STUDIES ON PROPAGATION BIOLOGY OF FOUR AND PHENOLOGY OF ONE MEDICINAL PLANTS

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ABREVATIONS

AAU	: Addis Ababa University
B5	: Gamborg Medium
BA	: Benzyladenine
BAP	: BenzylaminoPurine
IAA	: Indoleacetic acid
IPK	: Institute of Plant Genetics and Crop Plant Research
JU	: Jimma University
MPs	: Medicinal plants
MS	: Murashige and Skoog (1962)
NAA	: Naphtaleneacetic acid
SPSS	: Software Programme for Social Sciences
TDZ	: Tidiazuron
EHNRI	: Ethiopian Health and Nutrition Research Institute
GA	: gibberellins
IBA	: indole-3 butyric acid

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ABSTRACT

Studies on the phenology of *Plumbago zeylanica* L.*(*Plumbaginaceae*),* and propagation biology of other four highly threatened medicinal plants, namely *Echinops kebericho* Mesfin (Astraceae)*, Glinus lotoides* L. *(*Mol luginaceae) *Securidaca longepedunculata* Fres. *(*Polygalaceae) *and Taverniera abyssinica* L. (Leguminosae) were conducted with a view to conserving and developing these threatened medicinal plants. The objectives of this research were (1) to study the reproductive biology of *P. zeylanica;* and (2) to develop (i) seed-based propagation methods of *E. kebericho*, *G. lotoides* and *S. longepedunculata*, (ii) vegetative propagation by stem cuttings of *G. lotoides*, and (iii) *in vitro* regeneration of *T. abyssinica*.

Mature seeds were used as the starting plant material for all the studied species. Different parameters affecting seed germination and seedling establishment were studied for *E. kebericho*, *G. lotoides* and *S. longepedunculata*. Seedlings were only used for *T. abyssinica* and *G. lotoides* in tissue culture and vegetative propagation techniques, respectively. Studies on the phenology of *P. zeylanica* were conducted under glasshouse and nursery conditions using several parameters such as plant size, seasonal climate, and hormone application and mating system. A tissue culture protocol was tested from several explants, on two basic media and with hormone treatments for *T. abyssinica*.

Seeds of *E. kebericho* sterilized for 9 and 5 minutes in 70% ethanol and in 10% sodium hypochlorite, respectively, g erminated best (95.2 ± 1.2%) on Murashige and Skoog medium, supplemented with 10 g l^{-1} phytoagar. Further incre ases or decreases in sterilization time decreased percentage germination and increased contamination, respectively. Untreated seeds (control) were completely contaminated before the emergence of radicle as a result of fungi growth. Seed germination percentage declined with increasing storage time and dropped from 95 ± 0.4 % to 32.2 ± 1.2% in 15 months. Twenty-five degrees centigrade was an optimal temperature for best germination (94.6 ± 2.4%) of seeds compared to others. Seeds sown in pots containing a mixture of sand, nursery soil, and animal manure in a ratio of 0.5:2.5:0.5 respectively, germinated significantly ($p < 0.05$) compared to other soil ratios. Increases in sand or animal manure ratios decreased germination, while increases in nursery soil increased percentage and rate of germination. High percentage (96± 0.5%) germination was obtained with the seeds sown on nursery soil–surface mixed additives compared with the control. Seeds stored for less than 5 months, and at 25 ^{o}C, were the most suitable for *in vitro* and *ex vitro* germination of *E. kebericho*. Seedlings of nursery bed origin survived best compared

to those *in vitro* or pot origin seedlings.

Seeds of *G. lotoides* treated with water at 70° C for 10 to 30 minutes or GA_3 (10^{-3} and 10^{-4} M) did not show significant ($p<0.05$) difference in germination compared with the control. Seeds sown in pots containing a mixture of nursery soil, cattle dung, and sand in a ratio of 2:1:0.5, respectively, germinated best (91.6%) compared to other soil ratios, which showed rapid reduction in germination percentage with increases in cattle dung or sand. Seeds stored for 2 months gave best germination (93.7%) compared to ones stored for 5, 8 and 11 months, which showed decreases with increasing storage time. Apical stem cuttings gave the highest rooting percentages (90.2%), root number (8.02) and root length (6.18 cm) with or without hormone treatment than basal stem cuttings. In general, the number and length of roots decreased with applied indolebutyric acid (IBA) concentration. The highest rooting percentage (98.2%) was obtained in a rooting medium consisting of sand, nursery soil, and cattle dung, in equal proportions followed by 1.5:1:0.5 ratios of the same constituents. The percentage of survived rooted cuttings decreased with increasing age of stockplants from which the cuttings were derived. Rooted cuttings obtained without IBA treatment survived significantly ($P<0.05$). The study found that *G. lotoides* can effectively be propagated by both sexual and asexual means provided that germination media of specific are employed, and the apical cuttings derived from young seedlings are used for maximal rooting responses.

Seeds of *P. zeylanica* germinated best and vigorously grown (on a mixture of nursery soil and cattle dung in a ratio of 3:1 filled in pots (glasshouse) or on nursery bed-surface mixed cattle dung) as a prerequisite for vegetative and flowering phenological studies. Hypogeal germination characterizes the emergence of seedlings. Subsequent vegetative and flowering phenology between glasshouse and nursery seedlings showed significant difference ($p<0.05$) in terms of time, duration and yield. Glasshouse seedlings completed their phenophases (aseasonally) within 105 days while nursery seedlings extended to 225 days after seed sowing. Rainy season was the cause for the continuous damage of apical shoots, and consequently stunted vegetative growth of nursery seedlings. Plant size (≥ 95 cm in height), leaves number (33-38) and seasonal climate (wet season) were found to be the most signals for the initiation of flowering buds. Hundred ppm GA_3 was the most effective for early flowering (i.e., before 6 days) and production of higher number of flowers (32.6 ± 1.6%) compared to the control (22.5 ± 1.33%). The mode of reproductive biology appeared to be cross pollination and showed significance ($p<0.05$) compared to the control. The highest flowering (92.40 ± 0.52%) and/or seed-set (85.23 ± 3.55%) were obtained under glasshouse condition

compared to the nursery, which dropped as low as 50% in seed-set due to the damage of apical shoots during rainy and cold seasons, and differences of the adaptation of the species under both conditions.

In vitro and *ex vitro* seed germination, extent of seedling survival and establishment has been developed for *S. longepedunculata*. Seeds treated with gibberellic acid (10^{-3} M GA_3) germinated best ($\geq$94.6$\pm$1.32%) on Murashige and Skoog medium supplemented with 8 g 1^{-1} phytoagar or on the sterilized sand filled in a glass culture vessel. Seedlings germinated on MS survived best (81.6 ± 1.34%) and formed strong stems, multi-roots and many branchlets compared to the sand origin seedlings, which showed a high mortality rate upon transfer to glasshouse conditions due to hypocotyl elongation and a poorly developed root system. Seeds sown in pots containing a mixture of sand, nursery soil, animal manure in a ratio of (2:0.5:0.5, respectively) gave highest germination (94.6 ± 2.14%) compared to other ratios of soil mixture. Percentage germination decreased with decreases and increases of sand and nursery soil ratios, respectively. Only a poor germination (>50%) was obtained with seeds sown on the seedbed. Seeds stored at 25 ^{o}C germinated best compared to others and the seed viability was declined with storage time.

The best *in vitro* germination ((96 $\pm$ 0.6%) of *T. abyssinica* seeds and vigorous seedlings growth as a prerequisite for the development of tissue culture methods was obtained on Murashige and Skoog medium, supplemented with 12 g 1^{-1} phytoagar without sucrose. Light green compact calli from node, petiole and shoot meristem explants were efficiently induced on Gamborg medium containing 0.90 or 1.80 µM dichlorophenoxyacetic acid (2,4-D) combined with 2.22 µM 6-benzylaminopurine (BAP), and supplemented with 30 g 1^{-1} sucrose and 5 g 1^{-1} phytagel**.** Callus induction and plant regeneration has been established for dingetegna, *Taverniera abyssinica*. Light green compact calli from node, petiole and shoot meristem explants were efficiently induced on Gamborg medium containing 0.90 or 1.80 µM dichlorophenoxyacetic acid (2,4-D) combined with 2.22 µM 6-benzylaminopurine (BAP), and supplemented with 30 g 1^{-1} sucrose and 5 g 1^{-1} phytagel. Callus initiation from shoot meristems and nodes was faster and occurred with a higher frequency than callus initiation from petiole and leaf segments. A high frequency (100%) of shoot regeneration was obtained upon transfer of calli onto regeneration medium containing 8.88 µM BAP combined with 1.14 µM indoleacetic acid (IAA). Regenerated shoots were transferred to rooting medium, which turned out to be optimal when half strength B5 medium was supplemented with 9.84 µM indolebutyric acid (IBA). Upon transfer to glasshouse, 86% survived and grew vigorously.

The main results indicate that both *in vitro* and *ex vitro* seed-based can be used for the propagation of *E. kebericho, S. longepedunculata* and *G. lotoides* although *in vitro* seed culture of *G. lotoides* showed less germination even in the absence of contamination. *G. lotoides* was successfully propagated by stem cuttings without hormone treatments. Rainy season, plant size, leaves number, low temperature, cross pollination and glasshouse conditions were found to be the most determining factors for the phenology of *P. zeylanica*. Light green compact calli, high frequency of shoot regeneration and regenerated roots of *T. abyssinica* were successfully obtained and acclimatized upon transfer to glasshouse conditions. However, further studies on the development of tissue culture, genetic analysis and the ecological requirements are the next steps for the effective use of the propagation protocols developed by this study.

Key words: Ethnobotany Ethiopia, medicinal plants, seed germination, *in vitro* regeneration, reproductive biology, vegetative propagation.

1. INTRODUCTION

Plants have been used as medicines throughout history. According to the World Health Organization (WHO), approximately 85% of the world's population uses herbal medicines and the demand is still increasing in both developed and developing countries (Abramov, 1996; Rates, 2001). In developed countries this may be partly due to the dissatisfaction with conventional medicines while with the developing countries this is due to shortage of medical doctors, pharmaceutical products and their unaffordable prices (Farnsworth, 1990). This situation is supported by the fact that some 25% of drugs contain compounds obtained from higher plants (Farnsworth and Morris, 1976; Farnsworth, 1990). Moreover, the investigation of herbal drugs from plants to treat AIDS, cancer, and malaria, chronic complaints such as rheumatism, arthritis and asthma have been reported (Lee, 1998; Lee, 1999; Lee and Morris, 1999). Whatever the reasons, it can not be denied that herbal remedies are currently enjoying widespread popularity throughout the world (Sofowara, 1982; Viera and Skorupa, 1993; Lee, 1998; 1999; Lee and Morris, 1999; Afolayan and Adebola, 2004). However, still today, only 10% of medicinal plant species is cultivated, with by far the larger majority being obtained from wild collections. Harvesting from the wild, however, becomes problematic, as was seen in the case of many plant species become threatened, and leads to both loss of genetic diversity and habitat destruction (Canter *et al.,* 2005)..

It has been reported that the development of propagation methods is of major importance in medicinal plant biotechnology, due to improving agronomic and medicinal traits (Farnsworth and Morris, 1976; Saito, 1994). Thus, the domestic cultivation of medicinal plants is a well-accepted way in which to produce plant material both for conservation and treatment purposes. Moreover, such an approach helps to overcome other problems inherent in herbal extracts, such as the standardization of extracts, variability of the plant material, minimization of toxic constituents and contaminations, and increasing the content of the desired constituents (Rates, 2001; Canter *et al.,* 2005).

Ethiopia is one of the developing countries located in the horn of Africa with a total area of 1.13 million km^2 between the geographic coordinates 3^o and 15^o N_1 and 33^o and 48 ^{o}E. The altitude ranges from 125 m below sea level at the Dankil depression up to 4620 m at Mt. Ras Dejen. The landscape is characterized by plateaus, mountains, hills, deep gorges, rivers, incised valleys and sharp cliffs. The world's longest rift valley, about 6000 km, extends from Syria in the north passing through the Red sea to Mozambique in the south, thus, effectively dividing the country into two halves. Agriculture is the backbone of Ethiopian economy, about 85% of the nearly 77 million inhabitants depend on agriculture for their livelihood, which affects directly or indirectly the natural regeneration of medicinal plants. More than 85% of Ethiopian population relies on traditionally used medicinal plants (Dawit Abebe and Ahadu Ayehu, 1993).

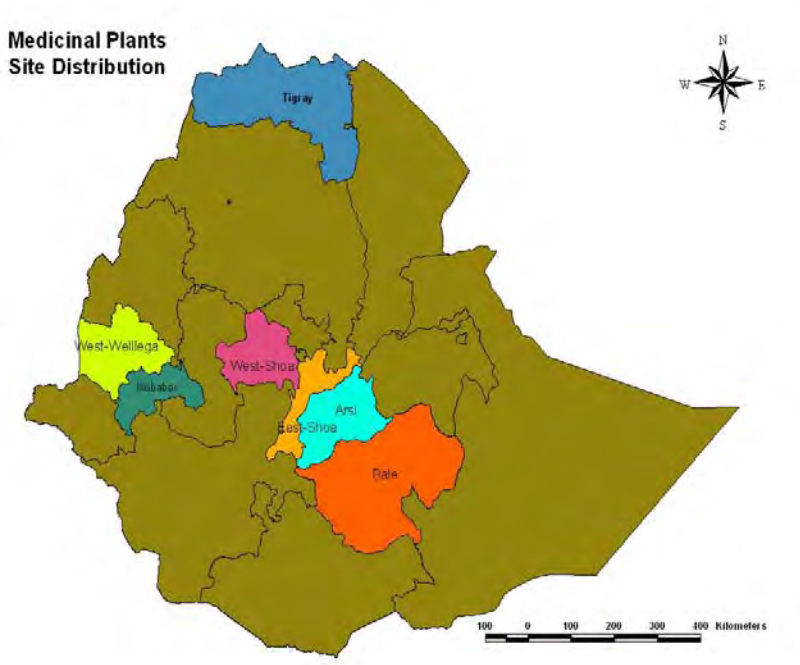


Figure 1 Studied medicinal plants distribution sites in Ethiopia: West wollega (*Securidaca longepedunculata* Fresen), Tigray (*Taverniera abyssinica* A Rich.), Bale *(Echinops kebericho* Mesfin), West Shoa (*Echinops kebericho* Mesfin), Illubabor (*Securidaca longepedunculata* Fresen), Arsi (*Glinus lotoides* L.), East Shoa (*Taverniera abyssinica* A Rich.)

Of the 1000 reported medicinal plant species (Endashew Bekele, 2007), most of those that were commonly used are becoming locally scarce for herbal preparations (McGuire, 1995; Dawit Abebe, 2001). For instance, *E. kebericho*, *P. zeylanica, T. abyssinica*, and *G. lotoides* are very scarce suggesting that these plants are indeed diminishing in almost all regions of Ethiopia (McGuire, 1995). *S. longepedunculata*, a plant well known for its' high methyl salicilate constituent is also one of the threatened medicinal plant species (McGuire, 1995).

The major reasons for the deterioration of these natural health resources for herbal preparations have occurred by a complex assemblage of natural, social and economic challenges: (1) uncontrolled use, not only to satisfy local needs but also to use it as a source of income; (2) decimation along with deforestation largely for agricultural purposes; 3)

environmental degradation, and 4) others including invasive alien species, fire, overgrazing, invasive alien species and urbanization appear to be the major threats to the medicinal plants of Ethiopia.

In order to reverse the scarcity of such valuable medicinal sources, it requires urgent and all-out actions for propagating, cultivating and domesticating medicinal plants species. This can only be achieved through rigorous studies on the phenology, seed polymorphism and physiology, and the development of propagation techniques. Studies on the phenology of *P. zeylanica* and development of propagation techniques of *E. kebericho* and *S. longepedunculata* (seed-based), *G. lotoides* (vegetative and seed-based) and *T. abyssinica* (*in vitro* regeneration) have been developed for the first time by this study.

1.1 Ecology, distribution, description, significances, and research status of studied medicinal plants

1.1.1 *Echinops kebericho* Mesfin (Asteraceae)

Ecology, distribution: - The 12 species of *E. kebericho* that occur in Ethiopia are confined to the highlands of the country between 7^{o} 30' N and 38^{o} 45' E and at altitudes between 1700 and 2900 m.a.s.l (Mesfin Tadesse and Berhanu Abegaz, 1990; Berhanu Erko, 2006) (Figure 1). *E. kebericho* grows in dry and stony lateritic soils. It is variable in habit and in dissection of the leaf blade. Populations from dry, stony lateritic soils are perennial herbs whereas those growing in deep vertisols are low shrubs (Mesfin Tadesse and Berhanu Abegaz, 1990; Berhanu Erko, 2006)

Description: - E. kebericho Mesfin belongs to the Asteraceae, commonly known as Kebericho. Taxonomically, the genus *Echinops* comprises 120 species, of which 12 are known to occur in Ethiopia. *E. kebericho* is an erect perennial herb or shrub, up to 1.2 m high, usually from a massive (C 30 – 50 x 10-20 cm) rootstock; stem-leafy throughout, sparsely arachnoid-pubscent, sometimes mixed with dark purple uniseriate hairs. Leaves elliptic, amplexicaul, basal ones up to 25 x 15 cm, pinnatisect with segments divided into triangular lobes all ending in sharp pungent spines or bipinnatisect with linear-lanceolate segments; upper surface arachnoid pubscent mainly along median vein, lower surface densely tomentose, dull, with uniseriate hairs along midvein. Flower head 3–4 in diameter, spineless peduncle up to 5.5 cm long, arachnoid, pubscent, 1-2mm wide. Capitalum 2-2.5 cm long. Outer phylarries 3–4 seriate, outer most row spathulate, 4–6mm long, with a few white, soft bristles along margins in the

lower half, middle row spathulate with apiculate apex, 6– 10 mm long, with several white, soft bristles on arraigns toward apex; inner most series lancelet, tapered at base and at apex, 9–14 mm long, 1mm wide, with bristles at margins in middle and toward tip; apex acute. Inner phyllaries oblong-linear, fused for up to ¾ of their length, fimbriate at apex. Corolla white or bright blue, C 12–15 mm long, tube glabrous except near base of limb where papules; anthers light brown, cypselas oblong-elliptic, 7.5–10.5 x 1.5–2.5mm covered with appressed silvery white ciliolate bristles pappus of numerous, continues, narrow, ciliolate scales that are connate at base.

Significance: - E. kebericho is a well-known medicinal plant to the majority of the rural population due to mainly its traditionally medicinal usages. Ethno-botanical surveys have documented that *E. kebericho* has long been traditionally employed by the local healers to prepare medicines against migraine, mental illness, heart pain, lung TB, leprosy, kidney disease, malaria, bilharzia, syphilis and amoebic dysentery (Dawit Abebe and Ahadu Ayehu, 1993; Belachew Desta, 1993; Balcha Abera, 2003). Rootstock of *E. kebericho* is one of the ten medicinal pants sold throughout the markets of the country, which is used as a source of income for a large number of local people and traditional healers (Kloos, 1976). Extracts such as sequiterpene lactones isolated from the rootstock have shown antitumor, antimutagenic and cytotoxic effects (Berhanu Abegaz, 1990). The bioactive extract of *E. kebericho* has been shown to have antimicrobial effects equal to or better than penicillin, especially against *Streptococcus beta-hemolytic, Escherichia coli, Klebsiela aerogenes* (Asfew Debela, 1993; Belachew Desta, 1993). Alkaloid extract of the roots of *E. kebericho* has been shown to have a very strong lethal activity against earthworm (Araya Hymete and Ayalew Kidane, 1991; Araya Hymete and Afiffi, 1996; Berhanu Erko, 2006). An enlarged rootstock of *E. kebericho* increases soil fertility, and also reduces soil erosion, especially when growing on mountainous slopes.

Research status: - The first report made by Kloos (1976) revealed the popularity of *E. kebericho* trade in all 19 markets of central Ethiopia. Thereafter, various ethno-botanical studies documented its' distribution, traditionally use of the species for various treatments, its medicinal parts and local methods of preparation. These studies were conducted at different times in various regions of the country (Belachew Desta, 1993; Dawit Abebe and Ahadu Ayehu, 1993; Balcha Abera, 2003). During 1990th various extracts have been isolated from the rootstock of *E. kebericho* and at the same time their effects have been investigated (Berhanu Abegaz, 1990; Araya Hymete and Ayalew Kidane, 1991; Araya Hymete and Afiffi, 1996). However, a treatment promoting seed-based propagation method for domestication and conservation purposes is developed for the first time by this study.

1.1.2 *Glinus lotoides* L. (Molluginaceae)

Ecology, distribution: - *G. lotoides* is distributed with impeded drainage sites, widespread in the tropics and subtropics. Its habitat is similar to that of various *Glinus* species, but tending to occur at higher altitudes, in East Africa it grows at 1600 m.a.s.l (Berhanu Abegaz and Berhane Tecle, 1980; Abebe Endale and Tsige GabreMariam, 1998*)* (Figure 1).

Description: - The genus *Glinus* belongs to Molluginaceae commonly known as Carpet weeds (Kloos, 1976; Abebe Endale *et al*., 1997; Abebe Endale and Tsige Gabre-Mariam, 1998). *G. lotoides* is a semierect to prostrate herb, most parts with stellate hairs. Stems upto 45 cm long. Leaves elliptic to obovate or suborbidular, 10–42 x 5–22 mm, base cuneate into petiole 1–11 mm long, tip subacute to rounded, margin sometimes ± wavy. Flowers 2–10 per node. Pediceles 1.5–4 mm long. Sepals enlarging to 5–8 x 2.5–4.5 mm in fruit, some times finged pink, staminodes 0 –9 + unear, deeply befid, inconspicuous, stamens 11–30. Capsules (3-15) locular. Seeds are small brown, bluntly papillate, 0.5–0.6mm long.

Significance: - Mature capsules of *G. lotoides* is harvested from the wild and used as a source of treatment for tapeworm infection throughout Ethiopia, mainly among rural population. The *in vitro* taenicidial activities of the extract of the seeds of *G. lotoides* on *Taenia saginata* and against *Hymenolepis nana* worms have been reported (Abebe Endale *et al*., 1997; Abebe Endale and Tsige GabreMariam, 1998). The cestocidal and pharmacological activities of the seeds of *G. lotoides* have been attributed to its saponin content (Abebe Endale *et al*., 2000). Triterpene glycoside with taenicidial properties has been isolated from the seeds of this plant (Birhanu Abegaz and Berhane Tecle, 1980).

Research status: - Despite its economic and medicinal importance, only a few studies have been carried out on *G. lotoides* and almost all researches have been focused on its medicinal properties and traditionally local uses (Mulatu Djote, 1978; Birhanu Abegaz and Berhane Tecle, 1980). *G. lotoides* was first documented as one of the medicinal plants traded in all 19 markets surveyed in central Ethiopia (Kloos, 1976). Although there are over 25 plant species in Ethiopia traditionally against tapeworm infection most of them are toxic (Birhanu Abegaz and Berhane Tecle, 1980). However, *G. lotoides* is claimed to have little or no side effect and it enjoys a considerable reputation as taenicide in the Ethiopian traditional medicine (Watt *et al*., 1962). The traditional mode of administration of *G.*

lotoides is to orally consume about 15-20 g of capsulated powdered seeds with some food to make it palatable (Mulatu Djote, 1978). This amount is believed to remove the tapeworms completely from the intestine. In the same year, preliminary pharmalogical studies on 'Meterie (local name), effects on gastrointestinal system and its taenicidal activity has been reported by Mulatu Djote (1978). A new triterpenoid glycoside from the seeds of *G. lotoides* has been reported by Berhanu Abegaz and Berhane Tecle (1980). Abebe Endale *et al*. (1997; 2000) have reported on *in vitro* activity of seed extracts on *Hymenolepis nana* worms, *in vivo* anthelminthic activity of the same extracts in albino mice infested with the same worm, and quantative determination of saponins in the extracts of seeds, respectively. Studies on the development of propagation techniques (seed-based and vegetative propagation by stem cuttings) have been reported by this study.

1.1.3 *Plumbago zeylanica* L. (Plumbaginaceae)

Ecology, distribution: - *Plumbago zeylanica* L. is an important medicinal plant native to South West Asia (Aditi *et al.,* 1999; Komaraiah *et al.*, 2003). In the recent decades, the plant is widely spread in tropical and sub tropical regions of Australia, Asia and Africa (Vijver and Looter, 1971), including Ethiopia (Figure 1). Nowdays, the plant is grown under the glasshouse of Science Faculty, Addis Ababa University (AAU) and at the medicinal plant garden of Ethiopian Health and Nutrition Research Institute (EHNRI), both located in Addis Ababa, Ethiopia.

Description: - *P. zeylanica* belongs to the Plumbaginaceae commonly known as leadwort. It is a much branched, evergreen shrub that reaches about 2 m height in nature, and grows more interwoven under the glasshouse conditions. Dark green leaves are ovate to 30 cm long by half as wide. The flowers are white in showy dense raceme. Individual flowers are up to ½ inch (a bit more than 1 cm).

Significance: - Traditionally, *P. zeylanica* has been used for the treatment of dermatological disorders including wounds, eczema, scabies, leishmaniasis and leprosy in Ethiopia (Dawit Abebe and Ahadu Ayehu, 1993).Though the root, root barks, and seeds of *P. zeylanica* are used medicinally, the root is the chief source of an acrid crystalline principle called plumbagin; a yellow naphtoquinone pigment, and also characteristic of plants in the tribe Plumbaginaceae including *Plumbago europea*, *P. rosea* (Aditi *et al*., 1999; Komaraiah *et al.,* 2003).

Research status: -Several studies have been reported on the development of propagation techniques ranging from

seed germination via vegetative by stem cuttings to plant tissue culture (Aditi *et al*., 1999). Ethno-botanical surveys documented that *P. zeylanica* is used locally for the treatment of dermatological disorders including wounds, eczema, scabies, leishmaniasis and leprosy in Ethiopia (Dawit Abebe and Ahadu Ayehu, 1993; Aditi *et al*., 1999). The chief source of an acrid crystalline principle called plumbagin; a yellow naphtoquinone pigment, has been extracted from different parts (root, root bark, seed) (Aditi *et al*., 1999; Komaraiah *et al*., 2003). However, the phenology of *P. zeylanica* has been reported for the first time by this study.

1.1.4 *Securidaca longepedunculata* Fresen (Polygalaceae)

Ecology, distribution: - Securidaca longepedunculata Fresen is a medicinal herb grows in many parts of Africa (Reinhard and Adimasu Adi, 1994). It occurs in semi arid and lowland savannah (most frequently between 500 and 1700 m.a.s.l.) from Ethiopia in the Southwest (Figure 1) to West Africa in Ghana, Senegal, Togo, Mali (Mouzou *et al.,* 1999; Georges *et al*., 2000; Jayasekara *et al*., 2000; Ancolio *et al*., 2002).

