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In vitro and *ex vitro* seed-based propagation methods of *Echinops kebericho* Mesfin: A threatened medicinal plant

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Abstract

Effects of seed sterilization, storage time, and temperature as well as extent of seeding survival and establishment under glasshouse versus nursery conditions were studied for *E. kebericho*. Seeds sterilized for 9 and 5 minutes in 70 % ethanol and in 10% sodium hypochlorite, respectively, germinated best (95.2 ± 1.2%) on Murashige and Skoog medium, supplemented with 10 g L^{-1} phytoagar. Further increases or decreases in sterilization time decreased germination percentage and increased contamination, respectively. Unsterilized seeds (control) were completely contaminated before the emergence of radicle as a result of fungal growth. Seed germination percentage declined with increasing storage time and dropped from 94.6 ± 0.4 % to 32.2 ± 1.2% in 15 months. 25 °C was an optimal temperature for best germination (94.6 ± 2.4%) of seeds. 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. Increase in sand or animal manure ratios decreased germination, while increase in nursery soil increased percentage and rate of germination. High percentage (96.0 ± 0.5%) germination was obtained with the seeds sown in nursery soil–surface mixed additives compared with the control. Seedlings of nursery bed origin survived best compared to those *in vitro* or pot origin seedlings. Ultimately, seedlings growth with vigorous and orthotropic developmental pattern was obtained under nursery conditions, compared to those in the glasshouse, which showed stunted and plagiotropic developmental pattern. The study found that seeds stored for less than 5 months, and at 25 °C, were the most suitable for *in vitro* and *ex vitro* propagation of *E. kebericho*.

Key words: *Echinops kebericho*, Kebericho (globe thistle), rootstock, seed-based propagation, Asteraceae

Introduction

Echinops kebericho Mesfin, commonly known as globe thistle belongs to the family Asteraceae. The genus *Echinops* comprises 120 species, of which 12 are known to occur in Ethiopia. The 12 species that occur in Ethiopia are confined to the highlands of the country between 7° 30′ N and 38° 45′ E and at altitudes between 1700 and 2900 m.a.s. (Tadesse and Abegaz, 1990; Erko, 2006). It grows in dry and stony lateritic soils. *E. kebericho* is variable in growth habit and in dissection of the leaf blade. Thus, populations from dry, stony lateritic soils are perennial herbs whereas those growing in deep vertisols are low shrubs. However, Tadesse and Abegaz (1990) noted that the chemical compounds isolated from both populations were identical.

E. kebericho is a threatened medicinal plant with an enlarged rootsystem, used as a source of medicaments (Hymete and Afifi, 1997; Wolde and Gebre-Mariam, 2002). 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, billharzia, syphilis and amoebic dysentery (Abebe and Ahadu, 1993; Desta, 1993; Abera, 2003). Extracts such as sequiterpene lactones isolated from the rootstock have shown antitumor, antimulagenic and cytotoxic effects. The bioactive extract of *E. kebericho* has been shown to have antimicrobial effects equal to or better than penicillin, especially against *Stereptococcus beta-haemolyticus, Escherchia coli, Klebsiela aerogenes* (Debela, 2002; Desta, 1993). Alkaloid extract of the roots of *E. kebericho* has been shown to have a very strong lethal activity against earthworm (Hymete and Kidane, 1991; Hymete and Afiffi, 1997; Erko, 2006). An enlarged roots of *E. kebericho* increases soil fertility, and also reduces soil erosion, especially when growing on mountainous slopes.

Kloos *et al.* (1978) have indicated that *E. kebericho* is one of the ten medicinal plants sold in all the 19 markets surveyed, including the capital city, Addis Ababa. However, despite its high health value, few studies have been undertaken for multiplication to conserve this threatened medicinal plant.

Although *Echinops* have no hard seed coat, which restricts water uptake and gaseous exchange, these species have pubscent hairs. These pubscent hairs are the pathway for the entrance of contaminant agents into seeds and tissues, which inhibits *in vitro* germination of *E. kebericho* seeds. Furthermore, length of seed storage time and temperature affect germination (Negash, 2004). However, also to what extent seed storage time and temperature affect germination of *E. kebericho* seeds are not yet known. In addition, since the natural regeneration of *E. kebericho* is restricted to a specific microclimate the domestication of this threatened medicinal plant species is still not easy. Thus, seed-based propagation method of *E. kebericho* provides the possibility to domesticate the population for conservation and sustainable usage. *In vitro* seed culture would constitute a basis for tissue culture development and approaches to genetic improvement.

