates of *Prunus africana* (Rosaceae)? *Biotropica* **40** (2): 218–224.

Fay, M. F. (1992). Practical Considerations in the development of a botanic garden Micropropagation Laboratory. The Proceedings of the Third International Botanic Gardens Conservation Congress, 25th October, 1992, Rio de Janeiro, Brazil, 10 pp.

Fotopoulos, S. and Sotiropoulos, T.E. (2005). *In vitro* rooting of PR 204/84 rootstock (*Prunus persica* x *P. amygdalus*) as influenced by mineral concentration of the culture medium and exposure to darkness for a period. *Agronomy Research* **3**(1): 3–8.

George, E.F. (1993). Plant Propagation by Tissue Culture: the Technology, Part One. 2nd eds. Exegetics Ltd, Edington, England, 355 pp.

Hartmann, H. T., Kaster, D. E., Davies, F.T. and Geneve, R.L. (2004). Plant propagation: Principles and Practices. 6th ed. Prentice Hall of India Private Limited, New Delhi, India, 770 pp.

Hedberg, O. (1989). Rosaceae. **In**: *Flora of Ethiopia,* Volume 3, Pittosporaceae to Araliaceae, pp. 31 – 44 (Hedberg, I. and Edwards, S. eds). Addis Ababa, Ethiopia, Uppsala, Sweden.

Kakudidi, E. K. (2007). A study of plant materials used for house construction around Kibale National Park, western Uganda. *Afr. J. Ecol*., **45**: 22–27.

Legesse Negash (1995). Indigenous Trees of Ethiopia: Biology, Uses and Propagation Techniques. SLU reprocentralen, Umea, Sweden, 285 pp.

Legesse Negash (2002). Review of research advances in some selected African trees with special reference to Ethiopia. *Ethiop. J. Biol. Sci.* **1**: 181 – 126.

Legesse Negash (2004). Rapid seed based propagation method for the threatened African cherry (*Prunus africana*). *New Forests* **27**: 215 – 227.

Mersha Gebrehiwot (2007). Gender Mainstreaming in Africa, Ethiopia. FAO, 46 pp.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiol. Plant.* **15**: 473-497.

Page, B. (2003). The political ecology of *Prunus africana* in Cameroon. *Area* **35**(4): 357–370.

Paul, J. R., Randlel, A. M., Chapman, C. A. and Chapman, L. J. (2004). Arrested succession in logging gaps: is tree seedling growth and survival limiting? *Afr. J. Ecol*., **42:** 245–251.

Pospíšilová, J., Ticha, I, Kadleček, P., Haisel, D. and Plzáková (1999). Acclimatization of micropropagated plants to ex vitro conditions. *Biologia Plantarum* **42** (4): 481- 497.

Reddy, B. O., Giridhar, P. and Ravishankar, G. A. (2001). *In vitro* rooting of *Decalepis hamiltonii* Wight & Arn., an endangered shrub, by auxins and root - promoting agents. *Curr. Sci*. **81**(11) 1479 – 1481.

Ruane, J. and Zimmermann, M. (2001). Agricultural Biotechnology for Developing Countries - Results of an Electronic Forum. FAO Research and Technology Papers series 8, http://www.fao.org/DOCREP/004/Y2729E/Y2729E00.HTM

Sacandé, M., Pritchard, H.W. and Dudley, A.E. (2004). Germination and storage characteristics of *Prunus africana* seeds. *New Forests* **27**(3): 239-250.

Savidge, A., Heald, J.K. and Wareing, P.F. (1982). Non-uniform distribution and seasonal variation of endogenous indol-3yl-acetic acid in the cambial region of *Pinus contorta* Dougl. *Planta* **155** (1):89-92.

Singh, R.J. (2003). Plant Cytogenetics. 2nd eds. CRC Press, 2000 N.W. Corporate Blvd., Boca Raton, Florida, 488 pp.

Spaeth, N. J. and Afanasiev, M. (1939). The effect of sterilization with calcium hypochlorite on germination of certain seeds *J. For.,* **37**(5):371-372.

Tesfaye Wubet, Weiß, M., Kottke, I., Demel Teketay and Oberwinkler, F. (2003). Molecular diversity of arbuscular mycorrhizal fungi in *Prunus africana*, an endangered medicinal tree species in dry Afromontane forests of Ethiopia *New Phytologist* **161**: 517–528.

Tileye Feyissa, Welander, M. and Legesse Negash (2005). Micropropagation of *Hagenia abyssinica*: a multipurpose tree. *Plant Cell, Tissue and Organ Culture* **80**: 119 – 127.

Tileye Feyissa (2006). Micropropagation, transformation and genetic diversity of *Hagenia abyssinica* (Bruce) J.F. Gmel. Doctoral Thesis No. 4. Faculty of Landscape Planning, Horticulture and Agricultural Science, Sweden, 36 pp.