Description: - S. longepedunculata belongs to the Polygalaceae, commonly known as tree violet. It is a semi-deciduous shrub or small tree that grows to 12 m tall, with an often flattened or slightly fluted bole. It is spiny and much branched, with an open, rather straggly looking crown. Leaves alternate or clustered on dwarf, lateral branchlets, simple, variable in size and shape, broadly oblong to narrowly elliptic, 1-5 x 0.5-2 cm with very fine hairs when young but losing these by maturity; apex rounded; base narrowly tapering; margin entire; petiole slender, up to 5 mm long. Flowers rather small, about 10 mm long, pink to lilac or purple, sweetly scented, on long slender stalks produced in beautiful profusion in terminal axillary sprays 3-5 cm long, appearing with the very young leaves; bisexual; sepals 5, unequal, the lateral 2 being petalloid, large and winglike; petals 3, free, the medium petal hooked; stamens 8, joined to form a split tube. Fruit is more or less a round nut, somewhat heavily veined, occasionally smooth, bearing a single, oblong, rather curved, membranous wing up to 4 cm long; purplish-green when young, becoming pale, straw-coloured when mature. The hatchetlike appearance of the fruit is referred to in the generic name, *Securidaca* while the specific name, '*longepedunculata*', refers to the long, slender stalks of the flowers.

Significance: - S. longepedunuclata has been traditionally employed for the treatment of various diseases: (1) the smoke of the root is employed as a fumigant to treat the insane; (2) the powder of the root bark is sniffed for migraine and headache, taken as fumigant against seizure, emetic and stomach distension. The use of the plant for

the treatment of paralysis is also indicated (Dawit Abebe *et al*., 2003). The medicinal importance of *S. longepeduncudata* has been recognized by the findings of different bioactive metabolites isolated from the bark yielding such as oleanolic acid, glycoside and alkaloid securiene, used in treating convulsion in children, increased blood pressure and paralysis following infectious disease (Oliver-Bever, 1986; Harborne and Baxter, 1993).

Research status:- Although the importance of *S. longepedunuclata* in traditional treatment has long been known amongst the rural population, ethno-botanical surveys have been made by various authors from different regions and countries at different times (Oliver-Bever, 1986; Harborne and Baxter, 1993; Dawit Abebe *et al*., 2003). These reported studies indicate the traditional uses as a fumigant to treat the insane, against seizure, emetic and stomach distension and for the treatment of paralysis. Different bioactive metabolites were isolated from the bark such as oleanolic acid, glycoside and alkaloid securiene, used in treating convulsion in children, and increased blood pressure have been reported by several authors (Oliver-Bever, 1986; Harborne and Boxter, 1993). However, a study on the development of seed-based propagation technique has been reported by this study.

1.1.5 *Taverniera abyssinica* A. Rich. (Leguminosae)

Ecology, distribution: - Taverniera *abyssinica* A. Rich. is a medicinal plant and known to occur in northeast Africa and Southeast Asia (Kloos, 1976). It is a threatened medicinal plant (Balcha Abera, 2003; Getachew Addis, 2003) usually grows in a bush land limestone areas with an altitude range of 1700 to 2300 above sea level (Thulin, 1989). Due to over-exploitation, *T. abyssinica* is now found as a remnant of isolated and scattered populations in the Ethiopian Shewa, Tigray and Welo regions (Ensarmu Kelbessa` *et al*., 1992; Fasil Kibebew and Getachew Addis, 1996) (Figure 1).

Description: - T. abyssinica belongs to the Leguminosae commonly and it is known as under the Amharic name *dingetegna*, literally meaning, remedy against sudden illness. It belongs to a small genus of 15 taxa. It is a shrub or shrub let growing up to 2 m high; young stems rather densely appressed pubescent. Leaves, 1 folio late, very rarely pinnately 3 folliolate ones present as well. Leaflets obovate oblong up to 20 (-23) x 10 (-13) mm, glabrous above, appressed pubscent beneath, petiole 1-10 mm long. Racemes 2-8 flowered, rhachis and peduncle together c 3-25 mm long. Calyx 5-7.5 mm long, appressed pubscent outside. Lobes equaling or longer than tube. Corolla 12-17 mm long, dark pink segments, stipitate; segments c 5-6.5 x 4.5-5 mm finely pubscent and with spines to c 1.5 mm long.

Significance: - T. abyssinica has been traditionally used for the treatment of various diseases in Ethiopia. A small bundle of the roots chewed and consequently the juice swallowed for immediate relief of fever, discomfort and pain (Kloos, 1976; Ermias Dange *et al*., 1990). Root extracts of *T. abyssinica* were used locally, as antipyretic and analgesic (Ermias Dange *et al*., 1990). Nematicidial and antimicrobial properties have confirmed the rational basis behind the ethno-botanical use of the species (Stadler *et al*., 1994).

Research status:- The trade of *T. abyssinica* in all 19 markets of central Ethiopia has been reported by Kloos (1976). Various ethno-botanical surveys indicated its distribution in Tigray and in Eastern Shoa of Oromia, Ethiopia (Figure 1). In addition, the same surveys have been reported that *T. abyssinica* has been traditionally used for the treatment of various diseases in Ethiopia revealing that the small bundle of the roots are chewed, and consequently the juice swallowed for immediate relief of fever, discomfort and pain and the root extracts are used, locally, as antipyretic and analgesic (Ermias Dange *et al*., 1990). Nematicidal and antimicrobial properties have been reported by Stadler *et al*. (1994). Other findings reported the isolation of different chemical compound from the rootstocks yielding four isoflavonoid derivatives and a new pterocarpan, 3, 4-dihydroxy-9-methoxy therocarpan (Noamesi and Ermias Dange, 1990). Consequently, pharmacological studies were also conducted using rootstock extracts on rats made hyperthermic with yeast injections (Ermias Dange *et al*., 1990). The aqueous extract of the roots was shown to antagonize the contractile responses of guinea pig ileum to acetylcolin and histamine. At least some of the analgesic properties of the root extracts have been attributed to the isoflavanoids, probably linked to the blocking of histamine receptors (Stadler *et al*., 1994). *In vitro* seed culture of *T. abyssinica* has been reported by Getachew Addis (2003). However, a study on the *in vitro* regeneration of *T. abyssinica* has been reported by this study.

1.2 Objectives

1.2.1 General objective

The general objective of this thesis has been to study seed- based, vegetative propagation by stem cuttings and tissue culture protocols for four and the phenology of one; medicinal plant from Ethiopia for their conservation and sustainable usages.

1.2.2 Specific objectives

1. To develop seed-based propagation techniques for *E. kebericho* Mesfin *S. longepedunculata* Fresen.

and *G. lotoides* L.

2. To study the phenology of *P. zeylanica* L.
3. To develop vegetative propagation by stem cuttings for *G. lotoides* L.
4. To develop *in vitro* regeneration for *T. abyssinica* A. Rich.

1. 3 LITERATURE REVIEW

1.3.1 Plant Propagation

Plant propagation is defined as the science and art of multiplication of plants by either sexual or asexual means. Sexual reproduction involves meiotic cell division that ultimately produces progeny (seedlings) with new or differing genotypes relative to their male and female parents (Hartmann *et al*., 2002). Most plants are highly *heterozygous*, that is, a relatively high number of genes on one chromosome of a Mendelian pair differ from those on the other chromosome. As a result, the progeny of plants grown from seed tend to exhibit a relatively high amount of genetic variation (Hartmann and Kester, 1983). For the purposes of this discussion, sexual reproduction is synonymous with propagation by seed, although not all embryos develop from sexual processes. Example, Apomixes results from the production of an embryo that bypasses the usual processes of meiosis and fertilization. So seed production is asexual and the clonal seedling plants are known as apomicts.

Asexual propagation by cuttings involves removing sections of stem or root tissue from the parent or donor plant, treating this tissue with plant growth regulators in order to induce adventitious root under controlled environmental conditions (Hartmann *et al*., 2002). Asexual propagation is reproduction from the vegetative parts of the donor plant through stem cuttings, grafting, budding, layering etc., and involves mitotic cell division in which the chromosomes duplicate and divide to produce two nuclei that are genetically identical to the original nucleus. This can occur through the formation of adventitious roots and shoots or through the combining of vegetative tissues, such as in grafting. This clonal process, in which the genotype of the parent plant is exactly duplicated, is made possible because of two unique plant characteristics: (1) *Totipotency* is the property of vegetative plant cells to carry all of the genetic information necessary to regenerate the original plant and (2) *Dedifferentiation* is the ability of mature (differentiated) cells to return to a meristematic condition and produce a new growing point (Hartmann and Kester 1983; Leaky, 1983; Leaky and Coutts, 1998; Hartmann *et al*., 2002; Legesse Negash, 2003a, b, c; 20004b). In the

context of this discussion, asexual reproduction refers to the induction of adventitious roots from stem cuttings and is synonymous with vegetative propagation.

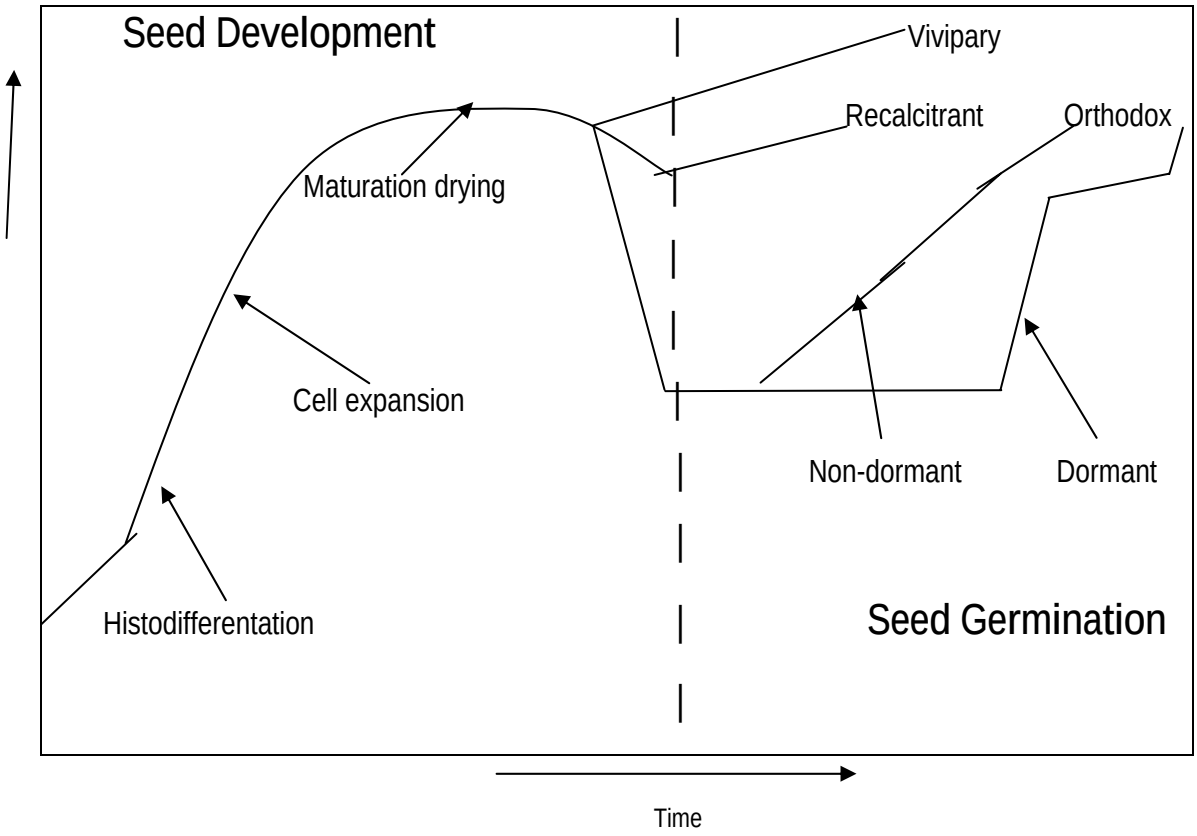


Figure 2 Transition from seed development to seed germination. Seeds may end seed development and display viviparous, recalcitrant, or orthodox seed behaviour. Viviparous and recalcitrant seeds germinate before completing the maturation drying stage of development. Whereas orthodox seeds continue to dry to about 10 % moisture and can be either non-dormant or dormant (Source: Taiz and Zeiger, 1998; Hartman *et al*., 2002)

1.3.2 Seed-based propagation

Since the beginning of agriculture, plants have been propagated by seed, which involves sexual recombination and

serve as the delivery system for the transfer of genetic materials from one generation to the next. The degree with which an individual plant establishes successfully is largely determined by the physiological and biochemical status of its seed (Legesse Negash, 1992; 2002; 2004a). The plants produced from seed are related, but not identical, to the parent plant (Bewley and Black, 1994). In plant propagation endeavors, the seed is considered as an inexpensive commodity, and in the majority of cases, an effective means of propagation started by means of seed germination followed by the emergency of seedlings. There are different definitions on seed germination (Copeland and McDonald, 1994). Seed physiologists define seed germination as the emergence of the radicle through the seed coat. A seed analyst also defines it as the emergence and development from the seed embryo of those essential structures, which for the kind of seed in question are indicative of the ability to produce a normal plant under favorable conditions (Figure 2). Others also consider seed germination as the active growth of the embryo, which results in the rupture of the seed coat and the emergence of young plant (Hartmann *et al*., 2002).

It has been reported that the seed remains inactive with low metabolic rate until it receives favorable environmental conditions that trigger the growth of the embryo (Bewley and Black, 1994). The response of seeds to favorable environmental conditions is different (Atwar, 1980; Bewley and Black, 1994). Some seeds are capable of germinating only a few days after fertilization and long before their normal harvesting time, while others are dormant and require an extended rest period or additional development before germination can occur. The seeds of all studied medicinal plant species are recalcitrant that germinate in providing appropriate conditions and require treatment to increase the percentage and rate of germination. But mostly it is species dependent and it may last for only a few days or for as long many years (Copeland and McDonald, 1994). Various studies have been reported on the factors imposing seed germination in general and often the inherited effects on tropical and sub tropical seed germination that show differences from those of the temperate species (Atwar, 1980; Berrie, 1984; Michael *et al*., 2003). These involve (1) seed germination mode; (2) morphological and physiological constraints; (3) the Orthodox-intermediate- recalcitrance continuum; and (4) seed polymorphism (Michael *et al*., 2003; Lodge, 2005).

1.3.2.1 Treatments breaking seed dormancy and germination conditions

Various effective pretreatments break further imposing seed germination (Hartmann *et al*., 2002; Michael *et al*., 2003). Of these pretreatments nickling, hot water soaking, and physical or acid scarification, hormone treatment

have all been used to good effect with the seeds of many legume species and other plants of tropical and subtropical origin (Michael *et al*., 2003) including medicinal plants. These treatments are expected to bring about physical and physiological changes within the seed coat and embryo that will enhance rapid germination (Legesse Negash, 2003a). These include: (1) activating enzymes; (2) changing stored foods to soluble forms; (3) changing germination inhibitor/promoter balance; and (4) softening hard seed coats (Bewley and Black, 1994; Hartmann *et al*., 2002).

Most experimental evidence supports the concept that specific endogenous growth promoting compounds are involved directly in the control of seed development, dormancy and germination (Black, 1981; Bewley and Black, 1994). Evidence for hormone involvement comes from the correlations of hormone concentration with specific developmental stages, effects of applied hormone, and the relationship of hormones to metabolic activities (Hartmann *et al*., 2002). Of the growth regulators (plant hormones) gibberellins (GA) compromise as one of the classes most directly implicated in the control and promotion of seed germination (Frank and Cleon, 1992). Germination conditions such as moisture, temperature and aeration also play a pivotal role for the germination of seeds and considered in this study. These conditions are expected to bring about enzyme activation, break down, translocation and use of storage material, denaturation of proteins, dormancy induction and release as well as in the oxidation processes (Frank and Cleon, 1992; Hartmann *et al.*, 2002).

1.3.3 Vegetative propagation

The goal of vegetative propagation is to reproduce progeny of plants identical in genotype to a single source plant. The biological process is known as cloning and the resulting population of plants is called a clone (Hartmann *et al*., 2002). There is now a growing consensus among scientists that easily propagated plants by stem cuttings, the method of vegetative propagation has numerous advantages (1) the method is inexpensive, rapid, and simple, and does not require the special techniques necessary in grafting , budding, or micropropagation; (2) many new plants can be started in a limited space from a few stock plants; (3) plants can be propagated by vegetative means at any time of the year; and (4) greater uniformity and superior growth characteristics of plantlets can be obtained compared to the seedlings (Leaky and Coutts, 1998; Legesse Negash, 2003b, c). A cutting can be defined as any vegetative plant part which, when detached from the parent, is capable of regenerating the missing organ or organs. It can be described as a method of propagating plants by the use of detached vegetative plant parts which, when placed under conditions favorable for regeneration, will develop into a complete plant, similar in all characteristics to the parent plant (Hartmann and Kester, 1983; Legesse Negash 2003b, c). According to Hartmann *et a*l. (2002)

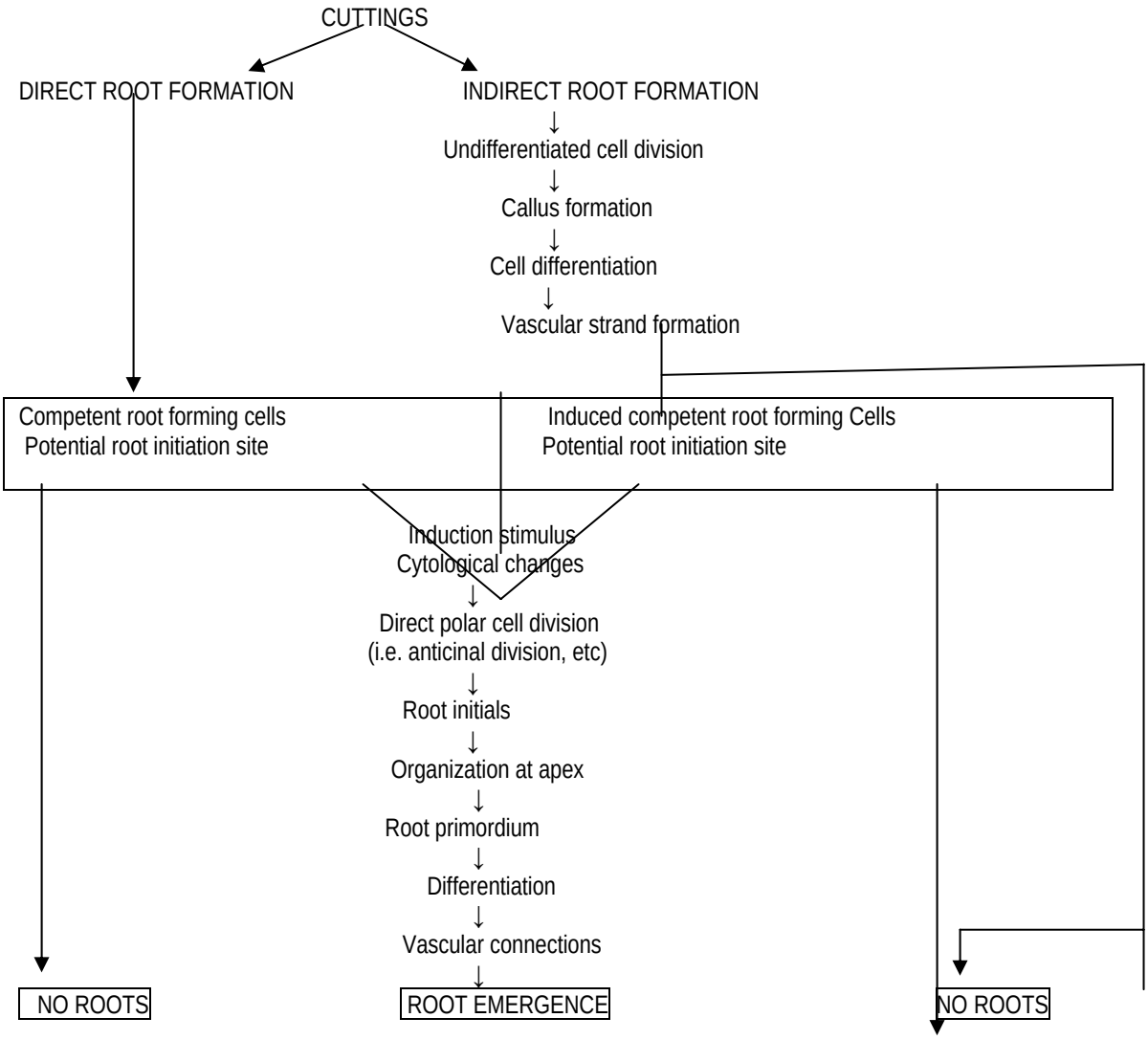


Figure 3 Flow diagram of adventitious root formation through direct (cells in close proximity to vascular system, more easy-to-root species) and indirect model (interim period of undifferentiated cell division, in more difficult–to-root species). When a potential root initiation site is already present the initial cell divisions lead to root production in situ. When a site is not present alternative routes leading to the creation of a site. However, rooting doesn't always occur. Source: Hartmann *et al*. (2002).

cuttings can be made from the vegetative portions of the plant, such as stem, modified stems (rhizomes, tubers, corms and bulbs), leaves or roots. Based on the part of the plant taken, cuttings can be classified as stem cuttings (hardwood, semi-hardwood, softwood and herbaceous), leaf cuttings, leaf-bud cuttings (single-eye or single-node cuttings) and root cuttings (Hartmann *et al.,* 2002).

In propagation by stem cuttings, segments of shoots containing lateral or terminal buds are obtained with the expectation that under the proper conditions adventitious roots will develop and thus produce independent plants (Leaky and Coutts, 1998). The formation of adventitious roots and buds is dependent on plant cells to differentiate and develop into either root or shoot system. The process of differentiation is the capability of previously developed, differentiated cells to initiate cell divisions and form a new meristematic growing point (Leaky, 1983) (Figure 4). However, the development of adventitious roots in a variety of plant species can be influenced by different factors such as stockplants age, cutting position, and rooting hormone, rooting medium, environmental and physical factors (Leaky, 1983; Leaky and Coutts, 1998). However, in this study, the effects stockplants age, cutting position, rooting hormone and medium were investigated in vegetative propagation of *G. lotides*

1.3.3.1 Factors influencing vegetative propagation

A number of studies have shown that cuttings derived from juvenile stockplants are easier to root than those derived from more mature stockplants (Desta Berhe and Legesse Negash, 1998; Wassner and Ravetta, 2000; Legesse Negash 2003b, c). However, the effect of cutting position seems to be species-dependant (Hansen, 1986; 1988; Hartmann *et al*., 1997) since in some species stem of basal cuttings root best (Leaky, 1983) compared to those stem of apical cuttings in other species (Wassner and Ravetta, 2000). In addition, other reported studies indicated that in some species the cutting position has no effect on rooting (Algere *et al*., 1998).

Rooting hormones are very important in the rooting process of cuttings (Wiessman-Ben and Tchoundjeu, 2000). Among the exogenous rooting hormones (inducing stimulus) (Figure 2), indole-3 butyric acid (IBA) and œ-naphthaleneacetic acid (NAA) are found to be reliable in root promotion of cuttings. IBA is widely applied for general use because it can remain non-toxic within a wide range of concentrations and improves root initiation of cuttings for most plants species (Hartmann *et al*., 1990; Al-Saqri and Alderson, 1996). Hartmann *et al*. (1997) reported that IBA might be toxic to certain cuttings taken from softwood plant species, which causes poor growth, no growth or

mortality of the cuttings. The purpose of treating cuttings with auxin is to increase the percentage of rooting, root initiation, number of roots, and uniformity of rooting (Al-Saqri and Alderson, 1996). It also accelerates the translocation of nutrients from the upper part of the cuttings to their basal ends by increasing the activity of enzymes. This increases hydrolysis of carbohydrates by providing enough energy in rooting respond of the cells (Arya *et al*., 1994). As reported by Al-Barazi and Schwabe (1982), occasionally, IBA treatment seems to stimulate cell division in the ray cells (Figure 4) between the primary bundles to improve root initiation and to increase uniformity of rooting. Eventhough there is no ideal or universal rooting medium for cuttings (Hartmann *et al*., 1997) no propagation method is going to work if the right media for growth is not used (Desta Berhe and Legesse Negash, 1998).

A medium is said to be suitable for propagation based on species, cutting type, season, propagation system used, its cost and availability (Hartmann *et al*., 1990). An ideal propagation medium has to provide the cuttings with good aeration, moisture, drainage, support, nutrients and must be free of disease causing pathogens (Hartmann *et al*., 1990). But most frequently used media contain combinations of sand, peat, sphagnum moss, vermiculite, pearlite, compost, and shredded bark/sawdust (Hartmann *et al*., 1997).

1.3.4 Plant tissue culture

Biotechnological tools are important in order to select, multiply and conserve the critical genotypes of medicinal plants. Plant tissue culture techniques are used as integrated approaches for the production of standardized quality phytomedicines, through the mass production of consistent plant material for physiological characterization, and the analysis of active ingredients. *In vitro* regeneration and micropropagation are the practices of rapidly multiplying stock plant material to produce large numbers of progeny plants. The integrated approaches of plant culture systems will provide the basis for the future development of novel, safe, effective, and high-quality products for consumers (Erica, 2000; Khanna, 2003).

Thus, plant tissue culture is the science or art of isolating plant cells, tissues and organs from the mother plant and growing on artificial media under aseptic condition (Erica, 2000). The idea of plant tissue culture was originally proposed in 1902 by German botanist Gottlieb Haberlandt, and successful *in vitro* growth of tissue was achieved by Philip White in 1934 from tomato root explants (Khan, 2003). The first whole plant regeneration from culture were carrots in 1958 by F.C. Stewart. Stewart also demonstrated the development of somatic embryos in liquid culture of

carrot cells. Today, the number of plant species in general, and of medicinal plants propagated by tissue culture techniques is large (Khan, 2003). Plant cell and tissue culture are used for clonal propagation, production of disease-free plants, germplasm conservation, secondary metabolite production, genetic transformation, haploid production, triploid production, *in vitro* pollination and fertilization embryo rescue, and somatic hybridization (Erica, 2000; Hartmann *et a*l., 2002; Tileye Feyissa, 2006).