The objectives of this study were (1) to evaluate seed pretreatments, and develop *in vitro* seed-based propagation; and (2) to examine appropriate *ex vitro* germination media, and consequently seedling establishment under glasshouse or nursery conditions.

Materials and methods

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), Addis Ababa, during August, 2005. The fruits were removed by hand from the flower head and stored at room temperature (ca 22 ºC). *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 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). The effect of storage time on germination was studied 1, 3, 5, 10, and 15 months after collection whereas the effect of temperature was evaluated with seeds stored at 15, 20, 25, 30 and 35 ºC.

***In vitro* seed germination**: Seeds of *E. kebericho* were released from the thin layers of seed coat by using a scalpel under laminar flow hood. Scarification was carried out opposite to the helium in order to avoid injury of the embryo tissue of the seed as suggested by Negash (1992). Seeds were surface sterilized in 70% ethanol for 5, 7, 9, 11, 13, 15 min, 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, gL^{-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 ºC for 20 minutes, and then cooled down in a water bath to about 50 ºC. The media (25 mL each) were then dispensed in glass baby jars (10 x 6 cm, height, width, respectively) following the method on the germination of Arabidopsis seeds (Kumlehn *et al.*, 2006). Twelve seeds per vessel were cultured with 10 replicates per treatment. Cultures were inocubated at 25 ºC under cool fluorescent (40 $\mu mol\ m^{-2}\ s^{-1}$: 16 photoperiod). Seed germination indicated by at least radicle emergence was recorded at days 5, 10, 15, 20, 25 and 30.

Pot experiments: Seeds (*i.e.* seeds with intact seed coat) were planted in plastic pots (mouth diameter 20 x depth 25 cm) filled with a mixture of sand, red soil, and animal manure 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. 17 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 (Negash, 2003; 2004). The mean minimum and maximum temperatures of the glasshouse during the study period were 11.8 ± 1.0 ºC (nights) and 28.5 ± 2.0 ºC (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).

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 decoated seeds were sown on each plot and the same numbers of intact seeds were used as control. The mean minimum and maximum temperatures of the nursery area during the study period were 10.8 ± 1.0 ºC (nights) and 24.5 ± 2.0 ºC (days), respectively and the relative humidity (RH) ranged from 64 to 75% throughout the experimental period.

Seedling survival and growth: One-month-old *in vitro*, pot (glasshouse) and nurserybed 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 germinants were divided into two (II-IV) and studied both under glasshouse and nursery conditions. 100-110 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.

Statistical analysis: Data were analyzed using SPSS. ANOVA, followed by Tukey Honest Significant Difference Test, was used for detecting significant differences among means. Test for ANOVA assumptions (*i.e.*, homogeneity of variance was run using Tukeys' homogeneity test).

Results

Effects of seed sterilization and phytoagar concentration: Both seed sterilization and phytoagar concentration influenced the germination of *E. kebericho* seeds (Table 1). 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 sterilization decreased germination. The germination of *E. kebericho* seeds was also influenced by the availability of water as was adjusted by the concentration of phytogar. Concentrations of 6 and 14 $g\ L^{-1}$ resulted in poor germination. The best germination was obtained at a concentration of 10 $g\ L^{-1}$ 15 after days of seed incubation. Further increases or decreases in phytoagar concentration decreased germination. All subsequent experiments

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

NaOCl (10%) (min)	70% Ethanol (min)	Phytoagar concentration	Germination (%) on different days				
			3	6	9	12	15
0	0	-	-	-	-	-	-
3	5	6	-	16 ± 0.9^{a*}	21 ± 0.6^{a*}	23 ± 0.4^{a*}	27 ± 1.2^{a*}
4	7	8	24 ± 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 (sand, red soil, horse dung) 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	30.2 ± 0.9^{c**}	43.4 ± 1.2^{c**}	62.4 ± 0.6^{c**}	76.7 ± 1.1^{c**}
B	54.3 ± 0.6^{d}	66.5 ± 0.3^{d}	76.1 ± 1.2^{d}	93.4 ± 1.2^{d}
C	13.5 ± 0.9^{a}	23.2 ± 1.2^{a}	28.4 ± 1.5^{a}	34.6 ± 1.2^{a}
D	21.3 ± 0.9^{b}	28.5 ± 0.6^{b}	33.5 ± 1.2^{b}	43.6 ± 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: 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).