Welander, M. and Snygg, J.O. (1987). Effect of Applied and Endogenous Auxin on Callus and Root Formation of In Vitro Shoots of the Apple. *Ann.Bot* **59**: 439-443.

Were, J., Munjuga, M., Simons, A. J., Dawson, I. and Ruigu, S. (2001). Reproductive biology of the endangered medicinal *Prunus africana* tree in central Kenya *E. Afr. Agri. For. J.* **67**: 1-2.

Young, H. C. (1919). Seed disinfection for pure culture work. *Ann. Miss. Bot. Gar.* **6**(2):147-158.

Zacchini, M. and Morini, S. (1998). Stomatal functioning in relation to leaf age in *in vitro*-grown plum shoots. *Plant Cell Reports* **18** (3):291 – 296.

Appendix 1. Compositions of stock solutions for MS basal medium

Components	Concentration (g/l)
Macronutrients	
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7
Micronutrients	
Fe-Na-EDTA	4.0
$ZnSO_4.7H_2O$	0.86
H_3BO_3	0.62
$MnSO_4.H_2O$	2.23
$CuSO_4.5H_2O$	0.0025
KI	0.083
$Na_2MoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.0025
Vitamins and other organic supplements	
Myo-inositol	1.0
Glycin (glycocoll)	0.2
Nicotinic acid	0.05
Pyridoxin (B6)	0.05
Thiamin (B1)	0.01

Appendix 2. Mean number of shoots per explant produced by different combinations of BAP and IBA

Treat's	Combinations of BAP and IBA in mg/l		Average shoot number per explant
	BAP	IBA	
1	0.1	0.00	0.9 ± 0.07^{bc}
2	0.1	0.01	1.1 ± 0.09^{bcd}
3	0.1	0.05	1.3 ± 0.11^{bcd}
4	0.1	0.10	1.0 ± 0.07^{bc}
5	1.0	0.00	4.6 ± 0.29^{f}
6	1.0	0.01	2.9 ± 0.24^{e}
7	1.0	0.05	3.0 ± 0.33^{e}
8	1.0	0.10	2.7 ± 0.26^{e}
9	5.0	0.00	1.4 ± 0.12^{cd}
10	5.0	0.01	1.6 ± 0.20^{d}
11	5.0	0.05	1.0 ± 0.13^{bc}
12	5.0	0.10	1.0 ± 0.11^{bc}
13	10.0	0.00	0.4 ± 0.09^{a}
14	10.0	0.01	0.8 ± 0.09^{ab}
15	10.0	0.05	0.8 ± 0.12^{ab}
16	10.0	0.10	0.4 ± 0.09^{a}
17	Control		1.0 ± 0.00^{bc}

Concentrations are expressed in mg/l

Means followed by the same letters are not significantly different at $P \leq 0.05$

Appendix 3. ANOVA table for shoot multiplication

Growth regulator	No	Mean no of microshoots	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
0.1 BAP + 0 IBA	30	0.933333	0.365148	0.066667	0.796985	1.069682
0.1 BAP + 0.01 IBA	30	1.133333	0.507416	0.092641	0.943861	1.322806
0.1 BAP + 0.05 IBA	30	1.266667	0.583292	0.106494	1.048862	1.484472
0.1 BAP + 0.1 IBA	30	1	0.371391	0.067806	0.86132	1.13868
1 BAP + 0 IBA	30	4.566667	1.612095	0.294327	3.9647	5.168633
1 BAP + 0.01 IBA	30	2.9	1.322224	0.241404	2.406274	3.393726
1 BAP + 0.05 IBA	29	3.034483	1.76236	0.327262	2.364117	3.704848
1 BAP + 0.1 IBA	31	2.709677	1.465004	0.263122	2.17231	3.247045
5 BAP + 0 IBA	30	1.4	0.674665	0.123176	1.148076	1.651924
5 BAP + 0.01 IBA	30	1.566667	1.104328	0.201622	1.154304	1.97903
5 BAP + 0.05 IBA	30	1.033333	0.718395	0.13116	0.76508	1.301587
5 BAP + 0.1 IBA	30	1	0.58722	0.107211	0.780728	1.219272
10 BAP + 0 IBA	30	0.4	0.498273	0.090972	0.213942	0.586058
10 BAP + 0.01 IBA	30	0.866667	0.507416	0.092641	0.677194	1.056139
10BAP + 0.05 IBA	30	0.766667	0.678911	0.123952	0.513157	1.020176
10 BAP + 0.1 IBA	30	0.4	0.498273	0.090972	0.213942	0.586058
Control (No BAP + IBA	30	1	0.232091	0.011120	0.825201	1.174799
Total	480	1.560417	1.446505	0.066024	1.430685	1.690148

Declaration

I, the undersigned, declare that this Thesis is my original work and has not been presented for a degree in any other University. All sources of materials used for the Thesis have been duly acknowledged.

Name: Shiferaw Abate

Signature: _____

This Thesis has been submitted for examination with our approval as University advisors.

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Legesse Negash (Professor) Tileye Feyissa (PhD)

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