Many new complete plants can be obtained from different explants through direct or indirect morphogenesis and through somatic embryogenesis. Direct morphogenesis is the production of shoots from explants without passing through callus (unorganized tissue) phase known as organ culture, which include meristem cultures, shoot cultures, embryo cultures and isolated root cultures. Whereas indirect morphogenesis refers to induction of shoots through callus phase grouped as unorganized tissue cultures, which include callus cultures, suspension or cell cultures or anther cultures (Khanna, 2003; Tileye Feyissa *et al*., 2005). Plant tissue culture is based on three cell doctrines (concepts): (1) plasticity (the flexibility of adaptability e.g. of tissue or cells to altered chemical and physical factors such as hormones, nutrient elements fixed carbon sources, light, temperature and culture vessels); (2) totipotency (capacity of cells or tissues to develop in to any of the structure of the plant; and (3) de-differentiation (in which differentiated cells get de-differentiated in order to pave way for a new line of development (Hartmann *et al*., 2002; Khanna, 2003

1.3.4.1 Effects of growth media, explants and growth regulators on tissue culture

The basic nutritional requirements of *in vitro* cultured plant cells are very similar to those utilized by plants in nature. However, the nutritional composition used *in vitro* varies depending on the type of protoplasts, cells, tissues, organs, and plant species. A nutrient medium is defined by its composition of mineral salts, carbon sources, vitamins, plant growth regulators, and other organic supplements. A particular medium is identified by its salt composition unless and otherwise identified. Other additives such as amino acids, vitamins, growth regulators, and other organic supplements can be added in any concentrations to a given salt concentrations to get the desired results (Murashige, 1974; Deen and Mohamoud, 1996). Although several media have been developed, the medium of Murashige and Skoog (1962) is widely used in different types of tissue culture systems. However, the most important factors that show recalcitrance of plant tissue culture depend on the species, used organic additives and the type and concentration of growth regulators (Erica, 2000). In addition, other additives such as activated charcoal,

ascorbic acid, acetic acid and polyamines and exogenous factors such as light, temperature, light quality and intensity are important in determining the response of some tissues in culture (Erica, 2000). In the maintenance of

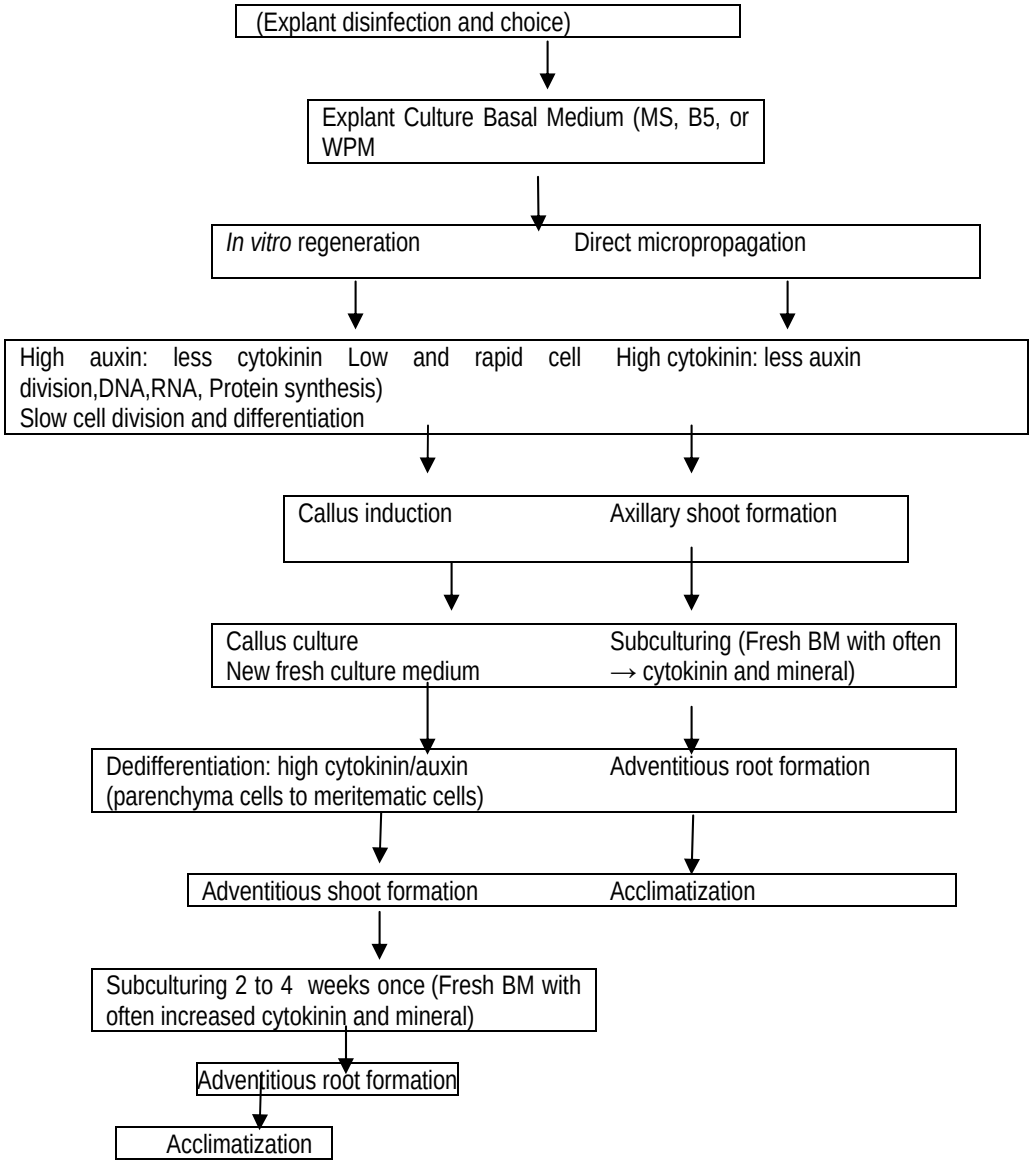


Figure 4 A general illustration of the stages of plant tissue culture (Source: Hartmann *et al*., 2002)

culture for long time, mostly physiological changes, but some genetic can cause abnormal phenotypes so that changing the economic and aesthetic value (Gupta, 2004). Therefore, it is important to monitor the performance of tissue cultured plants as they grow to maturity in greenhouse, garden or field.

Although all plants are composed of the basic unit of life; cells and all cells are not considered totipotent, only some cells are competent to divide and give rise to complete plant in tissue culture. Thus, not all plant species are equally amenable to tissue culture. Although production of perennial plants through tissue culture seems very attractive especially for cloning and genetic engineering the complex seasonal cycles and life cycles of those plants complicate the control of their growth in tissue culture. Although, the major cause of recalcitrance in perennial plants is the inability to establish fully established shoot cultures, still, shoot culture is one of the important approaches as a source of excellent cells, tissues and organs in protoplast generation, gene insertion and transeclone recovery. Establishing tissue culture involves several steps. Murashige (1974) generalized these into three steps; aseptic culture, establishment, multiplication and acclimatization. Gupta (2004) generalized into four stages. Isolation stage that involves explants collection and decontamination and growth of performed buds, stabilization stage that involve uniform and continuous shoot growth/ multiplication, optimization stage involving refining medium and environmental conditions and acclimation/rooting of the micropropagules (Figure 4).

1.3.5 Phenology

For centuries, people have observed annual variation in the dates at which buds break and flowers bloom (Sparks and Cary, 1995). Nevertheless, recently, the science of plant phenology become concerned with understanding the variability of vegetative and reproductive cyclic events in relation to their biotic and abiotic forcings (Leith, 1974). Therefore, phenology is defined as the study of the timing of biological events or phenophases in plants or animals and their relationship with seasonal climate (rhythm) (Maria *et al*., 2002). Studies on the phenology of medicinal plants is the first data to be obtained so as to develop a basic knowledge; the type of species (monoecious or dioecious), the right season of the collection of medicinal parts and propagules for effective treatment, and establishment of propagation techniques, respectively as well as in the analysis of the level of active principles during each phenological events (Maria *et al*., 2002).

In plants, the transition from vegetative growth to reproductive development is continued after embryogenesis and

seedling emergence. At the end of vegetative phase, the shoot apical meristem of adult plant undergoes a dramatic change and the inflorescence is initiated during minimum growth in height and production of leaves (Araki, 2001). Finally, in the late inflorescence flowers comprised of different whorls of floral organs are produced and terminated with fruit/seed-set. Thus, the superior plant phenological events are seedling emergence, vegetative growth (leaf flushing and leaf abscission), and reproductive development (flowering bud initiation, anthesis, fruit/seed-set). Although phenology is a valuable scientific and economic knowledge, researches on medicinal plants are scarce. However, some reports have been made on the investigation of the level of active principles for effective treatment during each phenophase associated with each seasonal climate (Santos et al., 1998; Maria *et al*., 2002). The duration of vegetative phase and the time of reproductive development are influenced by both exogenous (photoperiod, rainfall, temperature) (Maria *et al*., 2002) and endogenous (hormones, genes) ((Yong *et al*., 2000) signals. The phenology of tropical and subtropical plants is influenced by low temperature (Tan, 1983), rainy season, and water availability (Bie *et al*., 1998; Bach, 2002). In this study, the phenology of *P. zeylanica* was influenced by rainy and cold seasons under nursery, which extended to 225 days compared to 105 days under glasshouse conditions.

1.3.5.1 Factors influencing phenology

The transition of flowering is influenced by both endogenous and exogenous signals (Figure 5). The underlying genetic regulatory network that integrates and transduces these signals has been elucidated in *Arabidopsis.* Genes have been positioned in several pathways that promote or repress flowering, depending on environmental or autonomous conditions. Four major ways (photoperiod, autonomous, vernalization and gibberellins) converge on the meristem identity genes *LEAFY (LFY)* and *APETALA (API).* A key few genes *CONSTANS (CO*), five phytochrome genes *(PHYTOCHROME A-E, PHY A-E,)*, two cryptochrome genes *(CRYPTOGAME, CRY1 and CRY2*), *TIMING OF CAB 1 (TOC1)* stimulate expression of *LATE ELONGATED HYPOCOTYL (LHY)* and *CIRCADIAAN CLOCK ASSOCIATED 1 (CCA1*) (Tan, 1992). Other Genes have been described that also may function such as *EARLY FLOWERING* 3 (*ELF3*), *ZITULIPI (ZTL).* Genes in the autonomous pathway include *FCA, FY, FVE, FPA*, and *LUMINIDEPENDENS* (*LD)*, Vernalization pathway consists of floral repressor genes; *FLOWERING LOCUS (FLC*), leads to suppression of *FT* and *SOC I* and inhibition of flowering. FRIGIDA (FRI) enhances *FLC* function. A set of vernalization genes (*VRN 1 and VRN 2*) are involved in maintenance of *FLC* repression. Several major genes that have identified in gibberellins pathway are repressors of GA response, including *GIBBERELLIN SENSETIVE* and

REPRESSOR of *GA (RGA).* This pathway stimulates flowering by causing up-regulation of *SOC 1* and LFY, but not *FT* (Figure 5)

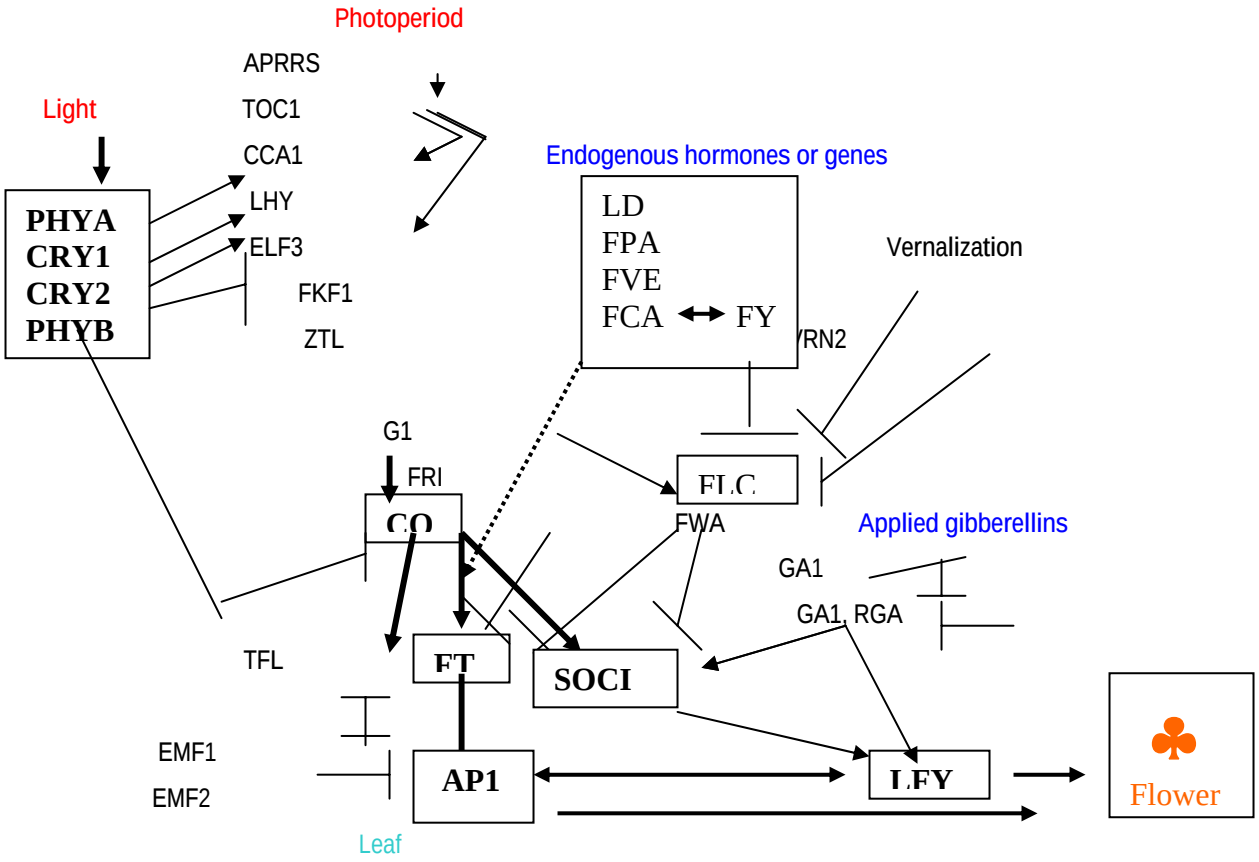


Figure 5 The regulatory pathway net work controlling flowering time in *Arabidopsis*. The positive (arrows) negative (bars) regulatory relationships are from reviews cited in the text. Genes in tinted boxes (FT, LFY, SOC1) integrate signals from multiple pathways. The dashed line dipcts a possible connection between the authonomous pathway and the photoperiod pathway that is independent of FLC (source: Stephen *et al.,* 2004)

1.3.5.2 Phenologiccal parameters

In addition to timing, duration and frequency, the following six reproductive and two vegetative phenological parameters were widely recommended to characterize ground-based study on the phenology of individual plant species (Maria *et al*., 2002). These include: (1) date of first flowering; (2) date of first fruiting; (3) date of peak flowering; (4) date of peak fruiting; 5) flowering duration; (6) fruiting duration; (7) date of peak leaf flush; and (8) date of peak leaf fall. Studies on the phenology of *P. zeylanica* (i.e., from seedling emergence via vegetative growth to reproductive development has been developed by this study under glasshouse versus nursery and seasonal climate. The effects of different growth hormones were also examined.

1.4 Importance of the Study and Future perspectives

The knowledge of phenology and development of propagation techniques is fundament in centers of biodiversity like Ethiopia, having numerous advantages. These include: (1) to identify the reproductive biology (mating system or whether the species is monoecious or dioecious); (2) to identify the time, duration and frequency of propagule production in the wild, and consequently their collection for propagation purposes; (3) to evaluate the effects of seasonal climate on the transition from vegetative growth to reproductive development; (4) to establish species-specific ecological requirements; (5) to determine the level of active principles (both quantitatively and qualitatively) in medicinal plants during each phenophase for effective treatments; (6) to use as an indicator of global climate change; and (7) to cultivate, domesticate and conserve of threatened and/or endangered plant species for sustainable use.

A study on the phenology of medicinal plants is scarcer due to the lack of consideration and complexity of phenological phenomenon being under array of interacting factors including various environmental and autonomous signals (Maria *et al*., 2002). Studies on the phenology of *P. zeylanica* conducted for the first time was limited as a result of time and financial limitations. Although the effects of glasshouse versus nursery conditions, seasonal climate, and plant size and hormone treatments have been developed, the underlying mechanisms and the influence of other factors are not known. More insight into these mechanisms and factors will enable the optimization of the effective use of *P. zeylanica*.

A rapid and effective phenological pattern (i.e., vegetative growth and reproductive development) of *P. zeylanica* and *G. lotoides* under glasshouse, and in contrast, their stunted growth under nursery conditions, revealed strong evidence that the suitability of glasshouse (controlled) conditions is species-dependant. This was also confirmed by *E. kebericho*, which showed the reverse phenological phenomenon. However, since phenology is determined by a variety of factors including autonomous signals (hormones and genes) further studies have a great contribution to determine the most suitable growth environment for the purpose of domestication in order to alleviate the harvest in the wild.

The studies on the development of propagation techniques were also limited on certain factors and/or methods due to the same reasons described in phenological studies. Although an *in vitro* regeneration of *T. abyssinica* has succeefully been developed, the same experiment conducted on *E. kebericho,* and *S. longepedunculata* have been established to callus induction (data not given). *G. lotoides* also showed difficulty due to its high pheonlic exudates (data not recorded). Thus, further studies on shoot regeneration of *E. kebericho,* and *S. longepedunculata,* and *in vitro* seed germination of *G. lotoides* can optimize the effective and efficient development of seed and tissue culture methods. *In vitro* seed-based propagation protocols developed by this study on *E. kebericho* and *S. longepedunculata* can be used for further investigation of pure medicaments rather from the wild stockplants widely exposed to the numerous contaminants in the nature. Ultimately, the studies on the ecological requirements and the establishment of protected areas are the next steps for the effective use of any of the propagation protocols developed by this study.

2 IN VITRO AND EX VITRO PROPAGATION OF *ECHINOPS KEBERICHO* MESFIN

2.1 Effects of various treatments on the seed germination, seedling survival and growth

Seed is a ripened ovule, which consists of an embryo, stored food and seed coat (Bewley and Black, 1994). Propagation by seed is one means of continuity of plant life and assuring of plant species survival (Copland and McDonald, 1994). Many authors define seed germination differently, and in this study the seed of *E. kebericho* is germinated with the emergence of radicle through the seed coat layers. However, seed germination is influenced by different factors. These are: (1) morphological characteristics of the seed coats including the presence of pubescent

hairs highly incorporating with pathogens, which inhibits seed germination (Hartmann *et al*., 2002). Thus, microbial disinfections (fungi, bacteria, viruses) can be either eliminated or reduced through sterilization using various detergents (ethanol, sodium hypochlorite) (Erica, 2000; Hartmann *et al*., 2002). (2) Evidence for hormone involvement in inducing seed germination comes from the correlations of hormone concentration with specific developmental stages, effects of applied hormone, and the relationship of hormones to metabolic activities (Hartmann *et al*., 2002). Of the growth regulators (plant hormones) gibberellin (GA_3) compromise as one of the classes most directly implicated in the control and promotion of seed germination (Frank and Cleon, 1992; Michael *et al*., 2003; Lodge, 2005); and (3) various germination conditions such as temperature, moisture, aeration and media (Hartmann *et al*., 2002). The use of appropriate germination media play a pivotal role to bring about nutrient availability, enzyme activation, break down, translocation and use of storage material, denaturation of proteins, dormancy induction and release as well as in the oxidation processes (Bewley and Black, 1994). However, the influence of these factors is not known yet for *E. kebericho.*

The main objectives of this study were to examine the effects of (1) disinfectants on *in vitro* germination; (2) germination substrates on *ex vitro* germination; and (3) glasshouse versus nursery on seedling growth and establishment for getting plant size.

2.1.1 Materials and methods

2.1.1.1 Plant material

Mature *E. kebericho* fruits were collected from two population sites: (1) from naturally growing population found in Tulu Baja peasant association, Gedo District, West Shoa Zone, Oromia, Southwest Ethiopia (160 km west of Addis Ababa), during October 2005; and (2) from the medicinal plant garden, located within the campus of Ethiopia Health and Nutrition Research Institute (EHNRI), found in Addis Ababa, during the month of August, 2005. The fruits were removed by hand from the flower head and stored at room temperature (ca 22^{o} C). *In vitro* germination experiment was conducted at the Institute of Plant Genetics and Crop Plant Research (IPK), Gateresleben, Germany, between September 2005 and February 2006. Glasshouse and nursery experiments were conducted both at the Faculty of Science (Addis Ababa University) (between September 2003 and August 2005) and at Jimma Agricultural College and Veterinary Medicine (Jimma University), (March to September 2006)

2.1.1.2 *In vitro* seed germination

Seeds of *E. kebericho* were released from the thin layers of fruit by hand using a scalpel, surface sterilized in 70% ethanol for 5, 7, 9, 11, 13, 15 minutes, then in 10% sodium hypochlorite solution for 3, 4, 5, 6, 7, 8, 9 minutes and rinsed several times using double distilled water under a laminar flow hood. The germination medium consisted of MS minerals and vitamins (Murashige and Skoog, 1962) supplemented with 6, 8, 10, 12, 14, g l^{-1} phytoagar (Sigma, St Louis MO, USA), whereas no sucrose was added. The different media were stirred; the pH adjusted to 5.75, autoclaved at 121^{O} C for 20 minutes, and then cooled down in a water bath to about 50^{0} C. The media (100 ml each) were then dispensed in glass culture vessels (100 mm x150 mm). Twelve seeds per vessel were cultured with 10 replicates per treatment. All cultures were inoculated at 25 ^{O}C under cool fluorescent (40 μmol m^{-2} s^{-1}: 16 photoperiod). Seed germination indicated by at least radicle emergence was recorded at days 5, 10, 15, 20, 25 and 30.

2.1.1.3 Pot experiments

Intact seeds (i.e. seeds with fruit) were planted in plastic pots (mouth diameter 20 x depth 25 cm) filled with a mixture of sand, red soil, and cattle dung in a ratio of (1.5:1.0:0.5, 0.5:2.5:0.5, 1.5:0.5:1.0, 1.0:1.5: 0.5, respectively), and maintained in a glasshouse. Seventeen seeds were cultured in each pot with 6 replicates per treatment. Dried grass stalks were laid horizontal on top of the pots for conserving moisture, and the system was watered once a day. The grass cover was removed at the onset of germination to prevent the bending of emerging seedlings. The mean minimum and maximum temperatures of the glasshouse during the study period were 11.8 ± 1.0^{o} C (nights) and 28.5 ± 2.0^{o} C (days), respectively. The relative humidity (RH) ranged from 61 to 73%, and was maintained roughly throughout the experimental period by sprinkling the floor of the glasshouse with water. RH was measured using a Humidity and Temperature Sensor (Type HP- 100-A., Umweltanalytische Mess-System GmbH, Munic, Germany).

2.1.1.4 Nursery experiment

A total of 100 m^2 nursery bed was prepared and divided in parallel into 4 similar split plots (each 25 m^2). Three plots were surface mixed with three types of additives (cattle dung, horse dung, and sand). Sowing seeds on a nursery soil without additives was considered as a control. 105 seeds were sown on each plot. The mean minimum and

maximum temperatures of the nursery area during the study period were 10.8 ± 1.0° C (nights) and 24.5 ± 2.0° C (days), respectively and the relative humidity (RH) was ranged from 64 to 75% throughout the experimental period.

2.1.1.5 Seedling survival and growth

One-month-old *in vitro*, pot (glasshouse) and nursery bed origin germinants were used to examine the survival and growth of the seedlings using five categories (I-V): (I) except nursery (which was only evaluated under nursery condition), each *in vitro* and pot origin germinant were divided into two (II-IV) and studied both under glasshouse and nursery conditions. Hundred to hundred ten seedlings were used for each treatment and regularly inventoried during the growing period. Inventories were made every second day during the first month after sowing, weekly at the end of the first growing season, and monthly during subsequent growing seasons. Data on the percentage survival and measurement on the growth height were scored per month.

2.1.2 Results

2.1.2.1 Effects of seed sterilization and phytoagar concentration

Both seed sterilization and phytoagar concentration influenced the germination of *E. kebericho* seeds (Table 8). Unsterilized seeds were drastically affected by fungal contamination, producing a whitish mycelium on the surface of the seeds 3 days after seed incubation (control). Seeds sterilized with 70% ethanol for 5 minutes and 10% sodium hypochlorite for 3 minutes showed poor germination (27 ± 1.5%) 15 days after seed sowing. Further, the growth of germinants was limited by the expansion of fungal growth throughout the culture media, thus resulting in the death of the germinants. The best germination was obtained with seeds sterilized for 9 and 5 minutes in 70% ethanol and 10% sodium hypochlorite, respectively. Further increases or decreases in time of sterilization decreased germination. The germination of *E. kebericho* seeds was also in uenced by the availability of water as was adjusted by the concentration of phytoagar. Concentrations of 6 and 14 g 1^{-1} resulted in poor germination. The best germination was obtained at a concentration of 10 g l^{-1} 15 after 28 days of seed incubation. Further increases or decreases in phytoagar concentration decreased germination. All subsequent experiments were therefore performed sterilizing seeds for 9 and 5 minutes with 70% ethanol and 10% sodium hypochlorite, respectively using 10 g 1-1 phytoagar. Seed germination indicated by at least radicle emergence was recorded at days 3, 6, 9, 12, and 15.

Table 1 Effects of sterilization and phytoagar concentration on the germination of *E. kebericho* seeds on MS medium

10 (%) NaOCl (min.)	70 (%) Ethanol (min.)	Phytoagar Conc.	Germination (%)				
			Day 3	Day 6	Day 9	Day 12	Day 15
0	0	-	-	-	-	-	-
3	*5*	6	-	$16 \pm 0.9^{a*}$	$21 \pm 0.6^{a*}$	$23 \pm 0.4^{a*}$	$27 \pm 1.2^{a*}$
4	*7*	8	$24 \pm 0.9^{b*}$	34 ± 0.6^{b}	46 ± 0.9^{b}	62 ± 0.6^{b}	75 ± 0.6^{c}
5	*9*	10	65 ± 1.2^{c}	78 ± 0.6^{d}	82 ± 0.6^{d}	88 ± 0.6^{d}	95 ± 1.2^{e}
6	*11*	12	43 ± 0.6^{d}	53 ± 0.6^{c}	72 ± 0.6^{c}	79 ± 0.5^{c}	84 ± 0.6^{d}
7	*13*	14	24 ± 1.8^{b}	33 ± 1.5^{b}	43 ± 2.2^{b}	47 ± 1.2^{b}	52 ± 0.9^{b}

.*) Means with standard deviation within the same column followed by different letters (a-e) are significantly different ($p < 0.05$).