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	14.3 ± 0.6^{a**}	22.7 ± 1.2^{a**}	33.0 ± 1.5^{a**}	42.6 ± 1.2^{a**}
A	63.6 ± 1.2^{d}	73.6 ± 0.9^{d}	85.0 ± 1.1^{d}	96.0 ± 0.5^{d}
B	40.6 ± 1.2^{c}	51.6 ± 1.7^{c}	63.3 ± 1.4^{c}	73.6± 2.1^{c}
C	32.3 ± 1.2^{b}	41.3 ± 0.9 ^{b}	51.3 ± 0.9^{b}	62.0 ± 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.

were therefore performed sterilizing seeds for 9 and 5 minutes with 70% ethanol and 10% sodium hypochlorite, respectively using 10 g L^{-1} phytoagar. Seed germination indicated by at least radicle emergence was recorded at days 3, 6, 9, 12, and 15.

Effects of soil mixture ratios: The germination of *E. kebericho* seeds was tested on different mixture of soil ratio (Table 2). 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 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 decoated and intact seeds on percentage and rate of germination of *E. kebericho* seeds.

Effects of additives: A significant (P<0.05) effect of animal manure was observed on the germination response of *E. kebericho* seeds (Table 3), which was influenced by surface-mixed animal manure and sand with a nursery soil (Table 3). Seeds cultivated on a nursery soil without additives (control) showed poor germination. The best germination (96.0 ± 0.5%) was obtained

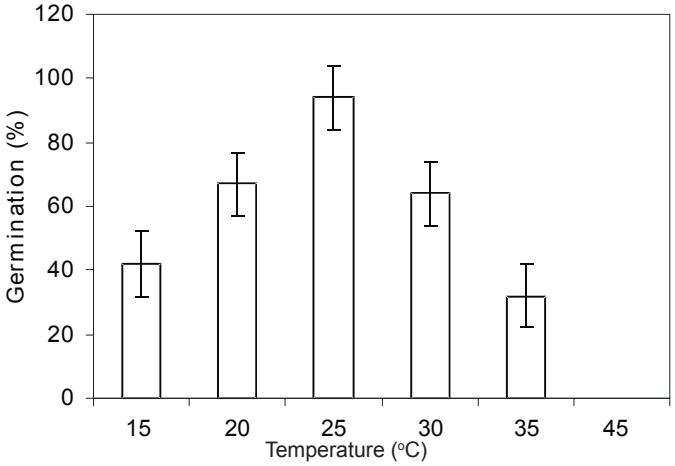

Fig. 1. Effects of temperature on *in vitro* germination of *E. kebericho* seeds. Bar represent ± S.D.

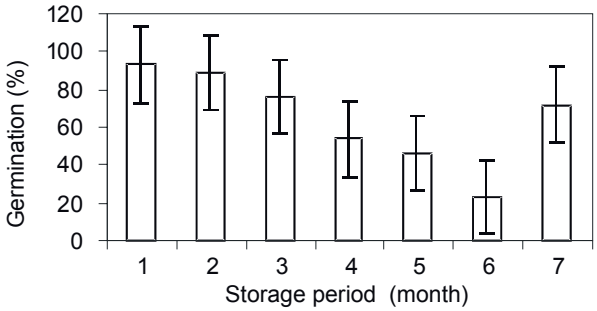

Fig. 2. Effects of storage time on in vitro germination of *E. kebericho* seeds. On the X-axis, 1, 2, 3, 4, 5 refer to the seeds stored for 1, 3, 5, 10 and 15 months while 6, and 7 indicates seeds stored at ca., 22 and 4 oC for 10 months, respectively. Bars represent ± S.D., 100-115 seeds per treatment.

from nursery soil surface-mixed horse dung followed by cattle dung (73.6 ± 2.1%). Seeds sown on a nursery soil surface-mixed sand and control also showed significant (P<0.05) with the least germination (42.6 ± 1.2%). Similar to the pot experiments, there was no significant difference between decoated and intact seeds on percentage and rate of germination of *E. kebericho* seeds.