Table 2 Effects of soil ratios on the germination of *E. kebericho* seeds sown in pots, maintained under glasshouse conditions.

Seeding media**	Germination (%)			
	day 7	day 14	day 21	day 28
A	$30 \pm 0.9^{c**}$	$43 \pm 1.2^{c**}$	$62 \pm 0.6^{c**}$	$77 \pm 1.1^{c**}$
B	54 ± 0.6^{d}	66 ± 0.3^{d}	76 ± 1.2^{d}	93 ± 1.2^{d}
C	13 ± 0.9^{a}	$23.\pm 1.2^{a}$	28 ± 1.5^{a}	35 ± 1.2^{a}
D	21 ± 0.9^{b}	28 ± 0.6^{b}	33 ± 1.2^{b}	44 ± 0.6^{b}

*) Means with standard deviation within the same column followed by different letters (a-e) are significantly different ($p < 0.05$).

**Seeding media: sand, red soil and horse dung (A. 1.5:1.0:0.5, B. 0.5:2.5:0.5, C. 1.5:0.5:1.0, D. 1.0:1.5: 0.5, respectively).

2.1.2.3 Effects of soil ratios

The germination of *E. kebericho* seeds was tested on different mixture of soil ratio (Table 9). Seeds sown in pots

containing a mixture of sand, nursery soil, horse dung soil in a ratio of (1.5:0.5:0.5; 1.0:1.5:0.5, respectively) showed poor germination. The highest percentage (93 ± 1.2%) germination was obtained in a mixture of sand, nursery soil and horse dung in a ratio of (0.5:2.5:0.5, respectively) 28 days after seed sowing. Decreased in germination was

Table 3 Interactive effects of additives and nursery soil on the germination of *E. kebericho* seeds

Additives**	Germination (%)			
	day 7	day 14	day 21	day 28
Control	14 ± 0.6^{a**}	23 ± 1.2^{a**}	33 ± 1.5^{a**}	43 ± 1.2^{a**}
A	64 ± 1.2^{d}	74± 0.9^{d}	85 ± 1.1^{d}	96 ± 0.5^{d}
B	41 ± 1.2^{c}	52 ± 1.7^{c}	63 ± 1.4^{c}	74 ± 2.1^{c}
C	32 ± 1.2^{b}	41 ± 0.9 ^{b}	51 ± 0.9^{b}	62 ± 0.6^{b}

*Means with standard deviation within the same column followed by different letters (a-e) are significantly different ($p < 0.05$). **Additives- A. nursery soil, horse dung, B. nursery soil, cattle dong, C. nursery soil, sand. Control- without additive

observed with an increment of both sand and horse dung. Increased ratio in horse dung not only reduced in germination but also steadily exposed to fungal contamination, and consequently caused the death of seedlings. No significant difference was observed between decarped and intact seeds on percentage and rate of germination of *E. kebericho* seeds.

2.1.2.4 Effects of additives

A significant ($p<0.05$) effect of animal manure was observed on the germination response of *E. kebericho* seeds (Table 10), which was in uenced by surface-mixed animal manure and sand with a nursery soil (Tab. 3). Seeds cultivated on a nursery soil without additives (control) showed poor germination. The best germination (96 ± 0.5%) was obtained from nursery soil surface-mixed horse dung followed by cattle dung (74 ± 2.1%). Seeds sown on a nursery soil surface-mixed sand and control also showed signi cant ($p<0.05$) with the least germination (43 ± 1.2%). Similar to the pot experiments, there was no significant difference between decarped and intact seeds on percentage and rate of germination of *E. kebericho* seeds.

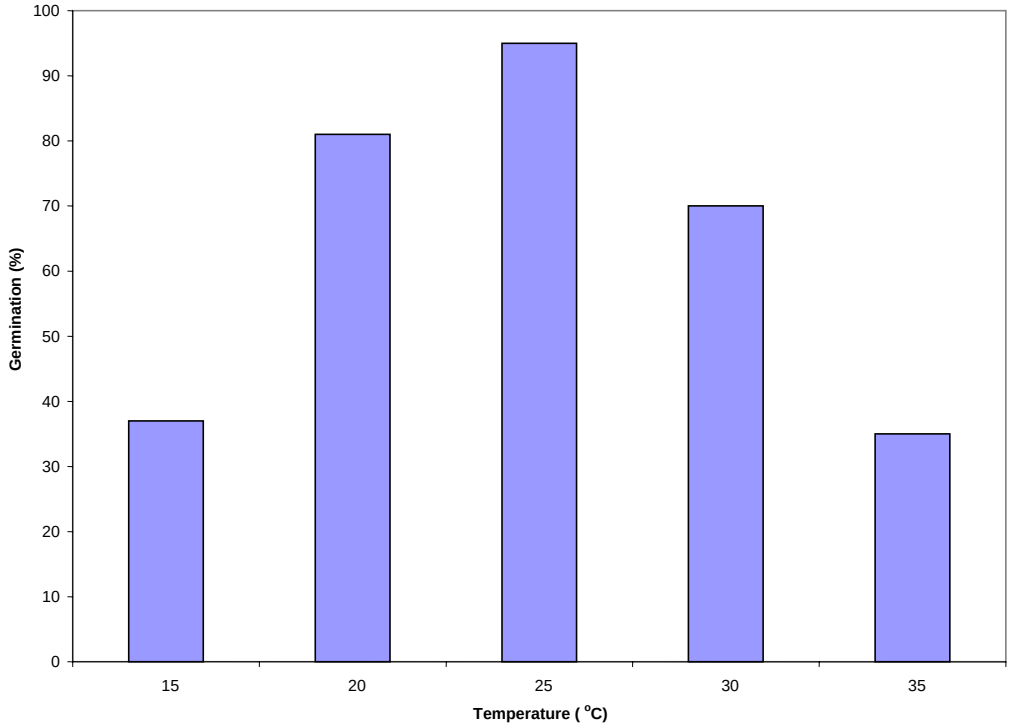


Figure 6 Effects of temperature on in vitro germination of *E. kebericho* seeds.
Bars represent ± S.D., (17 seeds were cultured in each pot with 6 replicates per treatment).

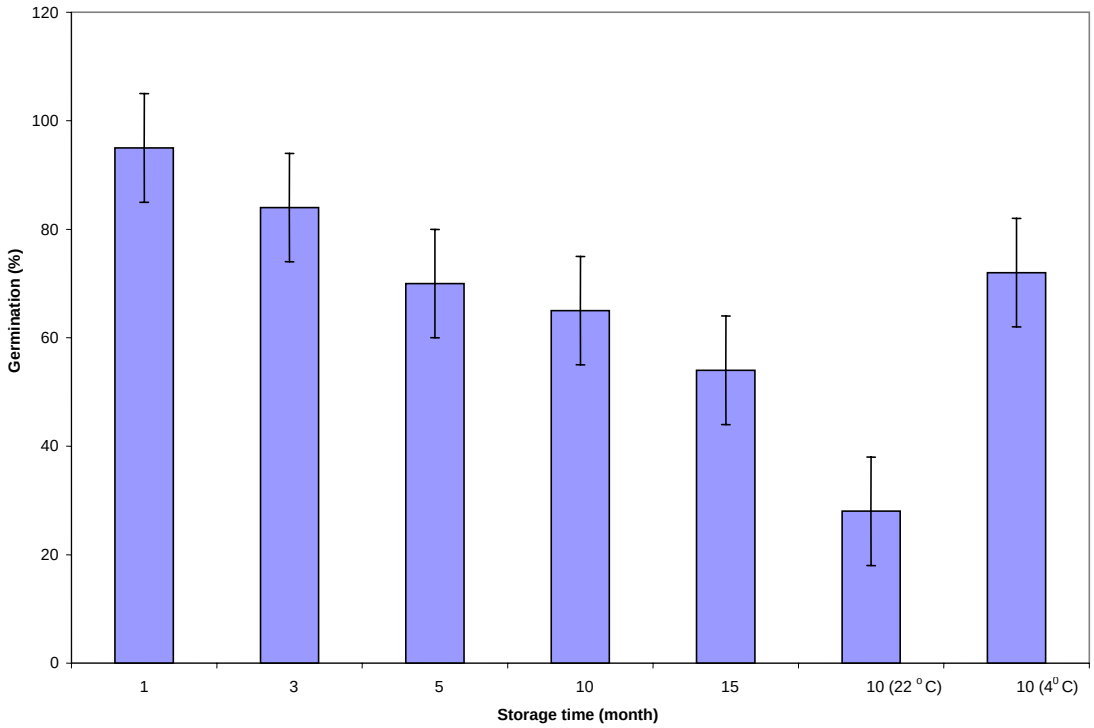


Figure 7 Effects of storage time on in vitro germination of *E. kebericho* seeds.

On the X-axis, 22 and 4° C indicate seeds stored for 10 months. Bars represent ± S.D., 17 seeds were cultured with 6 replicates per treatment.

2.1.2.5 Effects of temperature on seed germination

Seeds (i.e., propagules without fruit wall) germinated best (95 ± 2.4%) at 25° C, and percentage germination was significantly different ($p<0.05$) from those at 15, 20, 30 and 35° C (Figure 6). The least germination was obtained at 15 and 35° C.

2.1.2.6 Effects of storage time

Seed viability declined with storage time (Figure 7). The difference in seed viability stored at room temperature for 1,

3, 5 months and those stored for 10, 15, 20 months was signi cance ($p<0.05$). Seed germination at 1, 3, 5 and 10 months showed better than those stored for more than 10 months at 20^{o} C but not for 10 months at 4^{o} C. No experiment was conducted dry versus ripe seeds.

Figure 8 A) 8-month old seedlings of *E. kebericho* A) under glasshouse; and B) under nursery

2.1.2.7 Seedling survival and growth

Seedlings survival and growth depended on the type of germination media and subsequent growth environment (glasshouse versus nursery) (Table 11). Only 25 ± 15% and 32 ± 1.7% of *in vitro* origin upon transfer to the glasshouse and nursery, respectively survived 150 days after sowing.

Table 4 Percentage of seedling survival and height of *E. kebericho* seedlings germinated *in vitro*, in pots (glasshouse) and on a nursery soil, and maintained under glasshouse and nursery conditions for a period of five months (March-July 2006).

Seedlings	Seedling survival	Height of seedlings (cm)/days				
	(%)	30	60	90	120	150
A	25 ± 1.5^{a**}	5 ± 0.3^{a**}	10 ± 0.5^{b**}	13 ± 0.6^{ab**}	16 ± 0.8^{ab*}	20 ± 0^{bb*}
B	32 ± 1.7^{b}	4 ± 0.4^{a}	7 ± 0.2^{a}	11 ± 0.3^{a}	14 ± 0.5^{a}	15 ± 0.5^{a}
C	49 ± 2.2^{c}	7 ± 0.3^{b}	12 ± 0.3^{b}	16 ± 0.3^{b}	18 ± 0.5^{b}	21 ± 1.0^{b}
D	63 ± 1.9^{d}	13 ±1.0^{c}	17 ± 1.4^{c}	22 ± 1.3^{c}	26 ± 1.5^{c}	31 ± 1.9^{c}
E	94 ± 1.1^{e}	24 ± 0.8^{d}	35 ± 0.5^{d}	44 ± 0.8^{d}	55 ± 0.7^{d}	66.2 ± 0.5^{d}

*Means within each column followed by different letters (a-d) are significantly different at 0.05% probability.

****** Seedlings: A. *in vitro* germinated maintained under glasshouse; B. *in vitro* germinated and maintained under nursery; C. germinated in pots (glasshouse) monitored in the same; D. germinated in pots (glasshouse), maintained under nursery; E. germinated on a nursery, monitored under the same condition

A signi cant difference ($p<0.05$) in survival was observed between pot origin germinants divided and maintained under glasshouse and nursery conditions. Pot (glasshouse) origin germinants transferred to the nursery showed better survival than germinants evaluated under it's (the same) origin. *In vitro* germinated seedlings maintained under glasshouse and nursery was less survived compared to nursery germinated seedlings (Table 11, Figure 8).

2.1.3 Discussion

Seeds of *E. kebericho* are covered by overlapped fruit wall. The pubescent hairs are found between these walls and also on the surface of the cotyledon. This is generally the case that such overlapped walls facilitate the incorporation of pathogens, which caused seed contamination during *in vitro* germination. It is well known that many tropical species grow with pubescent hairs, which allows the penetration of pathogens into plant tissues that cause contamination. In this study, untreated seeds (control) were completely begun to be contaminated before the emergence of radicle, three days after seed sowing. It is known that the conditions *in vitro* which favor target seed germination (plant growth), i.e. high levels of nutrients, humidity and worm temperatures, also favour the growth of micro-organisms which multiply and grow rapidly affecting the germination potential (Bewley and Black, 1994; Khanna, 2003). Thus, contaminants affect plant growth /seed germination potential by growing on media, consequently reducing the pH below 3, metabolizing much of the nutrients (carbohydrate), and ultimately producing phytotoxic fermentation products such as ethanol and acetic acid (Bewley and Black, 1994; Khanna, 2003). This action will starve the carbohydrate of the plant tissue, make certain nutrients unavailable, and ultimately causes toxic effects through the production of secondary metabolites like phenol oxidates (Bewley and Black, 1994; Khana, 2003). However, sterilized seeds of *E. kebericho* with 10% sodium hypochlorite and 70% ethanol for five and nine minutes, respectively highly reduced the growth of fungi, and consequently best germination was achieved (Table 29).

Germination percentages and seedlings growth were highly variable for *E. kebericho* sown in pots containing different ratios of soil mixture maintained under glasshouse conditions (Figure 13). This germination variability is probably due to the (1) different ratios of soil properties, including the modifications in texture and structure; (2) confinement of the root system within a limited space (in pots) which retards the developmental status of the root and shoots as plants grow older; and (3) competition for light as plants grow bigger and start to crowd one another and with other species maintained under the same condition. This is in agreement with study report of Legesse Negash (2004a) on seed-based propagation of *Prunus africana.*

Uniform seed germination and orthotropic developmental pattern of the seedlings on a nursery bed under nursery condition (Fig. 1A) possibly due to the following major reasons: (1) the fertile nature of the soil of the study area (Kifle Berhanu, 1997) and surface-mixed animal manure (horse dung) might have enhanced the process of germination provided that in the presence of organic compounds in urea of this additive; (2) exposure of seedlings to unlimited light and other environmental conditions (humidity, temperature), may become appropriate resources; (3) with site preparation soil texture and structure may be modified such that pore size is increased, thus becoming available to bacterial decomposition and mineralization; (4) the increased soil temperature of prepared soil can affect the availability of soil nutrients positively, as decomposition of organic matter is increased by warm soil temperatures and by mixing with mineral soil; and (5) site preparation also modifies aeration by decreasing bulk density. However, sometimes these changes in soil properties can have both beneficial and detrimental effects on seedling morphology (Sutton, 1991; 1993). For example, high nutrient and moisture availability, as well as warmer soil temperatures benefit shoot and root biomass growth (Orlander *et al*., 1996; Sutton, 1991), whereas high soil bulk density can reduce height growth and root elongation. In addition, high Nitrogen promotes branch production and modifies biomass allocation patterns in seedlings. The change of soil texture and structure with site preparation has been reported by various authors in optimizing soil nutrient contents (Hassink, 1997; 1999; Meke *et al*., 2000), aeration (Ritaric and Lahde, 1978), temperature (Kubin and Kemppainen, 1994; Fleming *et al*., 1999), and water (Winsa, 1995) for plant growth.

In summary, *in vitro* and *ex vitro* germination and plant regeneration protocol was established that allows producing multiple seedlings. The value of such basic protocol for *E. kebericho* is many-fold. Above all, it can be used as a means to preserve germplasm of this endangered species of high medical importance. Further on, a rapid and efficient method of plant multiplication is of particular importance in perennial species such *as E. kebericho*.

3. Seed-based and vegetative propagation of *Glinus lotoides* L.

3.1 Effects of several factors on seed germination and vegetative propagation by stem cuttings

Seed is a mature ovule, which consists of an embryo, stored food and seed coat (Bewley and Black, 1994). Propagation by seed is one means of continuity of plant life and assuring of plant species survival (Copland and McDonald, 1994). Many authors define seed germination differently, and in this study the seed of *G. lotides* is

germinated with the emergence of radicle through the seed coat. However, seed germination is influenced by different factors. These are: (1) morphological and physiological dormancy, which inhibits water uptake and gaseous exchange (Bewley and Black, 1994; Hartmann *et al*., 2002); However, several scarification methods (mechanical, hot water and acid) and hormone treatments have been successfully used to break seed dormancy. Evidence for hormone involvement in breaking seed dormancy comes from the correlations of hormone concentration with specific developmental stages, effects of applied hormone, and the relationship of hormones to metabolic activities (Hartmann *et al*., 2002). Of the growth regulators (plant hormones) gibberellin (GA_3) compromise as one of the classes most directly implicated in the control and promotion of seed germination (Frank and Cleon, 1992; Michael *et al*., 2003; Lodge, 2005); (2) contaminants that release phenol exudates and inhibit germination, however, prevented by the use of antioxidants such as ascorbic aid acetic acid and charcoal; and (3) several germinations conditions such as temperature, moisture, aeration and media (Michael *et al*., 2003). These germination conditions play a pivotal role to bring about enzyme activation, break down, translocation and use of storage material, denaturation of proteins, dormancy induction and release as well as in the oxidation processes (Bewley and Black, 1994).

A cutting is another means of vegetative propagation in plants. It has many advantages such as being economical, not requiring much space and is rapid with simple technique. Cuttings can be made from the stem, modified stem, roots or leaves. In vegetative propagation by stem, cuttings can be made from the shoots, either from the apical or basal buds and terminal or lateral buds, which are capable of developing adventitious roots and then develop to a complete plant (Hartmann *et al*., 2002). However, the rooting success of cuttings is dependant on factors such as position of the cuttings on the shoots, rooting medium used, presence or absence of hormone and concentration, season when the cuttings were made as well as physical and environmental factors (Hansen, 1986; 1988; Leaky *et al*., 1997). The effect of these factors on germination and rooting of cuttings on *G. lotoides* is not known.

The aim of this study was therefore to: (1) study the effects of seed pretreatments, storage time, and optimal germination medium; and (2) examine the effect of cutting position, rooting medium and cutting treatments on the propagation of *G. lotoide.*

3.2 Materials and methods

3.2.1 Plant material

G. lotoides with white and green capsules were (1) purchased from the merchants, trade in Markato (Addis Ababa) throughout the experimental period and stored at room temperature (22 $\pm$ 1^{o} C) until used for the experiments. *In vitro* germination experiments with white capsules (mature) were selected after trials. The experiment was conducted at Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, (Germany) between September 2005 and February 2006. Whereas, *ex vitro* germination and vegetative propagation by stem cuttings were carried out in a glasshouse found with in a campus of the College of Jimma Agriculture and Veterinary Medicine (Jimma University) between March 2006 and February 2007. The effect of seed storage was conducted on seeds collected from the established seedlings followed by seed-set under glasshouse conditions.

3.2.2 *In vitro* seed germination

Seeds of *G. lotoides* were released from the capsules using a scalpel, cleared on a white paper, surface disinfected first in 70% (v/v) ethanol (3 min.), then in 10% (v/v) sodium hypochlorite solution (5 min.) and rinsed several times using double distilled water under a laminar flow hood. Two pretreatment experiments on seeds were conducted following preliminary trials. In the first experiment, the seeds were soaked under the surface of 10^{-3} and 10^{-4} M GA_3 solution for 16 hours. In the second experiment, the seeds were soaked in beaker (250 ml) containing 100 ml water at 70^{o} C for 10, 20, 30 or 40 minutes, and then cultured on the germination media. The germination medium consisted of MS minerals and vitamins {9} supplemented with 8 g l^{-1} phytoagar (Sigma, St Louis MO, USA), whereas no sucrose was added. The different media were stirred; the pH adjusted to 5.75, autoclaved at 121^{o} C for 20 minutes, and then cooled down in a water bath to about 50^{o} C. The media (100 ml each) were then dispensed in glass culture vessels (100 mm x 150 mm; length and width, respectively). Fifty five seeds per vessel were cultured with 2 replicates per treatment. All cultures were inoculated at 25^{o} C under cool fluorescent (40 μmol m^{-2} s^{-1}: 16 photoperiod). Seed germination indicated by at least radicle emergence was recorded at days 25, 30, 35, and 40.

3.2.*3 Ex vitro* seed germination

Unsterilized seeds of *G. lotoides* were obtained as described for *in vitro* germination, and sown in plastic pots (40 x 35

cm, length and width, respectively) containing a mixture of nursery soil, cattle dung, and sand in a ratio of (2.0:1.0:0.5, 1.5:1.0:0.5, 1.0:1.5:0.5, 0.5:2.0:0.5, respectively). Hundred to hundred twenty seeds per pot were sown per treatment. The pots were randomly arranged in the glasshouse. The mean minimum and maximum temperatures of the glasshouse during the study period were 10.8 ± 1.0° C (nights) and 24.5 ± 2.0° C (days), and the relative humidity (RH) was ranged from 64 to 75% throughout the experimental period. Seed germination indicated by at least radicle emergence was recorded at days 10, 13, 16, and 19. The effect of storage time was conducted with seeds stored for 2-, 5-, 8-, and 11-month-old. These were sown in pot containing a mixture of nursery soil, cattle dung, and sand with equal proportions that gave the highest percentage germination (Table 2). The mean minimum and maximum temperatures of the glasshouse during the study period were 11.8 ± 1.0° C (nights) and 28.5 ± 2.0° C (days), respectively. The relative humidity (RH) ranged from 61 to 73%, and was maintained roughly throughout the experimental period by sprinkling the floor of the glasshouse with water. RH was measured using a Humidity and Temperature Sensor (Type HP- 100-A., Umweltanalytische Mess-System GmbH, Munic, Germany)

3.2.4 Vegetative propagation experiments

IBA preparation, cutting source and treatments

A stock solution of 0.25% (W/V) indolebutyric acid (IBA) (SIGMA Chemical Company, St. Louis, MO, USA) was prepared by dissolving the hormone in a mixture of absolute ethanol and methanol in a 1:1 ratio. Concentrations of 0.05, 0.10, 0.15, and 0.20% were prepared from the stock solution by serial dilution. Selection of 2-, 3-, and 4-months-old pot (glasshouse) germinated seedlings was made on the basis of true-to-name and type, free of disease and insects and in proper physiological state as a source of cuttings. Shoots of 14 to 16 cm long were cut from the seedlings early in the morning (06:30 and 07:30 pm), wrapped with wet tissue paper followed by immediately placing them in plastic bags in order to keep them cool and turgid until taken to the laboratory. Apical and basal cuttings were trimmed to 2-3 mm in diameter, 70 – 80 mm in length. The base of each randomly allocated cutting was then treated with 10 µl IBA at concentrations of 0, 5, 10, 15, 20, µg IBA/ cutting. This range was selected on the basis of results from a preliminary experiment. The numbers of cuttings to each treatment ranged from 100 to 105, and the controls comprised comparable numbers of cuttings treated with the solvent only. The cuttings were separately packed in polyethylene bags, and taken to the glasshouse, and then inserted in perforated plastic pots (10 x 8 cm, length and width, respectively) containing a rooting medium. The rooting medium consisted of a mixture of sand, nursery soil and animal manure in a ratio of [(A) 1.00:1.00:1.00, (B) 2.00:1.00:1.00, (C) 1.00:2.00:1.00, (D) 1.00: 1.00:2.00 (E)

1.00:1.00: 0.00, respectively)]. The glasshouse condition during the study period was as described above for pot experiments. Assessments of the survival and rooting of cuttings were made 3 weeks after the start of the experiment. Data on the numbers of rooted cuttings, root number, and root length were collected just before transplantation.

3.3 Results

3.3.1 Effects of gibberellins and temperatureTable 5 Effects of pretreatments on *in vitro* germination of *G. lotoides* seeds.

The germination of *G. lotoides* seeds was influenced by the pretreatments of hormone and hot water as was adjusted by the concentrations of GA_3 and time-range scarifications, respectively (Table 12). Seeds treated with hot water (70^o C) for $\geq$ 40 minutes did not show seed viability. No significant difference was observed in germination percentage between seeds treated with GA_3 (10^{-3} and 10^{-4} M) and hot water (100^o C) for 10, 20 or 30 minutes compared to control 40 days after seed sowing (Table 5).

Table 5

Treatments	Germination (%)			
	Day 25	Day 30	Day 35	Day 40
Control	16 ± 0.54[a*]	18 ± 0.13[a*]	25 ±0.23[a*]	34 ± 0.23[a*]
10^{-3} M GA_3	17 ± 1.40[a]	17 ± 1.45[a]	25 ± 1.05[a]	34 ± 0.67[a]
10^{-4} M GA_3	15 ± 0.24[a]	19 ± 0.34[a]	25 ± 0.34[a]	32 ± 0.24[a]
10^{-5} M GA_5	17 ± 2.34[a]	23 ± 0.13[a]	28 ± 0.43[a]	33 ± 0.24[a]
10^{-6} M GA_3	17 ± 1.65[a]	23 ± 0.76[a]	26 ± 1.54[a]	31± 1.23[a]
Hot water (70^oC)/min.				
10	15 ± 0.43[a]	21 ± 2.3[a]	26 ± 0.54	32 ± 0.32[a]
20	16 ± 0.23[a]	22 ± 0.13[a]	28 ± 0.24[a]	34 ± 0.34[a]
30	15 ± 0.43[a]	26 ± 0.24[a]	30 ± 0.54[a]	35 ±0.23[a]
40	-	-	-	-

*) Means with standard deviations within the same column followed by same letters (a) are not significantly different ($P < 0.01$).

The germination of *G. lotoides* seeds was also influenced by the mixture of soil as this was adjusted by the ratios of nursery soil and cattle dung (Table 13). The germination percentage gradually decreased with increases with cattle dung or sand ratios, respectively. The best germination (91.6%) was obtained with the seeds sown in pot in a ratio of 2.00:1.00:0.50 (nursery soil, cattle dung, and sand, respectively). All subsequent experiments were therefore performed using this treatment to study the effect of storage time or as a source of cuttings for vegetation propagation methods.

The final germination percentage between seeds stored at room temperature (22 $\pm$ 1^{o} C) for 2-months, and those stored for 5, 8, and 11 months was significant ($P<0.05$). Seeds collected from glasshouse seedlings (under the experiment) and stored for 2 and 5 months gave better germination than seeds with white capsules purchased from Mankato and stored for more than 8 and 11 months. Mean germination percentage dropped as low as (31.6%) with seeds stored for 11 months (Table 14).

Table 6 Effect of storage time on the germination of *G. lotoides* seeds.

Months	Germination %			
	Day 10	Day 15	Day 20	Day 25
0	70 ± 1.4^{a*}	78 ± 1.56^{a*}	87 ± 1.40^{a*}	98 ± 2.31^{a*}
2	62 ± 1.23^{b}	75 ± 0.12^{b}	84 ± 0.03^{b}	94 ± 2.05^{b}
5	42 ± 1.23^{c}	54 ± 0.12^{c}	68 ± 0.02^{c}	77 ± 2.42^{c}
8	33 ± 0.23^{d}	50 ± 0.12^{d}	44 ± 0.03^{d}	53 ± 2.35^{d}
11	22 ± 0.23^{e}	26 ± 0.23^{e}	29 ± 0.02^{e}	32 ± 0.38^{e}
14	-	-	-	-

*) Means with standard deviations within the same column followed by different letters (a-e) are significantly different ($P < 0.01$)

Table 7 Effects of soil ratios on the germination of *G. lotoides* seeds sown in pot, maintained in the glasshouse

Soil ratio*	Germination (%)			
	Day 10	Day 15	Day 20	Day 25
A	$62 \pm 0.06^{a**}$	$75 \pm 0.34^{a**}$	$82 \pm 0.43^{**}$	$92 \pm 0.54^{a**}$
B	43 ± 0.23^{b}	55 ± 0.45^{b}	65 ± 0.25^{b}	72 ± 0.56^{b}
C	24 ± 0.05^{c}	33 ± 0.43^{c}	42 ± 0.42^{c}	47 ± 0.45^{c}
D	-	-	-	-

*A) 2.0:1.0:0.5 B) 1.5: 1.0: 0.5 C) 1.0: 1.5: 0.5, D) 0.5: 2.0: 0.5 (nursery soil, animal manure, sand, respectively).

**) Means with standard deviations within the same column followed by different letters (a-c) are significantly different ($P < 0.01$).

3.3.3 Interactive effects of cutting position and hormone treatment

G. lotoides cuttings were found to be sensitive to cutting position rather than to applied hormone treatment in terms of adventitious root development (Table 15). A significant difference ($P<0.01$) was observed between the apical and basal cuttings in rooting percentage, root number and root length in all treatments. In both cuttings the rooting percentage decreased with increasing IBA concentrations (i.e., from 0.05 to 1.5%). Similarly, the relative numbers of roots and root length obtained from apical cuttings were significantly different from basal cuttings in all treatments including control. However, the highest rooting percentage (90.2%), root number (8.02) and root length (6.18 cm) was obtained from apical cuttings without hormone treatment.

Table 8 Interactive effects of cutting position and hormone treatment on rooting percentage, root number and root length of *G. lotoides*.

IBA concentrations (µg)	Cutting position	Rooting % ± S.E	Root ± S.E	
			Number	Length (cm).
00	Apical	90 ± 0.02^{a*}	8 ± 0.09^{a*}	6 ± 0.06^{a*}
	Basal	64 ± 0.02^{b}	3 ± 0.117^{b}	3 ± 0.12^{b}
05	Apical	81 ± 0.03^{a}	14 ± 0.07^{a}	5 ± 0.07^{a}
	Basal	49 ± 0.02^{b}	8 ± 0.12^{b}	3 ± 0.12^{b}
10	Apical	76 ± 0.03^{a}	8 ± 0.12^{a}	3 ± 0.15^{a}
	Basal	35 ± 0.03^{b}	4 ± 0.10^{b}	3 ± 0.07^{b}
15	Apical	52 ± 0.03^{a}	6 ± 0.17^{a}	3 ± 0.07^{a}
	Basal	27 ± 0.02^{b}	3 ± 0.18^{b}	2 ± 0.09^{b}

*Means with standard deviations within the same column followed by different letters (a-b) are significantly different ($P < 0.01$).

3.3.4 Effect of rooting medium

Different ratios of soil were tested as a rooting medium. A significant difference ($p<0.01$) was observed with decreasing either sand or increasing nursery soil or cattle dung with respect to the percentage of rooted cuttings, root number and root length (Table 16). The rooting percentage, root number and root length obtained from A and B were significantly different ($P< 0.01$) from the C and D, which in turn showed significance. Equal proportions of soil ratios (1:1:1) showed best rooting in all variables followed by increasing sand or decreasing nursery soil or cattle dung. Whereas, increasing in a ratio of nursery soil or cattle dung showed deterioration in rooting percentage, root number and root length.

Table 9 Effect of rooting medium on the rooting percentage, root number and root length of *G. lotoides* without hormone treatment.

Age/month-old	Survival (%)			
	Apical		Basal	
	Without	With	Without	With
1	95 ± 0.45[a*]	64 ± 0.25[a*]	81 ± 1.67[a*]	54 ± 0.65[a*]
2	74 ± 0.46[b]	53 ± 0.25[b]	63 ± 1.45[b]	45 ± 0.65[b]
3	65 ± 0.56[c]	44 ± 0.42[c]	63 ± 1.54[c]	32 ± 0.23[c]
4	52 ± 0.65[d]	32 ± 0.43[d]	56. ± 1.43[d]	25 ± 0.54[d]

*Means with standard deviations within the same column followed by different letters (a-c) are significantly different (P < 0.01). ** (A) 1.00:1.00: 1.00 (B) 1.50: 1.00: 1.00 (C) 1.00: 0.50: 1.50 (D) 0.50: 1.50: 1.00 (Sand, nursery soil and cattle dung, respectively).

Table 10 Percentage survival of apical and basal rooted cuttings derived from 1-, 2-, 3-, and 4- month-old treated with 05 µg IBA or without hormone treatment.

Treatment **		Root	
	Rooting (%)	Number	Length (cm)
A	98 ± 2.35[a*]	12 ± 1.23[a*]	7 ± 1.30[a*]
B	95 ± 1.34[a]	11 ± 1.34[a]	7 ± 1.24[a]
C	64 ± 1.44[b]	5 ± 1.20[b]	5 ± 0.45[b]
D	42 ± 1.54[c]	3 ± 1.40[b]	5 ± 0.46[b]

*)Means with standard deviations within the same column followed by different letters (a-d) are significantly different (P < 0.01).

3.3.5 Survival of rooted cuttings

The percentage of survived rooted cuttings gradually decreased with increasing age of seedlings both in apical and basal cuttings. However, survived rooted cuttings without hormone treatment were significantly (P<0.01) higher than

those with hormone treatment (Figure 9).

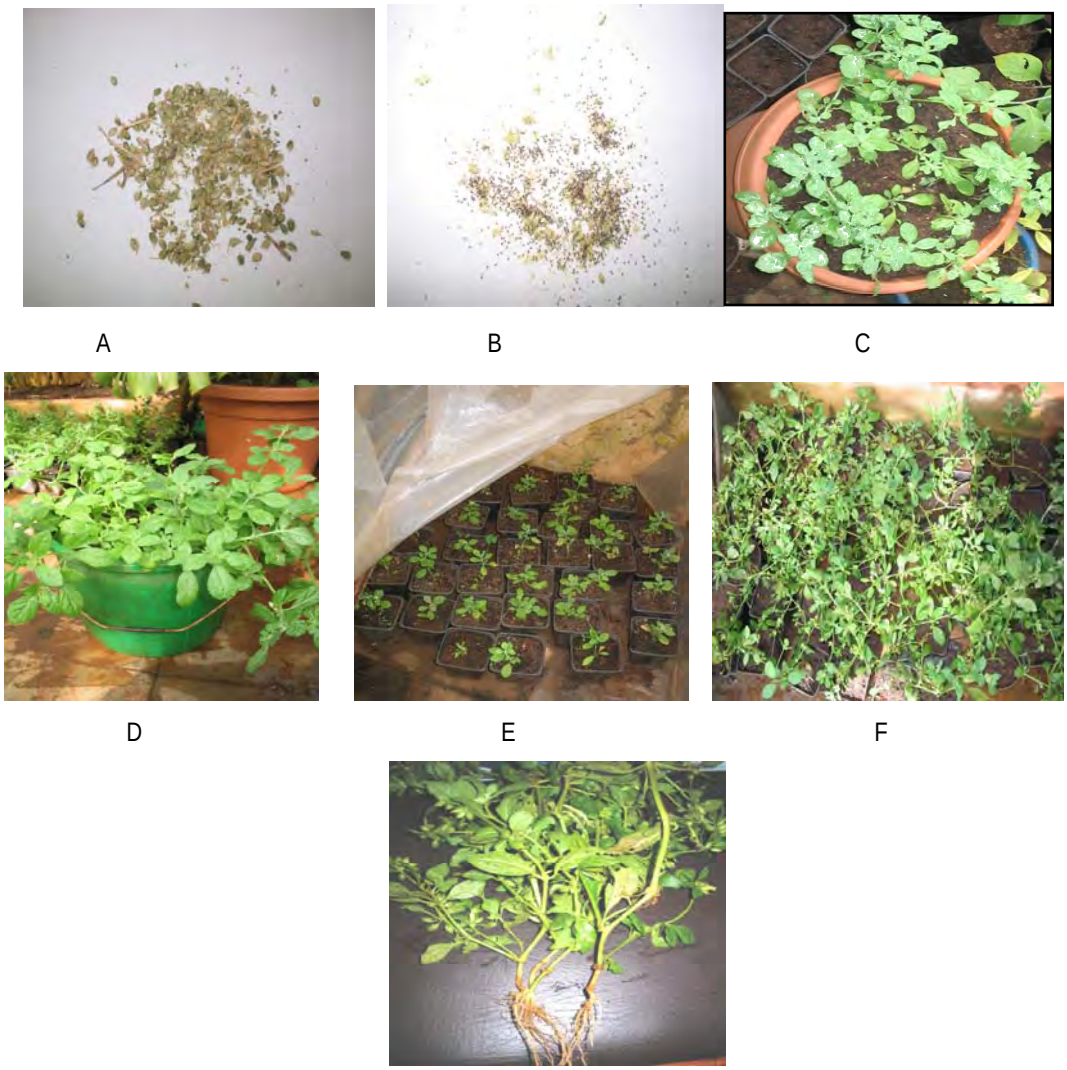


Figure 9 Seed-based (A-D) and vegetative (E-G) propagations of *G. lotoides*:

A) Capsules: B) seeds: C) one-month-old seedlings; D) two-month-old seedlings; E) Establishment of cuttings; F) rooted cuttings; G) rooted cuttings with fructification.

in all age groups. No significant difference was observed in the least survival of apical and basal rooted cuttings derived 4-month old seedlings (Table 17).

3.4 Discussion

The most important factor for the germination of *G. lotoides* seeds is the germination medium or conditions. This was probably the main cause for less *in vitro* germination (Table 1) eventhough no contamination was observed during seed culture. In contrast, the highest percentage and rate of *ex vitro* germination obtained in pot (glasshouse) containing 2:1:0.5 (nursery soil, animal manure, sand, respectively) may indicate that the germination process is enhanced in the presence of established similar microclimate. In addition, it may be associated with the availability of soil microorganisms (symbiotic germination) found in the same soil mixture. Similarly, numerous reported studies provided evidence that the germination of seeds of various plant species strongly depends on (1) the interaction of the seeds with soil microorganisms such as mycorrhizae (John, 1990), and (2) the presence of species own microclimate (Legesse Negash 2003). However, the *ex vitro* germination percentages, following the survival and growth of seedlings were highly variable for *G. lotoides*. This variability is probably due to the (1) different ratios of soil properties, including the modifications in composition, texture and structure in relation to the species *in situ* soil type ; (2) confinement of the root system within a relatively high ratio of sand (density), which drops aeration space, and consequently retards the developmental status of the root and shoots as plants grow older; and (3) competition for nutrients and light as plants grow bigger and start to crowd one another and with other species maintained under the same condition. This is in agreement with study report of Legesse Negash (2004a) on seed-based propagation of *Prunus africana.* On the other hand, uniform, rapid seed germination and orthotropic developmental pattern of the seedlings on a relatively high ratio of nursery soil or animal manure possibly due to the following major reasons: (1) the fertile nature of the nursery soil of the study area (Kifle Berhanu 1997) and surface-mixed animal manure may enhanced the process of germination provided that in the presence of organic compounds in urea of this additive; (2) exposure of seedlings to unlimited light and other environmental conditions (humidity, temperature), may become appropriate resources; (3) with soil preparation, the texture and structure may be modified such that pore size is increased, thus becoming available to bacterial decomposition and mineralization; (4) the increased soil temperature of prepared soil can affect the availability of soil nutrients positively, as decomposition of organic matter is increased by warm soil temperatures and by mixing with mineral soil; and (5) soil preparation also modifies aeration by decreasing bulk density. However, sometimes these changes in soil properties can have both beneficial and detrimental effects on seedling morphology (Sutton, 1991; 1993). For example, high nutrient and moisture availability, as well as warmer soil temperatures benefit shoot and root biomass growth (Orlander *et al*., 1996; Sutton, 1991), whereas high soil bulk density more inactive that can reduce height growth and root elongation. In addition, high Nitrogen promotes branch production and modifies biomass allocation patterns in seedlings. The change of soil texture and structure in soil preparation has been reported by various authors in optimizing soil nutrient contents (Hassink, 1997; 1999; Meke *et al*., 2000), aeration (Ritaric and Lahde, 1978), temperature (Kubin and Kemppainen, 1994; Fleming *et al*., 1999), and water (Winsa, 1995) for plant growth.

Lack of significant germination to applied GA_3 (Table 12) or hot water compared to control suggests that seeds of *G. lotoides* are non-dormant, and that induction of germination is not under the control of phytochrome system in agreement with Legesse Negash (2003a). It has been proposed that seeds whose germination is stimulated by applied GA_3 are regulated by the phytochrome. The effect of GA_3 on the synthesis of hydrolytic enzymes increase is well established (Bewley and Black, 1994; Hartmann *et al*., 2002).

Decreased percentage germination with increasing temperature (Figure 10) shows the effects of elevated temperature on the viability of *G. lotoides* seeds. It is reported that, in germinating seeds of other many plant species, high temperature causes greater membrane and embryo damages through lipid peroxidation (Berhanu Erko, 2006). A number of studies also indicated that different temperature optima for different plant species (Legesse Negash, 1992; 2003a).

G. lotoides are characterized by a short viability period when stored at room temperature, thus losing their capacity for germination (Figure 11). In this study, this was confirmed by comparing the germination of seeds collected from glasshouse seedlings (under the experiment) stored for 2- and 5- months with mature (white versus green) seeds purchased from Markato and stored for more than 5 months. Since seed longevity is determined by the interaction of seed moisture content and temperature (Bewley and Black, 1994; Hartmann *et al*., 2002) more extensive studies have to be conducted so as to determine (the range of) optimal storage temperature for *G. lotoides.*

The rooting ability of *G. lotoides* cuttings was sensitive to cutting position and age of stockplants rather to hormone treatments. In this study, apical cuttings derived from younger seedlings without hormone treatment recorded highest percentage of rooting, root number and root length per rooted cuttings relatively than the basal ones. It may be true for most herbaceous plants, since there are paranchymatous/ground tissues in the younger portion than dermal and vascular tissues in such plants. Similar studies were also reported by Palanisamy and Kumar (1997) in rooting of neem (*Azadirachta indica* A. Juss.), where cutting from the upper part of the branches derived from younger seedling cutting source rooted better than the lower ones. This was also supported in the propagation of *Grindelia chiloensis* where none of the basal cuttings rooted (Wassner and Ravetta, 2000). In addition, stem cuttings of *Rosmarinus officinalis* L. (Deen and Mahamoud, 1996), *Triplochiton scleroxylon* (Leaky, 1989), showed a gradual reduction in rooting percentage recorded with distance from the apex and older ages. This difference in rooting percentage of

apical and basal cuttings of *G. lotoides* could be due to high concentration of endogenous root promoting substances in the apical cuttings which arise from the terminal buds, and also" more cells" which are capable of becoming meristematic (Hartmann *et al*., 1983). In contrast, basal cuttings and old source cuttings could be too mature and highly lignified to develop roots than apical cuttings (Hartmann *et al*., 2002).

However, it has been reported that the basal cuttings are the best rooting materials in woody plants (Hartmann *et al*, 1983). This is probably due to the difference in the chemical composition of the shoots (position where the cuttings where taken; apical or basal), age of the stem, or due to carbohydrate accumulation or bud growth (especially basal cuttings may have root promoting substances from buds and leaves). In deciduous species where no carbohydrate or root promoting substances are present, cuttings from the soft shoot of the plant root best (Hartmann *et al*, 1983). Many authors (Leaky, 1983; Hansen, 1986; 1988) also reported results in agreement with the general statement of (Deen and Mahamoud, 1996) that the best rooting of cuttings is usually found from the basal portions of shoots in woody plants.

Many authors stated that rooting hormones such as auxins have an important role in the development of adventitious roots, increasing rooting percentage, improving quality of roots and uniformity in rooting of cuttings. When rooting hormones are applied especially in difficult to root plants, clear difference in rooting was reported (Hansen, 1986; 1988; Hartmann *et al.,* 1990). However, plants that root easily do not respond to the application of rooting hormone (Deen and Mahamoud, 199616). Rooting percentage of *G. lotoides* that root easily did not respond well to the application of rooting hormone. Similar study was also reported by (Ofori *et al.,* 1996) when no significance in rooting percentage was obtained in vegetative propagation of *Gnetum africanum* Welw., when IBA from 0-250 µg was applied. A similar result was also reported in *Milicia excelsa* with the application of IBA (Newton and Leaky, 1996). In this study, the negative response of *G. lotoides* cuttings to rooting percentage with rooting hormone application could be due to the high supplement of endogenous auxins in the shoots of the plant and these auxins (IBA) might interact negatively with the application of exogenous rooting hormones.

The necessity of establishing an optimum rooting medium depends on the species with respect to the development of adventitious root (Hartmann *et al*., 2002). Many authors suggested as there is no ideal rooting medium even for most species (AL-Saqri and Alderson, 1996; Ofori *et al*., 1996). This study also shows that the percentage of rooting, root

number and length of *G. lotoides* vary from each other depend on different rooting media. However, the highest ratio of sand provided positive results compared to other soils ratio. Perhaps the microclimate created by adding more sand than organic matter has enhanced the rooting, because the species normally grows in inundated habitat. Similarly, numerous reported studies support that a high proportion of sand component is more suitable for the rooting of cuttings due to its capacity to hold moisture and suitability for aeration (AL-Saqri F and Alderson, 1996; Newton and Leaky, 1996; Palanisamy and Kumar, 1997; Hartmann *et al*., 2002).

In summary, a rapid seed-based and vegetative propagation by stem cuttings protocol was established that allows the regeneration of many seedlings and plantlets, respectively. The value of such basic protocol for *G. lotoides* is many folds: Above all, it is used to preserve germplasm of this threatened species of high medicinal importance. It is also applicable for the establishment of commercial production under glasshouse conditions. Germination of seeds *in vitro* (eventhough less) is also used to obtain sterile seedlings for the investigation of medicaments.

4 Phenology in *Plumbago zeylanica* L.

4.1 Effects of glasshouse versus nursery conditions, seasonal climate, plant size and growth hormone application.

Phenology is defined as the study of the timing of biological events or phenophases in plants or animals and their relationship with seasonal climate (rhythm) (Maria *et al*., 2002). Plant phenological measurements/studies are currently made via two different approaches: (1) Ground-based observation phenology; it offers a high temporal resolution and detailed information about individual species (Ricotta and Avena 2000; Schwartz *et al*., 2002; White *et al*., 2005); (2) Satellite-derived phenology; as a complementary observation method, offers full spatial coverage on a global scale while providing temporal information about radiative characteristics of vegetation. This study is conducted on ground-based observation of *P. zeylanica* L. Ground-based observation of the phenology of medicinal plants has numerous advantages: (1) to identify the time and duration of propagule production in the wild versus controlled, and consequently their collection for propagation purposes; (2) to evaluate the effects of seasonal climate on the transition from vegetative growth to reproductive development; (3) to evaluate the effects of endogenous (hormones, genes) signals on plant development (Maria *et al*., 2002) and (4) to identify monoecious vs. monoecious. For instance, seedlings maintained under optimum conditions (depending on the species) or treated by their own microclimate can achieve a full plant size within a short period of time (Hartmann *et al*., 2002). At the end of vegetative phase, the shoot

apical meristem of adult plant undergoes a dramatic change and the inflorescence is initiated during minimum growth in height and production of leaves known as a plant size (Araki *et al*., 2001). In the late inflorescence flowers comprised of different whorls of floral organs are produced and terminated with fruit/seed-set. However, the fluctuation of seasonal climate brings about changes in water availability, rainy season, temperature, photoperiod and light that alter the phenological patterns of individual plants. On the other hand, it has been reported that growth hormone treatments proved to be the most effective in inducing flowering buds initiation and increasing the number of flowers per plants. Gibberellic acid (GA_3) alone or in combination with kinetin improved the time of flowering and increased the number of flowers in (McDonald *et al*., 1986; Bonner *et al*.., 1987; Awan, *et al*., 1999). However, the effects of these factors have not been developed for *P. zeylanica.*

The main objectives of this study were therefore to examine the effects of (1) glasshouse and nursery conditions, (2) seasonal climate, (3) plant size and (4) growth hormones application on the phenology of *P. zeylanica*. The identification of the mating system was also observed through selfing, cross, bagged, controlled and open pollinations.

4.2. MATERIALS AND METHODS

4.2.1 Study area

The study was carried out at the glasshouse and nursery found within the campus of the College of Agriculture and Veterinary Medicine (Jimma University), Jimma, Oromia Region. Jimma is located between latitudes 7^o 18'N and 8^o, 56'N and longitudes 35^o 52' E and 37^o 37'E in the South-western part of the country and region. The total area of Jimma zone is 18,415 km^2, where four distinct seasons are considered throughout the year based on the kop pen's system of classification (Kifle Berhanu 1997). These include tropical high land with a short dry, tropical climate, tropical high land with winter dry and arid climatic steppe. Specifically, the study was conducted where the seasonal climate is tropical high land with winter dry season, and the mean temperature during the coldest season ranges from 3 to 18^o C. This area is covered by forest, and the soil is very fertile, black in colour, and has a good retention capacity (Kifle Berhanu 1997) (Table 1).

Table 11 Seasonal climatic conditions of Jimma Zone, Southwest Oromia/Ethiopia

Season(s)	Temperature (°C)		Rainfall
	Maximum	Minimum	(ml)
Dry (Early December to late February)	20	26	500- 1000
Rainy (Late February. to early October)	8	24	1200 – 2800
Wet (cold) (Early Oct. to late Nov.)	3	18	1200 – 2300

Source: Kifle Berhanu (1997). Socio-economic profile of Jimma Zone, Oromia, Southwest Ethiopia

4.2.2 Plant material

Mature fruits of *P. zeylanica* were collected during the 2nd week of March, 2006 from: (1) the existing population established by Professor Legesse Negash under the glasshouse of Biology Department (Science Faculty, Addis Ababa University); and (2) the garden of medicinal plant found within a campus of Ethiopian Health and Nutrition Research Institute (EHNRI). The fruits were stored at room temperature (22 ± 1° C), and consequently used for the experiment conducted at the glass house and nursery found within a campus of Jimma Agriculture and Veterinary Medicine Faculty (Jimma University) during April 2006 to March 2007 (Figure 1).

4.2.3 Glass house experiment

A total of 300 intact seeds (that is, seeds with intact seed coats) were sown in 12 plastic pots (length 33 cm; mouth, 40 cm), each contained 25 seeds, on a mixture of nursery soil and cattle dung in a 3:1 ratio. The pots were placed in the glasshouse, and watered once a day. At a height of 6 – 7 cm each seedling was uprooted and transplanted in a bottom

perforated polyethylene bags (length 20 cm; width 9 cm) containing the same ratio of soil used for flower pots, and maintained under the same conditions.

4.2.4 Vegetative phenology

Three parameters were used to study the vegetative phenological patterns: (1) the growth height of the seedlings was measured per month; and (2) the time of leaf flushing and leaf abscission was recorded through monitoring

4.2.5 Flowering phenology

Four reproductive phenological parameters were used for the study of reproductive development (Luis, 2001; Margrit, 2002; Marco and Fernando, 2004): (1) onset (date first flower opened); (2) mean flowering date (peak of flowering: the mean of the census dates during which that individual was flowering, with each census date weighted by the number of flowers in that period; (3) duration (difference between date of first and last flower) and (4) reproductive output (fruit/ seed-set number per plant) under glass house and nursery conditions.

4.2.6 Floral morphology

In order to infer the correlation between floral morphology and the pollination type, the characteristics of both reproductive and non-reproductive flower organs were investigated on 20 flowers (one per plant).

4.2.7 Flowering time and duration

To investigate floral duration, a total of 20 flowers (one per plant) were marked before they were opened, and monitored daily for 30 days.

4.2.8 Effects of growth hormones on flowering

In order to study the effect of growth hormones on flowering, glass house and nursery seedlings at growth height of 50 to 54 cm were selected and marked according to their treatments. The treatments were started 2 and 6 months after seed sowing on glass house and nursery populations, respectively. Twenty seven µl of every hormonal treatment (100 ppm GA3, 100 ppm IAA, 20 ppm kinetin, 100 ppm GA3 + 100 ppm IAA, 100 ppm GA3 + 20 ppm kinetin, 100 ppm GA3

+ 100 ppm IAA + 20 ppm kinetin), was applied on the apical and lateral shoots of the plant 7 times until the initiation of flowering buds. This amount was considered from trials, and available to spray to the number of plant species used. The effect of hormones was studied on flowering that is, early or late flowering and number of flowers per plant and thus compared with control. The date of appearance of 1st flower was recorded. The total number of flowers per plant was also counted and compared with control. The mean of 10 flowers was taken and calculated as final (Steel and Torrie, 1981).

4.2.9 Determination of pollination system

Studies on the pollination system were conducted on nursery and glasshouse-grown seedlings based on reported methods (Luis, 2001; Margrit, 2002; Macro and Fernando, 2004; Mansor *et al*., 2004), and the flowers were treated as follows: (1) control pollination was done at stigma receptive stage (stigma lobes in open condition), and between 6.30 and 7.30 am using fresh pollen; (2) in bagging and controlled selfing, buds that were about to open were covered with paper bags. While in the former the buds/flowers were left as such till fruit formation, in the latter, after flower opening the bag was removed, stigma hand-pollinated with pollen from the same flower and rebagged, repeating the process 3 - 4 times; (3) in open cross and controlled cross, buds about to open in next couple of days were carefully emasculated. While in the former such buds were left open, in the latter the emasculated buds were hand pollinated with pollen from other seedlings, covered by paper bags and bagged, and the same process repeated 3-4 times; and (4) in open pollination, unopened buds were tagged and left for natural pollination without any artificial observation. The mean minimum and maximum temperatures of the glasshouse during the study period were 11.8 ± 1.0° C (nights) and 28.5 ± 2.0° C (days), respectively. The relative humidity (RH) ranged from 61 to 73%, and was maintained roughly throughout the experimental period by sprinkling the floor of the glasshouse with water. RH was measured using a Humidity and Temperature Sensor (Type HP- 100-A., Umweltanalytische Mess-System GmbH, Munic, Germany). Germination behavior was recorded through visual observation.

4.2.10 Field experiment

A hundred meter square field was dug up and prepared around the glasshouse. The same numbers of intact seeds sown pots were planted by hand at depth of 1 cm. The same procedure described for glasshouse experiment was followed to determine vegetative and flowering phenology. The effect of corresponding climatic conditions (rainy, hot,

and cold seasons) was observed during each phenophase only on field grown seedlings of *P. zeylanica*. Data record was carried out from seedling emergence via flowering bud initiation to fruit/seed-set per month. The mean minimum and maximum temperatures of the nursery area were 10.8 ± 1.0° C (nights) and 24.5 ± 2.0° C (days), and the relative humidity (RH) ranged from 64 to 75% throughout the experimental period. Germination behavior was observed through visual observation during the emergence of radicle.

4.3 RESULTS

4.3.1 Germination phenology

Data record on seed germination was not required since germination studies have been indicated by Route *et al*. (1999) and this experiment was established as a prerequisite for subsequent phenological studies (Table 2). Nevertheless, a mixture of soil filled in pot (glasshouse) in a 3:1 ratio of nursery soil and cattle dung, respectively, and cultivated soil (under field conditions) showed the most suitable germination substrates for *P. zeylanica* seeds (visual observation). Hypogeal germination of seedling emergence with hypocotyl elongation was observed 7 to 9 days after seed sowing, where 95-100% germination was obtained after 12 days.

4.3.2 Vegetative and flowering phenology

Effects of glasshouse and nursery conditions

A significant difference ($p<0.05$) was observed between glasshouse and field-grown population in terms of vegetative growth in height, transition time from vegetative to flowering phenology, and percentage of flowering and seed-set (Table 4). Vegetative growth of glasshouse seedlings showed fast and orthotropic developmental pattern, and consequently, the initiation of flowering bud was achieved within 105 days (15 March 2006 to 30 July 2006) after seed sowing while field-grown seedlings initiated flowering buds 225 days after seed sowing (15 March to 15 October 2006) due to the influence of rainy season, affecting the vegetative growth for about three months. The number of leaves per plant was directly proportional to plant growth. However, no significant difference ($p<0.05$) was observed in plant size (height ≥ 95 cm) and leaf number (32-37) during flowering bud initiation under both conditions. The highest and final flowering and seed-set percentages (95 ± 1.7%, 85 ± 1.6%, respectively) were obtained from glasshouse population compared to the field, which was dropped as low as 50% in both flowering and seed-set (Table 4).

Flowering time and duration

Flowering time was observed at 6:30 to 7:30 pm. Flowering started from the 3rd week of July and continued till the first week of October with the peak of 15 August to 20 September 2006 under glasshouse conditions while field-grown flowered from the first week of November to the end of December with the peak of 15 November to 10 December 2006 (Table 14) or during transition from cold to hot season (season dependant). Mean floral lifespan was 6.7 days (n=20 flowers). Anther dehiscence occurred when the flowers opened but maximum pollen germination did not occur until the third day. Most stigmas were not receptive until the third day (Table 13).

Table 12 Effects of glasshouse versus nursery conditions on the phenology of *P. zeylanica*

Phenological patterns	Observations	
	Glasshouse	Nursery
Germination	6 days after seed sowing	9 days after seed sowing
Germination behavior	hypogeal	Hypogeal
Flowering time	6:30 – 7:30 pm	6:30 – 7:30 pm
Floral life span	6-8 days	5-6 days
Fructification /seed-set	7 – 8 days after flowering	5 – 6 days after flowering

Table 13 Effects of seasonal climate on the phenology of nursery grown *P. zeylanica* seedlings (March 2006 to February 2007)

Season	Observations
Rainy (Late February to early October)	Stunted vegetative growth Apical shoot damage
Transitional period	Fast vegetative growth
Wet (cold) (Early October to late November.)	Leaf flushing Flowering bud initiation Flowering Fructification and seed –set
Dry (Early December to late February)	Leaf flushing

Table 14 Average growth in height, leaf number and flowering percentage of glasshouse and nursery seedlings of *P. zeylanica* L. for the first 3 and 7 months, respectively. These differences were calculated with time. Values are means of 110 seedlings for all variables.

Month	Glasshouse seedlings			Nursery seedlings		
	Height(cm)	Leave number	F1owering (%)	Height (cm)	Leave number	Flowering (%)
15 March	$10.2 \pm 1.23^{e*}$	$6 \pm 1.51^{c*}$	-	$11.1 \pm 2.43^{f*}$	$8 \pm 2.10^{f*}$	-
April	23.3 ± 1.64^{d}	15 ± 1.45^{b}	-	19.2 ± 1.50^{e}	11 ± 0.12^{e}	-
May	51.2 ± 0.21^{c}	21 ± 1.40^{b}	-	53.3 ± 1.34^{d}	16 ± 0.13^{d}	-
June	73.4 ± 1.62^{b}	32 ± 1.31^{a}	-	-	-	-
July	$\geq 95.2 \pm 0.34^{a}$	37 ± 1.42^{a}	$72. \pm 1.03^{c*}$	-	-	-
August	-	-	85 ± 1.30^{b}	57.6 ± 1.56^{c}	21 ± 1.56^{c}	-
September	-	-	95 ± 1.36^{a}	75.2 ± 1.40^{b}	34 ± 1.49^{b}	-
October	-	-	-	95.3 ± 1.71^{a}	40 ± 1.48^{a}	$30 \pm 1.62^{c*}$
November	-	-	-	-	-	44 ± 1.70^{b}
December	-	-	-	-	-	52 ± 1.90^{a}
January	-	-	-	-	-	
February	-	-				

*Means with standard deviations within the same column followed by different letters (a-f) are significantly different ($P < 0.05$).

Table 15 Flower morphology of *P. zeylanica*

Floral size(mm)	Mean ± SD	Range	Flowers Number
Corolla length	40 ± 1.04[a*]	38- 42	20
Corolla width	10 ± 0.20[c]	10–11	20
Style length	30 ±0.32[b]	28 - 32	20
Filament length	25 ±0.0 2[d]	23 -2 6	20

*Means with standard deviations within the same column followed by different letters (a-d) are significantly different ($P < 0.05$).

Table 16 Effect of growth hormones on flowering time and number of flowers of *P. zeylanica* per treatment after 48 days

Treatments	Appearance of 1st flower bud (days)	Number of flowers per plant
Control	59	22 ± 1.33[d*]
100 ppm GA_3	53	32 ± 1.07[a]
100 ppm IAA	57	20 ± 0.76[d]
20 ppm kinetin	58	25 ± 1.54[c]
100 ppm GA_3 + 100 ppm 1AA	57	27 ± 2.63[b]
100 ppm GA_3 + 20 ppm kinetin	57	22 ± 1.36[d]
100 ppm GA_3 + 100 ppm IA.A. + 20 ppm kinetin	55	28 ± 1.42[b]

*Means with standard deviations within the same column followed by different letters (a-d) are significantly different ($P < 0.05$).

4.3.3 Effects of seasonal climate

The vegetative growth of nursery populations was stunted by rainy season compared to the glasshouse, which showed uninterrupted phenological transitions being under controlled conditions (Table 13). During rainy season (late February to early October), the shoot buds (apical shoots) of field-grown populations were continuously damaged by heavy rainy fall, and the maximum growth was limited to less than 55 cm in height, when glasshouse seedlings achieved a height of ≥ 95 cm and begun flowering bud initiation. Fast vegetative growth and leaf flushing of nursery seedlings was observed during the transition period of rainy to wet season. The initiation of flowering buds and flower opening occurred during cold season, which was accompanied by low temperature, especially in early morning to 3° C (early October to late November). However, ultimately, a relative low flowering, and consequently low fructification and/or seed-set were obtained on field compared to glasshouse population (Table 14).

4.3.4 Effects of growth regulators on time and number of flowering

Time of flowering

The date of appearance of first flower bud was noted in treated as well as in control plants. The first flower bud appeared after 59 days in control. In 100 ppm GA_3 treated plants it appeared 6 days earlier in comparison to control. On the other hand, the flowering in IAA treatments was delayed only for 2 days when compared with control. In the kinetin treatments it appeared after 58 days. In the mixed doses of 100 ppm GA_3 +100 ppm IAA, 100 ppm GA_3 + 20 ppm kinetin and 100 ppm GA_3 + 100 ppm IAA + 20 ppm kinetin the first flower bud was observed after 57 days. In the combination of 100 PPM/AA +20 PPM kinetin the flowering was delayed for 4 days when compared to control. Extraneous IAA application did not reveal any significant effect when compared with control (Table 16).

Number of flowers per plant

The numbers of flowers per plant in treated as well as in control counted after 65 days. In control the mean numbers of flowers were 22.5. The 100 ppm GA_3 proved to be most effective thus increasing the number to 32.6 when compared to control. In the mixed dose the 100 ppm GA_3 + 100 ppm IAA an increase of a 25.7 was observed over the control. The 100 ppm GA3 + 20 ppm kinetin showed an increase in comparison to control, which was 26. The 100 ppm IAA + 20 ppm Kinetin treatments proved to be ineffective. When all the three hormones were applied simultaneously, the number of flowers was 28 in comparison to 22.5 in control (Table 16).

4.3.5 Floral morphology

Mean morphological measurements are shown in Table 15. The mean length of the corolla tube was 40.2 and the style slightly exceeded the rim of the corolla. The filaments remain 10.1mm below the stigma. Thus, pollinators encounter the stigma first and then anthers before reaching nectar that are secreted at the bottom of the corolla. Access to the interior of the flower for pollinators is only possible from the apex. The narrow diameter of the corolla only allows an access to those pollinators with sharp bill (Table 15).

Table 17 Effects of pollination methods on *P. zeylanica*

Treatment	Glasshouse		Nursery	
	Fruit set (%)	Seed set (%)	Fruit set (%)	Seed set (%)
T1(Natural pollination)	87±0.42^{b*}	81 ±2.43^{b*}	67 ±0.65^{b*}	55 ±0.76^{b*}
T2 (bagging)	0.00	0.00	0.00	0.00
T3 (controlled selfing (xenogamy)	7±0.56^{c}	5 ±3.42^{c}	3 ±0.45^{c}	2 ± 0.23^{c}
T4 (Open cross)	87 ±0.34^{b}	81 ±4.31^{b}	67 ±0.63^{b}	63 ±2.43^{a}
T5 (controlled cross)	92 ±0.52^{a}	85 ±3.55^{a}	71 ±0.67^{a}	66 ±2.54^{a}

*) Means with standard deviations within the same column followed by different letters (a-c) are significantly different ($P < 0.05$).

4.3.6 Pollination system

Response of *P. zeylanica* to different pollination methods was tested (Table 17). The highest percentage fruit (92 ± 0.52%) and seed-set (85± 3.55%) was obtained in controlled cross-pollination compared to open pollination [fruit (87±0.42 %) and seed-set (81±2.43%)] and open cross pollination [fruit 87±0.3% and seed-set 81±4.314%)], which showed less fruit and/or seed-set. There was no significant difference observed between open cross (T_4) and controlled cross-pollination. However, 6 ± 0.56% fruit and/or 5 ± 3.42% seed-set in controlled selfing indicates that some self-pollination is still possible. Simillar corresponding results were also observed under nursery conditions compared to the percentages indicated above under glasshouse conditions. Mature seeds were harvested, after which the achene dried one-month after flowering.

4.4 DISCUSSION

The most important factors influencing the phenology of *P. zeylanica* is the growth environment (e.g., glasshouse versus nursery), plant size and seasonal climate. The study found that the seeds have no hard seed coat and other germination barriers that either delayed or prevented the transition of germination phenology to vegetative phenology provided germination media of specific are employed. A mixture of nursery soil and animal manure filled in pots (glasshouse) or cultivated field under natural conditions provided the highest germination for subsequent phenological studies. Hypogeal germination characterizes the emergence of seedlings with hypocotyl elongation. Meanwhile, leathery layers of seed coat were attached to the surface of elongated hypocotyl, and were dropped with the expansion of leaf primordia.

The identification of appropriate growing conditions suitable for fast transition of vegetative growth to reproductive development was central to the establishment of a valuable method in this study. This study shows that subsequent vegetative and flowering phenological patterns were quite distinct between glasshouse and nursery populations of *P. zeylanica.* Glasshouse population with orthotropic developmental patterns completed their vegetative phenology within a short period. It is well known that seedlings maintained under optimum conditions can achieve a full plant size within a short period of time although depending on the species (Hartmann *et al*., 2002). Whereas field-grown which showed a rest period of vegetative growth during rainy season (about three and half months), the flowering time was elongated to 225 days. This was due to the continuous damage of apical shoot buds by heavy rainfall. Apical shoot bud is the main meristematic region where active cell division occurs for the growth of plants (Hartmann *et al*., 2002).

After rainy season, the appearance of healthy apical shoots showed a dramatic change in vegetative (stem) growth and leaf flushing. This was followed by the initiation of flowering buds and flower opening (anthesis) during wet season accompanied by low temperature (3^{o} C, especially early in the morning). In addition, leaf abscission after fructification followed by leaf flushing during hot season indicates that the phenology of *P. zeylanica* is climatic dependant. On the other hand, the similarity of plant size and leave number during flowering bud initiation indicates the requirement of a minimum growth in height that determined the transition from vegetative to flowering phenology in *P. zeylanica* in

agreement with other reported studies (Childs, 1989; White *et al*., 1997; Bie *et a l*., 1998; Bach, 2002). It is well known that the presence of a minimum number of leaves is critically required as a mediator in receiving climatic signals for further flowering bud initiation, and consequently flower opening in many plant species.

The effects of GA_3 application on flowering of sunflower and rice showed early flowering (Hernandez, 1997; Awan *et al*., 1999). Similarly, in the present study this hormone induced early flowering. Moreover, increased number of flowers was obtained when compared with control. The IAA treatments showed no significant delay in flowering and likewise no effect on the number of flowers was observed compared with control. According to Lang (1952) and Livermann (1955) auxins inhibit floral initiation in some plants. Moreover, low concentrations of auxins are ineffective (Chaudhry, 1997). However, no significance was registered in this experiment. Nakayama *et al*., (1962) have reported that flowering can be enhanced by cytokinins. Similarly, the application of kinetin showed early flowering and an increase in the number of flowers in comparison to control (Table 16).

The combined doses of GA_3 + IAA and GA_3 + kinetin should early flowering which was accompanied by more number of flowers in comparison to control. This may be attributed to the applied mixed dose of GA_3 because IAA has no positive effect. This is further proved by the reports of Thompson and Gutteridge (1959). The combined dose of IAA and kinetin should induce late flowering in comparisons to control and proved to be ineffective in increasing the number of flowers. This delay in flowering may be attributed to the mixed dose of IAA (Chaudhry, 1997). When all the three hormones i.e., GA_3 + IAA + kinetin were applied simultaneously they caused early initiation of flowers. These treatments proved to be the most effective in increasing the number of flowers per plants (Table 17). This clearly indicated the effect of GA_3 and kinetin on the initiation of flowering. Sachs *et al*. (1959) have reported that each of the hormones can flowering in certain plants. Furthermore, these hold required for flowering may have been acquired earlier with the combination of these three hormones.

Response of *P. zeylanica* to different pollination methods tested showed it to be strongly cross compatible as was evident by significant fruit and seed-set in controlled cross pollination. Absence of fruit/seed-set in bagged flowers may be due to the inflorescence type that prevents self-pollination. A similar phenomenon has also been reported in *Gentiana newbery* L., an alpine perennial species in which no fruit and seed set was reported in caged plants (Spira and Pollak, 2002). The fact that *P. zeylanica* is cross-pollinated is further strengthened by the observation that open-pollinated (T_1) and open cross-pollinated (T_4) flowers performed statistically at par with each other with regard to all parameters studied (Table 17). The presence of anthers (in open-pollinated flowers) or their absence (due to emasculation in open cross-pollinated flower) does not make any significant difference for seed and fruit set in open and open cross-pollinated flower, respectively. In flowers of *P. zeylanica,* it has been observed that the anthers are grouped around the stigmatic region when the corolla is still closed and move towards the periphery when the corolla opens, creating a small gap between the anthers and the stigma.

Although *P. zeylanica* seems to be chiefly cross-pollinated, as indicted by no fruit and seed set in bagged flowers and statistically similar results obtained in open and open cross-pollinated flowers, 4.62% fruit set (Table 17) in controlled selfing (selfing effected manually at stigma receptive stage) indicates that some self-pollination is still possible.

The rest period of vegetative growth during rainy season, the initiation of flowering buds and flower opening during cold season under natural condition showed that the phenology of *P. zeylanica* is climate dependent. Thus, the most important factors influencing the phenology of *P. zeylanica* is the growth environment, plant size and seasonal climate. The glasshouse condition is the most effective for fast and vigorous seedling growth, flowering bud initiation, and flower opening, ultimately, where high fructification and seed-set can be obtained within 4 months at any time of the year.

5 *In vitro* and *ex vitro* seed-based propagation of *Securidaca longepedunculata* Fresen

5.1 Effects of various factors on the seed-based propagation of *S. longepedunculata*

Seed is a ripened ovule, which consists of an embryo, stored food and seed coat (Bewley and Black, 1994). Propagation by seed is one means of continuity of plant life and assuring of plant species survival (Copeland and McDonald, 1994). Many authors define seed germination differently, and in this study the seed of *S. longepedunculata* is germinated with the emergence of radicle through the seed coat layers. However, seed germination is influenced by different factors. These are: (1) morphological characteristics of the seed coats including the presence of pubescent hairs highly incorporating with pathogens, which inhibits seed germination (Hartmann *et al*., 2002). Thus, microbial disinfections (fungi, bacteria, viruses) can be either eliminated or reduced through sterilization using various detergents (ethanol, sodium hypochlorite) (Erica, 2000; Hartmann *et al*., 2002). (2) Evidence for hormone involvement in inducing seed germination comes from the correlations of hormone concentration with specific developmental stages, effects of applied hormone, and the relationship of hormones to metabolic activities (Hartmann *et al*., 2002). Of the growth regulators (plant hormones) gibberellins (GA_3) compromise as one of the classes most directly implicated in the control and promotion of seed germination (Frank and Cleon, 1992; Michael *et al*., 2003; Lodge, 2005); and (3) various germination conditions such as temperature, moisture, aeration and media (Hartmann *et al*., 2002). The use of appropriate germination media play a pivotal role to bring about nutrient availability, enzyme activation, break down, translocation and use of storage material, denaturation of proteins, dormancy induction and release as well as in the oxidation processes (Bewley and Black, 1994). However, the influence of these factors is not known yet for *E. kebericho.*

The main objectives of this study were to examine the effects of (1) several disinfectants on *in vitro* germination; (2) different germination substrates on *ex vitro* germination; and (3) glasshouse versus nursery on seedling growth and establishment for getting plant size.

5.2 Materials and methods

5.2.1 Plant material

Mature winged and yellow-colored fruits of *S. longepedunculata* were collected from Chewaka area (Resettlement Station (7^{o} 55' N 34^{o} 50'E), Chewaka District, Ilubbabor Zone, Oromia, Southwest Ethiopia during September 2004. The fruits were stored at room temperature (ca 22^{o} C) until used for experiments. Glasshouse (pot) and *in vitro* (sand medium) experiments were conducted at the Faculty of Science (Addis Ababa University) between September 2003 and August 2005 and at Jimma Agricultural College and Veterinary Medicine (Jimma University), March to September 2006. *In vitro* germination (MS medium) was conducted at the Institute of Plant Genetics and Crop Plant Research (1PK), Gatertsleben (Germany) between September 2005 and February 2006.

5.2.2 In vitro experiments

For surface sterilization, mature seeds of *S. longepedunculata* were released one by one cracking the hard-coats using a surgical blade. Seeds were scarified opposite to the helium to avoid embryo injury as suggested elsewhere by Legesse Negash (1995). The seeds were kept in a 200 ml glass beaker containing 5-7 ml of distilled water so that they did not dehydrate until the completion of the operation. The seeds were then surface disinfected in 10% sodium hypochlorite for 3 minutes and 70% ethanol for 5 minutes, and rinsed three times using double distilled water under laminar flow hood. The seeds were soaked in a 250 ml Erlenmeyer flask containing either distilled water (control) or different concentrations of GA_3 (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} M) for 16 hours using a shaker. Germination solution was filter-sterilized before shaking. The germination medium consisted of MS minerals and vitamins (Murashige and Skoog, 1962) supplemented with 8 g l^{-1} phytoagar (Sigma, St Louis MO, USA), whereas no sucrose was added. The media were stirred; the pH adjusted to 5.75, autoclaved at 121° C for 20 minutes, and then cooled down in a water bath to about 50 $^{\circ}$C. The media (100 mm each) were then dispensed in glass culture vessels (100 x 150 mm; length, width, respectively). Fifteen seeds per vessel were cultured with 7 replicates per treatment. All cultures were inoculated at 25^{o} C under cool fluorescent (40 μmol m^{-2} s^{-1}: 16 photoperiod). Seed germination indicated by at least radicle emergence was recorded at days 7, 14, 21, 28 and 35 on MS medium and at 3, 7, 11 and 15 on sand medium.

5.2.3 Seedbed experiments

A seed bed 3 x 1 x 0.5 m (length, width, and depth, respectively) setup in a glasshouse was filled with different ratios of soil mixture in a ratio of 2:1:1, 1:2:1, 1:1:1, 1:1:2 (sand, red soil, cattle dung, respectively) at equidistant. 102 decoated seeds were sown per treatment and intact seeds sown were considered as control. Dried grass stalks were laid horizontal on top of the seedbed for conserving moisture, and the system was watered once a day. Monitoring throughout the onset of germination the grass cover was removed to avoid the bending of the emerging seedlings. The mean minimum and maximum temperature of the glasshouse during the study period were 11 ± 1^{O} C (nights) and 22±2^{o} C) days), respectively. The relative humidity (RH) ranged from 65 to 75% was maintained roughly at this level throughout the experimental period of sprinkling the floor of the glasshouse with water. RH was measured using a

humidity and Temperature sensor (type HP-100-A, Umuleltanalftische Mess-system GMbH, Munic, Germany). Seed germination indicated by at least radicle emergence was recorded at days 7, 14, 21 and 28.

5.2.4 Pot experiments

Decoated seeds (i.e., seeds without fruit wall) and intact seeds (control) were planted in conical pots (mouth, diameter, 20 cm; depth, 20 cm) filled with a mixture of soil in a ratio of 0.5:2.0:0.5, 1.0:1.5:0.5, 1.5:1.0:0.5, 2.0:0.5:0.5 (sand, red soil, cattle dung respectively). Fifteen seeds per pot were planted with 7 replicates. The pots were randomly arranged in the glasshouse and maintained under conditions similar to those of the seedbed. Seed germination indicated by at least radicle emergence was recorded at days11, 21, 33 and 44.

5.2.5 Seedling survival and growth

The survival and growth of *in vitro*, pot and nursery germinated seedlings were tested maintaining under glasshouse and nursery conditions using four categories (I-IV). (1) one-month old *in vitro* germinated seedlings were removed from the medium, washed with running tap water and planted in plastic pots (diameter, 15: length, 25 cm) filled with a mixture of red soil and cattle dung in a 3:1 ratio. Of these, half of the seedlings were transplanted on a nursery soil (I) while the rest were maintained under glasshouse conditions (II); (2) one-month old pot germinated seedlings were partly transferred to a nursery soil (III) while the rest continuously monitored under the glasshouse (IV). Data on the percentage seedlings survival and growth were scored upon continued growth and production of 3 to 4 leaves. Seedling growth under all categories was evaluated for five months and the system was watered once a day.

5.3 Results

5.3.1 Effects of gibberellic acids

In vitro germination of *S. longepedunculata* seeds was influenced by the concentration of gibberellic acid (GA_3) (Table 18 and 19). Seed viability was not observed with the seeds treated with sterile water neither on MS nor on sand. Concentration of 10^{-6} M GA_3 resulted in poor percentage and rate of germination. The best germination was obtained at a concentration of 10^{-4} M GA_3 (95 ±1.6%) and there was no significant difference between 10-3 and 10-4 M GA_3 3 and 7 days after seed incubation on sand and MS medium, respectively. Thus, percentage and rate of germination was faster and higher on sand than on MS medium. Epigeal germination was observed during seedling emergence.

Table 18 Effects of different concentrations of GA_3 on the germination of *S. longepedunculata* seeds cultured on MS medium.

GA_3 (M)	Time /days			
	3	7	11	15
Control	-	-	-	-
10 -7	15 ± 1.23c*	25 ± 1.41c*	34.± 1.62c*	42 ± 2.60c*
10 -5	42 ± 1.67b	52 ± 2.41b	66 ± 1.06b	82 ± 1.04b
10 -3	57 ± 1.67a	66 ± 1.81a	75 ± 1.71a	95 ± 1.06a
10 -1	42 ± 2.47b	51 ± 2.14b	68 ± 1.21b	78.± 1.24b

*) Means with standard deviations within the same column followed by different letters (a-c) are significantly different ($P < 0.05$

Table 19 Effects of GA_3 on the germination of *S. longepedunculata* seeds cultured on sand medium

GA_3 (M)	Time/days				
	7	14	21	28	35
Control	-	-	-	-	-
10^{-6}	15 ± 0.53c*	26 ± 1.32c*	35 ± 1.04c*	45 ± 1.04c*	53 ± 1.32c*
10^{-5}	45 ± 0.81b	55 ± 1.31b	66 ± 1.05b	77 ± 2.01b	88 ± 1.47b
10^{-4}	56 ± 0.53a	67 ± 0.41a	74 ± 1.04a	83 + 2.41a	95 ± 1.32a
10^{-3}	43 ± 0.41b	54 ± 0.81b	62 ± 1.62b	74 ± 1.05b	80 ± 1.67b

*) Means with standard deviations within the same column followed by different letters (a-c) are significantly different ($P < 0.05$)

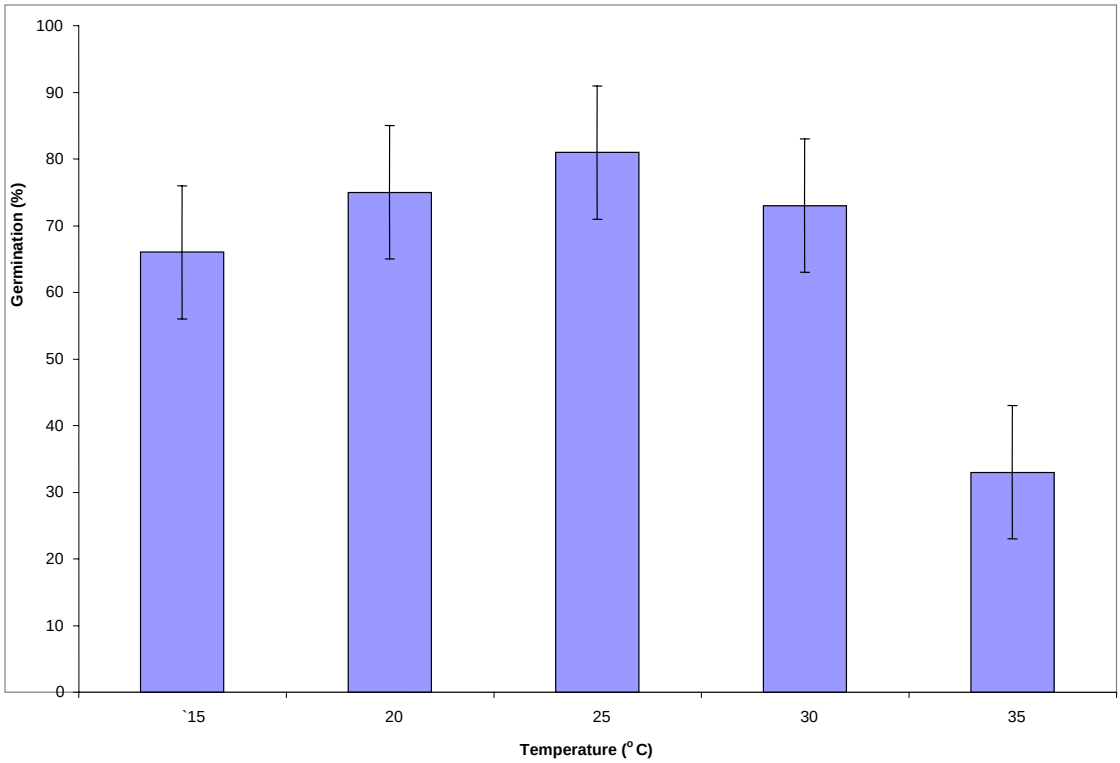


Figure 10 Effects of temperature on in vitro germination of *S. longepedunculata* seeds 2 months after seed collection. Bars represent ± S.D., 15 seeds per pot were planted with 7 replicates.

5.3.2 Effects of temperature

Although seeds (i.e., propagules without seed coat layers) germinated best (86.3 ± 0.44%) at 25° C, no significant difference ($p<0.05$) from those at 15, 20, and.30° C (Figure 10). The least germination was obtained at 35° C

5.3.3 Effects of storage time

Seed viability also declined with storage time (Figure 11). The difference in seed viability between seeds stored at room temperature for 1, 3, 5 months and those stored for 10, 15, 20 months was significant ($p<0.05$). A storage time of 20 months lowered germination capacity close to 20. Mean percentage germination dropped to as low as 42% after storing seeds for 10 months at room temperature. On the other hand, seeds stored for 1, 3, and 5 months showed better germination than those stored for 10 and 15 month.

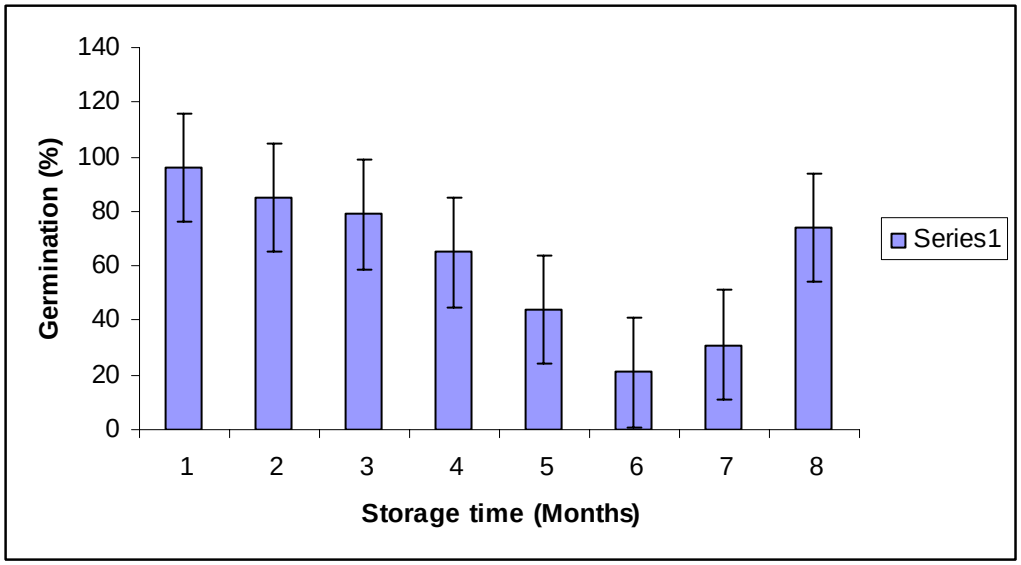


Figure 11 Effects of seed storage time and temperature on *in vitro* germination of *S. longpedunculata* seeds.
X-axis: Number 1= 1, 2=3, 3=5, 4= 10, 5=15, 6=20 months, respectively and 7=10 months at 20 ^{o}C, 8=10 months at 4 ^{o}C, respectively
Bars represent ± S.D., 15 seeds per pot were planted with 7 replicates.

5.3.4 Effect of soil ratios on the germination of *S. longepedunculata* seeds on pots

Ex vitro germination of *S. longepedunculata* seeds (pots) was influenced by mixture of soil ratios tested in pots maintained under glasshouse conditions (Table 20). Seeds planted in pots containing a mixture of soil in a ratio of 0.5:2.0:0.5 (sand, red soil, horse dung, respectively) not only resulted in poor germination but also decayed by fungal contamination; producing a whitish mycelium on the seed surface. However, percentage germination was increased or decreased with decreasing or increasing red soil and sand ratios, respectively. Thus, the final highest germination was obtained with the highest ratio of sand 35 days after seed sowing.

Table 20 Effect of soil ratios on the germination of *S.longepedunculata* seeds planted on pot under glasshouse conditions

Seeding medium**	Time /days			
	3	7	11	15
A	53 ± 1.20[a*]	63 ± 0.34[a*]	79 ± 1.40[a*]	95 ± 2.14[a*]
B	45 ± 1.34[b]	55 ± 0.34[b]	63 ± 1.42[b]	78 ± 1.07[b]
C	33 ± 2.3[c]	43 ± 1.42[c]	55 ± 0.61[c]	62 ± 1.81[c]
D	21 ± 1.21[d]	26 ± 1.61[d]	29 ± 1.32[d]	35 ± 0.60[d]

*) Means with standard deviations within the same column followed by different letters (a-c) are significantly different (P 0.05).

**A. 2.0: 0.5: 0.5; B. 1.5: 1.0: 0.5; C. 1.0: 1.5: 0.5; D. 0.5: 2.0: 0.5 (each treatment with sand, red soil and cattle dung, respectively).

Table 21 Effects of soil ratios on the germination of *S. longepedunculata* seeds planted on the seedbed.

Seeding medium**	Time /days			
	3	7	11	15
A	24 ± 0.14[a*]	34 ± 1.42[a*]	41 ± 1.71[a*]	56 ± 1.62[a*]
B	15 ± 1.21[b]	23 ± 1.39[b]	32 ± 1.23[b]	43 ± 1.71[b]
C	10 ± 1.30[c]	22 ± 1.45[c]	29 ± 0.76[c]	34 ± 1.67[c]
D	-	-	-	-

*) Means with standard deviations within the same column followed by different letters (a-c) are significantly different (P < 0.05).

**A. 2.0: 0.5: 0.5; B. 1.5: 1.0: 0.5; C. 1.0: 1.5: 0.5 (each treatment with sand, red soil and cattle dung, respectively).

Table 22 Survival of *S. longepedunculata* seedlings germinated in vitro on MS, Sand and under glasshouse on pot and seedbed 15, 20, and 40 days after final germination, respectively.

Seeding medium	Seedlings survival (%)						
	Day 15	Day 20	Day 35	Day 40	Day 50	Day 60	Day 70
Sand (*in vitro*)	95 ±2.14^{a*}	77± 1.21^{b*}	34.±1.36^{c*}	-	-	-	-
Pot	-	84 ± 1.41^{a}	75±1.41^{b}	72 ± 1.52^{b}	-	-	-
MS	-	94 ±1.52^{a}	85 ±1.23^{a}	82 ±1.34^{b}	-	-	-
Seedbed	-	-	-	56 ±1.52^{a}	44 ± 1.52^{b}	33 ± 1.41^{c}	30 ± 1.52^{c}

*) Means with standard deviations within the same row followed by different letters (a-c) are significantly different ($P < 0.05$).

5.3.5 Effect of soil ratios on the germination *S. longpedunculata* seeds on the seed bed

Germination response of *S. longpedunculata* was influenced by mixture of soil ratios tested on a seedbed under glasshouse conditions (Table 21). No seed viability was totally observed on plots containing the highest ratio of red soil which is similar to pots. The less germination obtained (<50%) on the seedbed was affected not only as a result of soil ratios similar to the results on pots, but also by the seedbed conditions itself.

5.3.6 Seedling survival and growth

The survival of seedlings germinated *in vitro* (MS, Sand) and glasshouse (pot and seedbed) were tested under glasshouse and nursery conditions (Table 22). A high significant difference was observed in percentage survival of seedlings germinated on MS and sand, and also between seedlings germinated in pot and on seedbed. Seedlings germinated *in vitro* (on MS) survived best and grown vigorously compared to seedlings germinated on sand, which showed hypocotyl elongation with a few root hairs and resulted in high mortality rate upon transferring to a nursery condition.

5.4 Discussion

Seeds of *S. longepedunculata* have hard seed coats. Such hard seed-coats appeared to regulate germination by restricting water entry, gaseous exchange, and outward diffusion of possible endogenous germination inhibitors (Hartmann *et al*., 2000; Legesse Negash, 2004a). In this study results presented in Table 22 shows that the rate of germination (i.e., the number of days required to produce a given germination percentage, for instance T_{50}) of intact seeds (i.e., seeds with hard seed-coat) took a long time compared to the decoated seeds (i.e., seeds released from seed-coat) planted on the same germination media. Thus, 50 % of the decoated and intact seeds germinated within 10 and 45 days, respectively. Legesse Negash (1992) noted that the possession of hard seed-coats has an advantage in terms of the perpetuation of the species under extreme environmental conditions (alternating wet rainy seasons and often extended hot dry seasons) which also characterize Ethiopia.

The best *in vitro* germination percentage of seeds was obtained on sterilized sand or on MS (>95%) both treated with GA_3 may possibly be due to the following reasons. (1) the suitability of sand for *in vitro* germination of large seeds (Michael *et al*., 2003). (2) the establishment of an optimum sterilization and an appropriate MS medium supplemented with GA_3 may enhance the process of germination. It is well known that an appropriate medium and pretreatments are the most important factors, which determines the germination potential and further morphological appearances in plant growth cycle. MS medium is the source of nutrients in supplying necessary elements while GA_3 pretreatments break either coat-imposed and/or embryo dormancy (Berhanu Erko, 2000).

Although there was a significant difference in germination response of treated seeds compared to the control (<40%)

the seeds of *S. longepedunculata* are non-dormant. The induction of germination of this seed is therefore not under the control of phytochrome system. However, it has been proposed that seeds whose germination induced by applied GA_3 are regulated by phytochrome system (Borthwick *et al*., 1964; Grubisic *et al*., 1988; Legesse Negash, 2004a). Also, seed dormancy in various positively photoblastic seeds may be broken by the application of GA_3 and that the pfr form of phytochrome increases the sensitivity of seeds to GA_3 (Berrie, 1984; Bewley and Black, 1994; Legesse Negash 2004). The effect of GA3 on the synthesis of hydrolytic enzymes in cereals is well established (Plag, 1965; Bewley and Black, 1994; Hartmann *et al*., 2002; Legesse Negash, 2004a).

In this study, the least germination of seeds was obtained on the seedbed compared to others, which probably due to the following reasons: (1) an imperforated seedbed may hold excess water, and consequently maximizes the uptake of water by the seed, which affects germination potential; (2) uneven vertical and horizontal distribution of soil mixed by hand. Similarly, Legesse Negash (2004a) noted that the possible reason for poor or gradual germination of the seeds of *Prunus africana* on the seedbed was uneven horizontal and vertical distribution of seeds sown by hand; (3) exposure of the seedbed may steadily increases evaporation, and at the same reduces the soil moisture required for germination; and (4) the feature of the seed (large and soft) endosperm may steadily absorb a high amount of water, and consequently excess water in the seed may also disrupt germination.

After germination, the developmental patterns and the morphological growth (appearance) of the seedlings was central to the establishment of valuable method in this study. A high mortality of *in vitro* germinated seedlings on sand was more pronounced than on MS treated with the same concentrations of GA_3. In addition, hypocotyls elongation with a few root hairs characterized seedlings obtained from sand. In contrast, seedlings obtained *in vitro* on MS showed fast growth with strong- branched stems and production of green leaves during transplantation. The difference was probably due to the adaptation of seedlings to different seeding media. In fact, there is a great difference between MS and sand as a medium in supplying sufficient nutrients for further survival and continuous growth of healthy seedlings (Michael *et al*., 2003).

In summery, *in vitro* (MS) and *ex vitro* (pot, glasshouse) germination was established that allows producing seedlings. The value of such protocol for *S. longepedunculata* is many-fold: Above all it is used to preserve germplasm of this threatened species of high medicinal value. *In vitro* germinated sterile seedlings can be employed for the extraction of pure bioactive metabolites. Further, a rapid and efficient method of plant multiplication is of particular importance in perennial species such as *S. longepedunculata.*

6 *In vitro* regeneration of *Taverniera abyssinica* A. Rich.

6.1 Effect of explants, culture media and hormone treatments.

Many new complete plants can be obtained through direct or indirect morphogenesis and through somatic embryogenesis (Gamborg and Witter, 1975). Direct morphogenesis is the production of shoots from explants without passing through callus (unorganized tissue) phase known as organ culture, which include meristem cultures, shoot cultures, embryo cultures and isolated root cultures. Whereas indirect morphogenesis refers to induction of shoots through callus phase grouped as unorganized tissue cultures, which include callus cultures, suspension or cell cultures or anther cultures. In this study, *in vitro* regeneration of *T. abyssinica* has been developed through callus induction or indirect morphogenesis. Callus-mediated free of genetic off-types of plants regeneration has been successfully developed for a large number of plant species (Manickam *et al*., 2000; Siddique *et al*., 2003; Vinod *et al*., 2004). However, the ability to propagate plants through callus induction, free of genetic off-types, is often dependent on the technique used for micropropagation (Dew, 1991). These include: (1) selections of appropriate explants disinfect either with activated charcoal or antioxidants; (2) the kinds of explants used vary with the purpose of the culture, and the species; 3) the medium selected varies with the species, cultivar and type of explant to be used; and (4) the levels of auxin and cytokinin applications for callus induction (with less relative concentrations), shoot and root regeneration (Erica, 2000; Hartmann *et al*., 2002). However, the effect of these factors is not known for *T. abyssinica*.

The main objectives of this study were therefore to examine the (1) effect of explants, (2) culture media, and (3) hormone treatments in *in vitro* regeneration of *T. abyssinica*.

6.2 Materials and methods

6.2.1 Plant material

Mature pods of *T. abyssinica* were: (1) Collected from Melka Konture area, Kersana Malima District, East Shoa zone, Oromia (65 km from Addis Ababa to Butajira road) at an altitude of about 2100 m a.s.l. during March 2005. The pods were then transported to Germany on 28 August 2005, and consequently the experiments were conducted at the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany.

6.2.2 *In vitro* seed germination

After preliminary experimental trials, the effect of seed polymorphism was observed on germination, and the seeds were categorized into white-large and brown-small ones after the husk was removed using a scalpel. The seeds were soaked in hot water (70^{o} C) for 15, 20, 25, 30 or 35 minutes, surface sterilized with 70% ethanol for 3 minutes, and10% sodium hypochlorite solution for 5 minutes and rinsed three times using sterile double distilled water. The germination medium consisted of MS minerals and vitamins ((Murashige and Skoog, 1962) supplemented with 6, 8, 10, 12, 14, 16

g l^{-1} phytoagar (Sigma, St Louis MO, USA), whereas no sucrose was added. The pH was adjusted to 5.75, autoclaved at 121^{o} C for 20 minutes, and then cooled down in a water bath to about 50^{o} C. The media (100 ml each) were then dispensed in 100 mm x 150 mm glass culture vessels. Twelve seeds per vessel were cultured with 10 replicates per treatment. All cultures were inoculated at 25^{o} C under cool fluorescent (40 μmol m^{-2} s^{-1}, 16 h photoperiod). Seed germination indicated by at least radicle emergence was recorded at days 5, 10, 15, 20, 25 and 30.

6.2.3 Callus induction

Two-month-old *in vitro* germinated seedlings were used as sources of explants. Stem segments (0.5 cm) including a node, petiole segments (0.5 cm), leaf segments as well as shoot (apical and lateral) meristems were used for callus induction. The explants were inoculated in 9 cm Petri plates containing MS or B5 (Gamborg and Witter, 1975) media, supplemented with auxin (0.90, 1.80 μM 2, 4-D, 5.37 μM NAA) in combination with cytokinin (2.22 μM BAP), sucrose (30, 60 g l^{-1}), and phytagel (3, 5, 7 g l^{-1}) (Sigma, St Louis MO, USA). Fifteen explants were cultivated per plate with 7 replicates per treatment. The plates were incubated either in the dark or in the light (control) for 3 weeks. Then, all explants were transferred to fresh media and incubated for another 3 weeks under light conditions. Data on the percentage of callus-forming explants as well as fresh and dry callus weight scored after 6 weeks of culture.

6.2.4 Shoot regeneration

After 2 passages on callus induction medium, all calli were transferred to shoot induction media for 2 passages of 3 weeks. B5 and K4N (Kumlehn *et al*., 2006) media, supplemented with BAP (2.22, 4.44, 6.66, 8.88 μM) or TDZ (2.21, 4.42, 6.63, 8.84 μM) individually, and BAP (8.88 μM) or TDZ (8.84 μM) in combination with IAA (1.14 μM), sucrose (30 g l^{-1}), and Phytagel (5 g l^{-1}) were used for shoot regeneration. Light green, compact callus was selected and distributed onto ten different shoot regeneration media. Three replicates comprising at least 20 calli (about 0.5 cm in diameter) were cultured in each medium. After 6 weeks of culture on shoot regeneration media, the percentages of shoot forming calli and the number of shoots per callus were recorded.

6.2.5 Rooting

The regenerated shoots were cultured on half-strength B5 media supplemented with IBA (2.46, 4.92, 7.38, 9.84 μM) to induce root formation. At least three replicates with 20 shoots each were conducted for each of these media. The percentage of root-forming shoots, and the number and length of roots per shoot were scored after one month of culture on rooting medium.

6.2.6 Acclimatization

Twenty five plantlets (6-8 cm long) regenerated from each explant were removed from the medium, the roots were carefully washed with running tap water to remove the phytagel. The plantlets were then planted into pots filled with a mixture of loam and sandy soil in equal proportions and acclimatized for 5 months under natural diffuse sunlight and

70% humidity in a glasshouse.

6.3 Results

6.3.1 Seed germination

The germination of *T. abyssinica* seeds was influenced by the availability of water as was adjusted by the concentration of phytoagar (Table 24). Concentrations of 6 and 16 g 1^{-1} resulted in poor germination. The best germination (96 ± 0.6%) was obtained at a concentration of 12 g 1^{-1} after 30 days of seed incubation. The germination of *T. abyssinica* seeds was also influenced by seed polymorphism as was adjusted by hot water (70° C) treatments for different times (Table 23). No seed viability was obtained with large-white or small-brown seeds under control treatment. Scarification of brown-small seeds for 15 to 35 minutes and large-white seeds for <20 or > 40 minutes resulted in poor germination. The best germination (95 ± 0.34%) was obtained with white-large seeds treated with hot water (70° C) for 25 minutes 28 days after seed]incubation(Table 23).

Table 23. Effects of seed polymorphism on seed germination of *T. abyssinica*

Treatments	Seed polymorphism	
	Brown-small Germination (%)	White-large Germination (%)
Control	-	76 ± 0.76
70° C (hot water/minutes		
15	-	-
25	-	95 ± 1.56
35	25 ± 0.45	82 ± 1.34
45	31 ± 0.47	56 ± 0.87

Table 24 Effect of phytoagar concentration on seed germination of *T. abyssinica*

Phytoagar concentration ($g1^{-1}$)	Germination %					
	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
6	16 ± 0.6[b]	21 ± 0.6[a]	28 ± 0.6[a]	31± 0.6[a]	35 ± 0.7[a]	41 ± 0.3[a]
8	27 ± 0.6[c]	33 ± 0.9[b]	44 ± 0.6[b]	57± 0.6[c]	65± 0.9[c]	68 ± 0.6[c]
10	38 ± 0.6[e]	56 ± 0.6[d]	64 ± 1.8[d]	74 ± 0.6[e]	78 ± 0.6[d]	85 ± 0.5[e]
12	43 ± 0.6[f]	57 ± 0.6[d]	66 ± 0.6[d]	79 ± 0.6[f]	85 ± 0.6[e]	96 ± 0.6[f]
14	33 ± 0.6[d]	43 ± 0.6[c]	57 ± 0.6[c]	61± 0.7[d]	68 ± 0.6[c]	72 ± 0.5[d]
16	13 ± 0.6[a]	23 ± 0.6[a]	30 ± 0.6[a]	38 ± 0.6[b]	43 ± 0.6[b]	47 ± 0.6[b]

Means with standard deviations within the same column followed by different letters (a-f) are significantly different ($p<0.05$).

All subsequent experiments were therefore, performed using this treatment as a source of explants used for the development of tissue culture.

6.3.2 Callus induction

Callus was formed from all explant types tested, except from leaf segments which only poorly responded. Light green compact callus was obtained upon culture in the dark on B5-based media supplemented with 2, 4-D and BAP. The highest frequency (94 ±-0.8%) of callus induction was obtained from shoot meristem explants followed by petiole (91 ± 0.6%) cultured on B5 medium supplemented with 1.80 µM 2, 4-D, 2.22 µM BAP, 30 g 1^{-1} sucrose and 5 g 1^{-1} phytagel. The data on callus formation from node, petiole and shoot meristem explants are presented in tables 24, 25 and 26, respectively. Culture on MS media resulted only in the formation of dark brown calli and the accumulation of polyphenols in the medium. Explants exposed to light during culture did not form callus at all (data not shown) while culture on medium supplemented with 5.37 µM NAA generally resulted in light brown callus not capable of forming shoot buds. Media containing 1.80 µM 2, 4-D appeared to be superior in producing light green callus formation compared with that containing 0.90 µM. Moreover, 30 g 1^{-1} sucrose and 5 g 1^{-1} Phytagel turned out to be more suitable for regenerable callus formation than the other concentrations of these compounds tested. Under these conditions, also shoot bud formation was most abundant. The highest callus fresh weight (554.3 ± 4.2 mg) resulted from petiole segments (Table 24).

Table 25 Effects of medium modifications on callus formation from node explants of *T. abyssinica* recorded after 6 weeks of culture.

Particularities media	Callus formation (%)	Callus colour	Callus fresh weight (mg)	Callus dry weight (mg)
Standard *	73 ±2.60^{d}	Light green	400 ± 3.30^{e}	59 ± 4.21^{a}
0.90 µM 2,4-D	83 ± 0.82^{e}	Light green	484 ± 5.81^{d}	70 ± 3.90^{b}
1.80 µM NAA	64 ±1.23^{c}	Light brown	252 ± 4.52^{b}	57 ± 4.34^{a}
60 g1^{-1} sucrose	75 ± 0.91^{d}	light green	326 ± 3.92^{c}	59 ± 4.23^{a}
3 g1^{-1} phytagel	44 ± 1.12^{b}	light green	194 ± 2.03^{a}	58 ± 4.22^{a}
7 g1^{-1} phytagel	34 ± 0.63^{a}	light green	193 ± 1.74^{a}	58 ± 4.41^{a}

* Standard conditions: B5 minerals and vitamins, 0.90 µM 2, 4-D, 2.22 µM BAP, 30gl^{-1} sucrose, 5g 1^{-1} phytagel Means with standard deviations within the same column followed by different letters (a-e) are significantly different ($p<0.05$).

Table 26 Effects of medium modifications on callus formation from petiole explants of *T. abyssinica* recorded after 6 weeks of culture.

Particularities of media	Callus formation (%)	Callus colour	Callus fresh weight (mg)	Callus dry weight (mg)
Standard *	82 ± 0.6^{d}	Light green	450 ± 3.3^{c}	60 ± 4.32^{a}
0.90 µM 2,4-D	91 ± 0.6^{e}	Light green	554 ± 4.2^{d}	61 ± 4.93^{b}
1.80 µM NAA	46 ± 0.6^{b}	Light brown	436 ± 3.5^{c}	60 ± 4.94^{a}
(without 2,4-D) no BAP				
60 g1^{-1} sucrose	74 ± 2.3^{c}	light green	370 ± 4.5^{b}	61 ± 4.95^{a}
3 g1^{-1} phytagel	35 ±1.6^{a}	light green	232 ± 2.5^{a}	59 ± 4.22^{a}
7 g1^{-1} phytagel	44 ±1.1^{b}	light green	252 ± 2.7^{a}	59 ± 4.33^{a}

*) Standard conditions: B5 minerals and vitamins, 0.90 µM 2, 4-D, 2.22 µM BAP, 30 gl^{-1} sucrose, 5 g1^{-1} phytagel Means with standard deviations within the same column followed by different letters (a-e) are significantly different ($p< 0.05$).

Table 27 Effects of medium modifications on callus formation from shoot meristem explants of *T. abyssinica* recorded after 6 weeks of culture.

Particularities of media	Callus formation (%)	Callus color	Callus fresh weight (mg)	Callus dry weight (mg)
Standard *	92 ± 1.2^{d}	Light green	$372.\pm 3^{c}$	60 ± 3.0^{a}
0.90 µM 2,4-D	94 ± 0.8^{d}	Light green	454 ± 3^{d}	64 ± 3.0^{a}
1.80 µM NAA	55 ± 1.5^{ab}	Light brown	343 ± 4^{b}	63 ± 2.0^{a}
(without 2,4-D) no BAP	-	-	-	-
60 $g1^{-1}$ sucrose	77 ± 2.3^{c}	light green	358 ± 3^{b}	63 ± 3.0^{a}
3 $g1^{-1}$ phytagel	56 ± 2.1^{b}	light green	227 ± 4^{a}	62 ± 3.0^{a}
7 $g1^{-1}$ phytagel	48 ± 1.4^{a}	light green	238 ± 4^{a}	61 ± 2.8^{a}

*) Standard conditions: B5 minerals and vitamins, 0.90 µM 2, 4-D, 2.22 µM BAP, 30 g l^{-1} sucrose, 5 g 1^{-1} phytagel

Means with standard deviations within the same column followed by different letters (a-d) are significantly different ($p<0.05$).

6.3.3 Shoot regeneration

Shoot buds were formed at the surface of light green callus within 3 to 4 weeks of culture. At the end of the callus induction period, the first leaf primordial appeared. The K4N-based media turned out not to be suitable for shoot formation (data not shown). While both shoot formation frequency and shoot number per callus increased with the BAP concentration within a range of 2.22 to 8.88 µM, no shoots were formed on any of the media supplemented with TDZ (Table 28). On B5 medium supplemented with 1.14 µM IAA and 8.88 µM BAP, shoots were formed from all calli derived from node and shoot meristem explants. The maximum number of shoots (5.6 ± 0.4%) was regenerated from callus cultured on B5 medium supplemented with 8.88 µM BAP and 1.14 µM IAA (Table 27).

6.3.4 Rooting

The percentage of root-forming shoots and the number of roots per shoot increased with the concentration of IBA within a range of 2.46 to 9.84 µM. Of these, 9.84 µM produced the highest percentage (98%) of roots and root number (4.0 ± 0.44), but the longest root (3.0 ± 0.3 cm) was (3.0 ± 0.3 cm) was obtained at 7.38 µM. Shoots grown using higher IBA concentrations seemed to form shorter roots, yet this effect was not statistically significant (Table 28).

Table 28 Effect of growth regulators in B5 media on shoot formation from calli of *T. abyssinica*.

Growth regulator(s) (μM)	Node		Petiole		Shoot meristem	
	Shoot-forming callus (%)	Shoot number/ callus	Shoot-forming callus (%)	Shoot number/ callus	Shoot-forming (%)	Shoot number/ callus
2.22 BAP	51	2 ± 0.3^{a}	43	4 ± 0.3	39	4 ± 0.4^{a}
4,44 BAP	63	3 ± 0.4^{b}	48	5 ± 0.3^{b}	45	4 ± 0.3^{a}
6.66 BAP	64	4 ± 0.9^{c}	55	5 ± 0.4^{c}	65	5 ± 0.3^{b}
8.88 BAP	73	4 ± 0.5^{c}	71	5 ± 0.4^{d}	85	5 ± 0.5^{b}
2.21 TDZ	-	-	-	-	-	-
4.42 TDZ	-	-	-	-	-	-
6.63 TDZ	-	-	-	-	-	-
8.84 TDZ	-	-		-	-	-
8.88 BAP + 1.14 IAA	100	6 ± 0.4^{d}	76	5 ± 0.3^{b}	100	7 ± 0.5^{c}
8.84 TDZ + 1.14 IAA	-	-	-	-	-	-

Values within the same column followed by different letters (a-d) are significantly different ($p<0.05$).

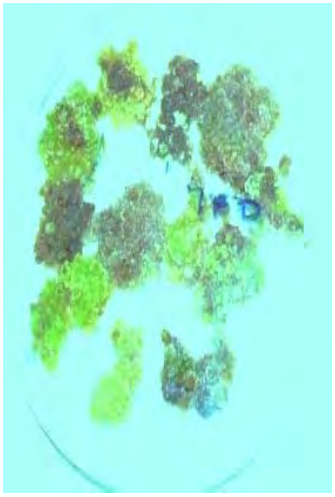
Table 29 Effect of IBA concentration in half-strength B5 media on rooting of shoots derived from *T .abyssinica* calli

IBA concentrations (μM)	Node			Petiole			Shoot meristem		
	Root-forming shoots (%)	Root number	Root length (cm)	Root-forming shoots (%)	Root number	Root length	Root-forming shoots (%)	Root number	Root length
2.46	24[a]	3 ± 0.39[a*]	3 ± 0.9[a]	33[a]	3 ± 0.38[a*]	3 ± 0.3[a]	50[a]	3 ± 0.27[a]	3 ± 0.3[a*]
4.92	36[b]	3 ± 0.37[a]	3 ± 1.0[a]	56[b]	3 ± 0.38[a]	3 ± 1.2[a]	75[b]	4 ± 0.36[b]	3 ± 0.3[a]
7.38	47[c]	3 ± 0.33[a]	3 ± 0.9[a]	68[c]	3 ± 0.30[a]	3 ± 1.2[a]	88[c]	4 ± 0.35[b]	3 ± 0.3[a]
9.84	98[d]	4 ± 0.41[a]	2 ± 1.1[a]	77[d]	4 ± 0.44[a]	2 ± 0.9[a]	100[d]	4 ± 0.25[b]	3 ± 0.3[a]

Values within the same column followed by different letters (a-d) significantly different ($p<0.05$).

6.3.5 Acclimatization

Among the 75 plantlets transferred to greenhouse, 86% plants survived and grown vigorously during 5 months after planting while the rest (14%) showed aberrant phenotypes and stunted growth (Figure



A

B

C

D

Figure 12 In vitro regeneration of *T. abyssinica;*

A. callus induction after 6 weeks on B5 medium supplemented with 1.80 µM 2, 4-D, 2.22 µM BAP, 30 g 1^{-1} sucrose and 5 g 1^{-1} phytagel; B. shoot regeneration after 9 weeks on B5 medium supplemented with 1.14 µM IAA and 8.88 µM BAP, formed from all calli derived from node and shoot meristem explants; C. root formation on B5`medium supplemented with 9.84 µM IBA; and D. seedlings acclimatization under greenhouse condition.
Bars; a) 3.7 mm, b) 1 cm, c) 3.5 cm, d) 4.5 cm

6.4 Discussion

Optimal water availability provided by appropriate concentration of gelling agent is one of the most important factors for *in vitro* germination of seeds either to obtain sterile seedlings for tissue culture or to produce plantlets for its conservation (Anjali *et al*., 2000). This study indicates that MS minerals supplemented with 12 g l^{-1} phytoagar showed the highest percentage (96 ± 0.6%) germination of seeds of *T. abyssinica* after 30 days of seed inoculation. Similarly, numerous published studies have provided evidence that *in vitro* germination of seeds can strongly depend on the concentrations of the gelling agent (Legesse Negash 1992) which influences not only the water status but also mineral and carbohydrate availability (Erica, 2000).

Jurado *et al*. (2001) have reported that the characteristics of seed (seed polymorphism) including seed size, shape and color play a major role in germination potential in many plant species. The result of this study also indicates that the percentage and rate of germination between large-white and small-brown seeds showed highly significant difference ($p<0.05$) (Table 23). The highest percentage (95.4%) germination was obtained with white-large compared to small-brown, which resulted in poor germination even with increased time scarification.

In this study, the maximum amount of light green compact callus was obtained from shoot meristem and petiole explants cultured on B5 media containing different concentrations of 2, 4-D combined with BAP. According to previous reports, 2,4-D in combination with BAP proved to be effective plant growth regulators for callus induction amongst others in many medicinal plants (Anjali *et al*., 2000; Chen *et al*., 2000; Manickam *et al*., 2000; Azad *et al*., 2004; Vinod *et al.,* 2004). Successful callus induction results from the combined interaction of plant genotype, plant physiological condition, explant source as well as the nutritional and regulatory conditions provided by the specific medium used (Azad *et al*., 2004). The poor induction of callus from the leaf segments in contrast to other explants employed in this study may be at least in part due less amount of parenchyma mature tissues. Straub *et al*. (1992) have also reported that in *Sporobolus virginicus,* young leaf tissue as well as shoot meristem, node and petiole explants frequently produced callus, yet the type of callus varied and the leaves produced the least amount.

Browning of both explants and media was the major impediment when *T. abyssinica* explants were cultured in MS-based media containing the same supplements as the B5 media that was successfully used in this study. This significant difference between B5 and MS was probably due to the relatively high concentration of ammonium ions and their high proportion among the total nitrogen provided by the MS medium which is not tolerated by quite a number of plant species and explant types (Murashige and Skoog, 1962; Hartmann *et al*., 2002; Ayan *et al.;* 2004).

The identification of culture conditions suitable for multiple shoot formation from calli was central to the establishment of a valuable method in this study. The promoting effect of BAP on shoot initiation is in agreement with results on other medically important plant species reported earlier by Ang and Chan (2003) in *Spilanthes acmella*, Siddique *et al.*

(2003) in *Hemidesmus indicus*, Nikam and Shitole (1993) in *Guizotia abyssinica* as well as by Kaul *et al*. (1992) in *Anogeissus sericea*. Plant regeneration in *T. abyssinica* appeared to occur by organogenesis rather than through somatic embryogenesis. This was supported by the observation that adventitious roots were only formed from well developed shoots after being placed on rooting medium.

In summary, *in vitro* plant regeneration protocol was established and this allows production of multiple plantlets per single seed via callus formation. The value of such basic protocol for *T. abyssinica* is many-fold. Above all, it can be used as a means to preserve germplasm of this endangered species of high medicinal importance, when low concentration of growth regulators used in the treatment. Callus or suspension cultures may be used to identify and comprehensively analyze the function and significance of the bioactive metabolites as well as to produce biomass potentially employed for the extraction of the medically relevant substances. Furthermore, a rapid and efficient method of plant multiplication is of particular importance in perennial species such as *T. abyssinica*. Also, the method established may enable diverse approaches to genetic improvement of this species.

7 Summary of results and discussion

Ethiopia has a large number of medicinal plants, estimated to 1000 species (Endashew Bekele, 2007). Although the population of these medicinal plant species were once abundant, currently, the most commonly used are seriously threatened due to their high demand, and consequently harvest in the wild to satisfy local markets for traditional treatments and as a source of food security by the rural community (Endashew Bekele, 2007). In addition to this, extensive land deforestation for agricultural purposes in Ethiopia is another major devastating factor for the decimation of medicinal plants. Therefore, studies on their reproductive biology and development of efficient propagation techniques are needed. This can only be achieved in establishing alternative propagation methods, and studying on their reproductive biology.

In this study, the initial activity was the use of treatments (scarification, sterilization, hormone application, and appropriate germination medium) promoting *in vitro* and/or *ex vitro* seed germination for the following two major activities: (1) to develop seed-based propagation protocol, and (2) as a prerequisite for tissue culture method, studies on the reproductive biology and for the development of vegetative propagation by stem cuttings. Seeds of *E. kebericho* sterilized, and cultured on Murashige and Skoog medium (1962) germinated well (95 ± 1.2%) 15 days after seed sowing. The most important factor was the range of seed sterilization time (minutes) with detergents, which was used to prevent the contamination of seed culture. *E. kebericho* is one of the plant species with pubescent seeds. These are also widely distributed within the thin layers of seed coat, which facilitate the incorporation of microorganisms (contaminants such as fungi). It is well documented that most of the tropical plant species are pubscent, which greatly affects *in vitro* culture, and consequently retards propagation activities (Bewely and Black, 1994). Moreover, the growth, survival and establishment of *in vitro* and nursery origin seedlings of *E. kebericho* were very high under nursery conditions compared to pot (glasshouse) origin seedlings, which showed stunted growth with thin leaflets (Figure 13) . This result agrees with Hartmann *et al.,* (2002) that the growth of plant under optimum (greenhouse) condition is species-dependant.

Seeds of other medicinal plants viz., *S. longepedunclata* and *T. abyssinica* (except *G. lotoides*) sterilized in 70% ethanol and in 10% sodium hypochlorite for 3 and 5 minutes, respectively germinated best and showed fast and healthy growth on Murashige and Skoog medium (1962), supplemented with phytoagar (10 and 12 $g1^{-l}$, respectively). Upon transfer to nursery condition, the seedlings of *S. longepedunculata* survived, but showed a stunted growth, because of outside its natural regeneration that is in warm savannah, below 1800 m a.s.l or habitat difference. The most important factor in *in vitro* germination of *T. abyssinica* seeds is the size of its seed, which is identified as one of the seed polymorphisms. The percentage and rate of germination of large-white seeds were higher than those seeds with small-brown one. Seed polymorphism is one of the factors that influence seed germination of many plant species not only in percentage, but also in the rate of germination (Michael *et al*., 2003). These *in vitro* origin seedlings of *T.*

abyssinica were used as a source of several explants for *in vitro* regeneration on B5 medium, supplemented with different growth hormones in callus induction, shoot regeneration and root formation. B5 medium was the most suitable compared to the MS medium, which gave browning of culture. Stem node, apical shoot meristem and petiole explants (except leaf) provided green compact callus, and regenerated shoots, when treated with different concentration of growth regulators (Figure 13).

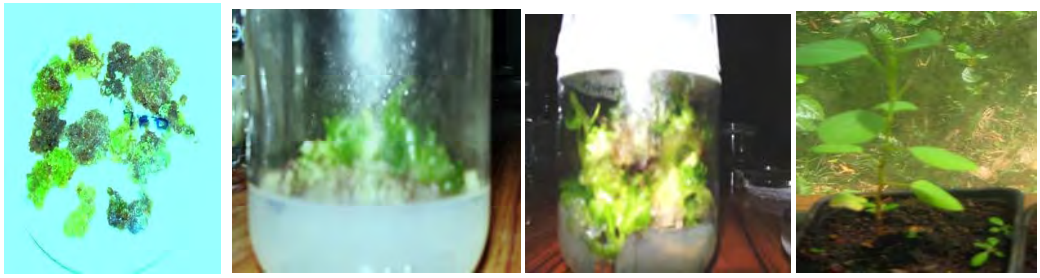
Other important factors for ex *vitro* seed germination; seedling growth and establishment were the germination substrates (soil type and soil ratio). Since there is no standard germination media even for most plant species (Hartmann *et al*., 2002), the ratio of soil (sand: nursery soil: animal manure) applied in this study varied from each other depend on the type of species. For instance, the highest ratio of sand is most suitable for the germination of *S. longepedunculata* seeds. Seeds of *E. kebericho* were best germinated on the highest ratio of nursery soil while equal proportions provided maximum germination of *G. lotoides* seeds.

A B C D

Seed-based and vegetative propagation of *G. lotoides*: A) one-month-old seedlings; B) Establishment of cuttings; C) rooted cuttings; D) rooted cuttings with fructification. *Bars*; a) 3.7 mm, b) 1 cm, c) 3.5 cm, d) 4.5 cm

A B

Eight-month-old *Echinops kebericho* under: (A) nursery; and (B) glasshouse conditions *Bars*; a) 3.7 mm, b) 1 cm

- 

A B C D

In vitro regeneration of *T. abyssinica*; A) callus induction after 6 weeks on B5 medium supplemented with 1.80 µM 2, 4-D, 2.22 µM BAP, 30 g 1^{-1} sucrose and 5 g 1^{-1} phytagel; B) shoot regeneration after 9 weeks on B5 medium supplemented with 1.14 µM IAA and 8.88 µM BAP, formed from all calli derived from node and shoot meristem explants) root formation on B5`medium supplemented with 9.84 µM IBA; and D) seedlings acclimatization under greenhouse condition. *Bars*; a) 3.7 mm, b) 1 cm, c) 3.5 cm, d) 4.5 cm

A B C

Seed-based propagation of *S. longepedunculta*: A) Fruit; B) seed; C) 3-month-old *ex vitro* germinated seedlings maintained under glasshouse conditions. *Bars*; a) 3.7 mm, b) 1 cm, c) 3.5 cm

Figure 13 Summary of results in picture shows propagation techniques of studied medicinal plants.

Table 29 Average growth in height, leaves number and flowering percentage of glasshouse and nursery of *P. zeylanica* seedlings for the first 4 and 6 months, respectively. These differences were calculated with time. Values are means of 110 seedlings for all variables

Month	Glasshouse seedlings			Nursery seedlings		
	Height(cm)	Leave number	F1owering (%)	Height (cm)	Leave number	Flowering (%)
15 March	10.2 ± 1.23^{e} *	$6 \pm 1.51^{c*}$	-	$11.1 \pm 2.43^{f*}$	$8 \pm 2.10^{f*}$	-
April	23.3 ± 1.64^{d}	15 ± 1.45^{b}	-	19.2 ± 1.50^{e}	11 ± 0.12^{e}	-
May	51.2 ± 0.21^{c}	21 ± 1.40^{b}	-	53.3 ± 1.34^{d}	16 ± 0.13^{d}	-
June	73.4 ± 1.62^{b}	32 ± 1.31^{a}	-	-	-	-
July	$\geq 95.2 \pm 0.34^{a}$	37 ± 1.42^{a}	$72 \pm 1.03^{c*}$	-	-	-
August	-	-	85 ± 1.30^{b}	57.6 ± 1.56^{c}	21 ± 1.56^{c}	-
September	-	-	95 ± 1.36^{a}	75.2 ± 1.40^{b}	34 ± 1.49^{b}	-
October	-	-	-	95.3 ± 1.71^{a}	40 ± 1.48^{a}	$30 \pm 1.62^{c*}$
November	-	-	-	-	-	44 ± 1.70^{b}
December	-	-	-	-	-	52 ± 1.90^{a}
January	-	-	-	-	-	
February	-	-				

*) Means with standard deviations within the same column followed by different letters (a-f) are significantly different ($P < 0.05$).

Table 30 Percentage survival of *S. longepedunculata* seedlings germinated in vitro (MS, Sand) and glasshouse (pot and seedbed) days after final germination

Seeding medium	Time/days						
	15	20	35	40	50	60	70
Sand	$95 \pm 2.14^{a*}$	$77 \pm 1.21^{b*}$	$34. \pm 1.36^{c*}$	-	-	-	-
Pot (soil)	-	84 ± 1.41^{a}	75 ± 1.41^{b}	72 ± 1.52^{b}	-	-	-
MS	-	94 ± 1.52^{a}	85 ± 1.23^{a}	82 ± 1.34^{b}	-	-	-
Seedbed (soil)	-	-	-	56 ± 1.52^{a}	44 ± 1.52^{b}	33 ± 1.41^{c}	30 ± 1.52^{c}

*) Means with standard deviations within the same row followed by different letters (a-c) are significantly different ($P < 0.05$).

An alternative propagation protocol has also been developed for *G. lotoides.* Although seed-based propagation in pots with specific germination medium employed gave a good result, vegetative propagation by stem cuttings of *G. lotoides* showed better, and provided the possibility to obtain many plants from a single stockplant. On top of this, higher rooting percentages can be obtained without hormone treatment from apical stem cuttings of younger stockplants within a short period of time under glasshouse conditions and at any time in a year (Figure 13).

The development of appropriate growth conditions for the seedlings was central and a valuable method in this study. The survival of the seedlings, their vegetative growth and reproductive development depended on the interaction between the plant species and the specific growth environment. For instance, the vegetative growth of *E. kebericho* seedlings was fast and rapid under nursery establishment than glasshouse condition (Figure 13). On the contrary, the growth of *P. zeylanica* showed vice-versa (Table 29). In addition, although the seeding medium was similar, the germination of *G. lotoides* seeds was not observed under nursery while the highest germination was obtained in pots, followed by rapid growth of the seedlings. *E. kebericho* has grown well in Addis Ababa while others showed stunted growth since for *E. kebericho* its habitat overlaps Addis Ababa altitude while not for the rest of studied species.

8 GENERAL DISCUSSION

Ethiopia has a large number of medicinal plants, as one of the centers of biodiversity (Pavlov, 1962). Although these medicinal plant species were once abundant, at present, the most commonly used are seriously threatened due to their high demand, and consequently harvest in the wild both to satisfy local markets for traditional treatments and as a source of food security for the rural community. In addition to this, deforestation for agricultural purposes in Ethiopia is another major devastating factor for the decimation of medicinal plants. Therefore, studies on their reproductive biology and development of efficient propagation techniques contribute a lot in conservation and improvement of these species.

In this study, the initial activity was the use of treatments (scarification, sterilization, hormone application, and appropriate germination medium) promoting *in vitro* and/or *ex vitro* seed germination for the following two major activities: (1) to develop seed-based propagation protocol, and (2) as a prerequisite for tissue culture method, studies on the reproductive biology and for the development of vegetative propagation by stem cuttings.

Seeds of *E. kebericho* sterilized, and cultured on Murashige and Skoog medium (1962) germinated well (95 ± 1.2%) 15 days after seed sowing. The most important factor was the range of seed sterilization time (minutes) with detergents, which was used to prevent the contamination of seed culture. *E. kebericho* is one of the plant species with pubescent seeds. These hairs are also widely distributed on the thin layers of seed coat, which facilitate the incorporation of microorganisms (contaminants such as fungi). It is well documented that most of the tropical plant species have pubscent seeds, which greatly affects *in vitro* culture, and consequently retards propagation activities (Bewley and

Black, 1994). Moreover, the growth, survival and establishment of *in vitro* and nursery origin seedlings of *E. kebericho* were very high under nursery conditions compared to pot (glasshouse) origin seedlings, which showed stunted growth with thin leaflets (Figure). This result agrees with Hartmann *et al*. (2002) that the growth of plant under optimum (greenhouse) condition is species-dependant.

Seeds of other medicinal plants viz., *S. longepedunclata* and *T. abyssinica* (except *G. lotoides*) sterilized in 70% ethanol and in 10% sodium hyjpochlorite for 3 and 5 minutes, respectively germinated best and showed fast and healthy growth on Murashige and Skoog medium (1962), supplemented with phyto-agar (10 and 12 $g1^{-l}$, respectively). Upon transfer to nursery condition, the seedlings of *S. longepedunculata* survived, but showed a stunted growth, which requires further study on acclimatization. The most important factors in *in vitro* germination of *T. abyssinica* seeds are the size and colour of its seed, which is identified as one of the seed polymorphisms. The percentage and rate of germination of white-large seeds were higher than those seeds with brown-small one. Seed polymorphism is one of the factors that influence seed germination of many plant species not only in percentage, but also in the rate of germination (Michael *et al*., 2003). These *in vitro* origin seedlings of *T. abyssinica* were used as a source of several explants for *in vitro* regeneration on B5 medium, supplemented with different growth hormones in callus induction, shoot regeneration and root formation. B5 medium was the most suitable compared to the MS medium, which gave browning of culture. Stem node, apical shoot meristem and petiole explants (except leaf) provided green compact callus, and regenerated shoots, when treated with different concentration of growth regulators.

Other important factors for ex *vitro* seed germination; seedling growth and establishment were the germination substrates (soil type and soil ratio). Since there is no standard germination media even for most plant species (Hartmann *et al*., 2002), the ratio of soil (sand: nursery soil: animal manure) applied in this study varied from each other depend on the type of species. For instance, the highest ratio of sand is most suitable for the germination of *S. longepedunculata* seeds. Seeds of *E. kebericho* were best germinated on the highest ratio of nursery soil while equal proportions provided maximum germination of *G. lotoides* seeds.

An alternative propagation protocol has also been developed for *G. lotoides.* Although seed-based propagation in pots with specific germination medium employed gave a good result, vegetative propagation by stem cuttings of *G. lotoides* showed better, and provided the possibility to obtain many plants from a single stockplant. On top of this, higher rooting percentages can be obtained without hormone treatment from apical stem cuttings of younger stockplants within a short period of time under glasshouse conditions and at any time in a year.

The development of appropriate growth conditions for the seedlings was central and a valuable method in this study. The survival of the seedlings, their vegetative growth and reproductive development depended on the interaction between the plant species and the specific growth environment. For instance, the vegetative growth of *E. kebericho* seedlings was fast and rapid under nursery establishment than glasshouse condition. On the contrary, the growth of *P.*

zeylanica showed vice-versa. In addition, although the seeding medium was similar, the germination of *G. lotoides* seeds was not observed under nursery while the highest germination was obtained in pots, followed by rapid growth of the seedlings.

9 CONCLUSIONS

Based on the findings of this study, the following conclusions could be made: This study was the first of its kind on the reproductive biology/phenology of *Plumbago zeylanica* L and development of alternative propagation techniques for four threatened medicinal plants viz *Echinops kebericho* Mesfin*, Glinus lotoides* L. *Securidaca longepedunculata* Fresen. *and Taverniera abyssinica* L. from Ethiopia.

1. Glasshouse condition is more preferable for fast vegetative growth, reproductive development and high yield in *P. zeylanica L*. and *Glinus lotoides* L. compared to nursery conditions, which showed slow growth as a result of apical shoot damages (during rainy and cold seasons) and less germination potential, respectively. Whereas, the reverse germination potential was identified both in *Echinops kebericho* and *Securidaca longepedunculata* Fresen. This indicates that the suitability of glasshouse (controlled) and nursery (field) conditions in vegetative growth and reproductive development is species-dependent.

2. All the seeds of studied medicinal plants can be germinated *in vitro* on MS medium [(supplemented with different concentrations of phytoagar) after pre-treatment (except *Glinus lotoides* L. , which showed the least germination potential even in the absence of contamination)], and *ex vitro* on specific medium employed again except *Glinus lotoides* especially under nursery soil conditions with additional additives. However, the most suitable and alternative propagation method for *Glinus lotoides* developed by this study was the use of apical stem cuttings from younger stockplants without hormone treatment.

3. The highest percentage *in vitro* germination of *T. abyssinica* L. seeds was obtained with white-large compared to brown-small, which resulted in poor germination even with increased time scarification. In this study, the maximum amount of light green compact callus was obtained from shoot meristem and petiole explants cultured on B5 media containing different concentrations of 2, 4-D combined with BAP

10 LIMITATIONS OF THE STUDY

The major problem in the study we faced was the identification of the threat status of medicinal plants (critically endangered, endangered and vulnerable), which was alleviated through up and down efforts, followed by the shortage of transport supply to reach where anticipated to collect propagules. In addition, the shortage of light and water supply in the country limited the laboratory activities to continue with tissue culture of three medicinal plants that were developed to callus at Leibniz Institute of Plant genetics and Crop Plant Research (IPK), Gatersleben, Germany.

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Declaration

I, the undersigned, declare that the thesis is my original work, has not been presented for a degree in any other university, and that all sources of material used for the thesis have been duly acknowledged.

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