Effects of temperature on seed germination: Seeds (*i.e.*, propagules without seed coat layers) germinated best (94.6 ± 2.4%) at 25°C, and percentage germination was significantly different (P<0.05) from those at 15, 20, 30 and 35 °C (Fig. 1). *In vitro* germination was least at 15 and 35 °C and was significantly different from all other treatments (P<0.05). There was no significant difference in germination between seeds at 15, 20 and 30 °C.

Effects of storage time: Seed viability declined with storage time (Fig. 2). The difference in seed viability stored at room temperature for 1, 3, 5 months and those stored for 10, 15, 20

Table 4. Percentage of seedling survival and height of 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)				
		30	60	90	120	150
A	24.6 ± 1.5^{a**}	5.0 ± 0.3^{a**}	10.4 ± 0.5^{b**}	13.2 ± 0.6^{ab**}	16.2 ± 0.8^{ab*}	19.8 ± 0^{bb*}
B	31.8 ± 1.7^{b}	4.2 ± 0.4^{a}	7.6 ± 0.2^{a}	10.8 ± 0.3^{a}	13.8 ± 0.5^{a}	15.4 ± 0.5^{a}
C	49.0 ± 2.2^{c}	7.0 ± 0.3^{b}	12.2 ± 0.3^{b}	15.8 ± 0.3^{b}	18.0 ± 0.5^{b}	21.0 ± 1.0^{b}
D	63.4 ± 1.9^{d}	13.0±1.0^{c}	17.2 ± 1.4^{c}	22.2 ± 1.3^{c}	26.2 ± 1.5^{c}	30.8 ± 1.9^{c}
E	93.9 ± 1.1^{e}	23.8 ± 0.8^{d}	34.6 ± 0.5^{d}	44.4 ± 0.8^{d}	55.0 ± 0.7^{d}	66.2 ± 0.5^{d}

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

months was significant (P<0.05). A storage time of 20 months lowered germination capacity close to 0. 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 (96.4 ± 0.4, 85.2 ± 0.3, 75.4 ± 1.4%, respectively) showed better germination than those stored for more than 10 months.

Seedling survival and growth: Seedlings survival and growth depended on the type of germination media and subsequent growth environment (glasshouse versus nursery) (Table 4). Only 24.6 ± 15 and 31.8 ± 1.7% of *in vitro* origin upon transfer to the glasshouse and nursery, respectively survived 150 days after sowing. A significant 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 survived less as compared to nursery germinated seedlings, continuously maintained under the same condition. The highest percentage (93.9 ± 1.1%) survival of the seedlings was obtained from nursery origin evaluated under nursery conditions. Similarly, seedlings growth with vigorous and orthotropic developmental pattern (Fig. 3) was recorded under nursery conditions with a maximum growth in height (66.2 ± 0.5 cm) compared to those in the glasshouse, which showed stunted and plagiotropic developmental pattern (Fig. 3).

Discussion

Seeds of *E. kebericho* are covered by overlapped seed coat layers. These seed coat layers contain pubescent hairs. This is generally the case that such overlapped seed coat layers 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, unsterilized seeds (control) cultured *in vitro* 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 favour target seed germination (plant growth), *i.e.* high levels of nutrients, humidity and warm temperatures, also favour the growth of micro-organisms which multiply and grow rapidly affecting the germination potential (Bewley and Black, 1994; Khana, 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; Khana, 2003). This action 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 1).

Fig. 3. 8-month-old seedlings under nursery; and 8-month-old seedlings under glasshouse conditions.

Germination percentages and seedlings growth were highly variable for *E. kebericho* sown in pots containing different ratios of soil mixture maintained under glasshouse conditions (Fig. 3). 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 Negash (2004) on seed-based propagation of *Prunus africana.*

Uniform seed germination and orthotropic developmental pattern of the seedlings on a nurserybed under nursery condition (Fig. 1A) possibly due to the following major reasons: (1) the fertile nature of the soil of the study area (Kifle, 1997) and surface-mixed animal manure (horse dung) 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 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; Meke *et al.*, 2002), aeration (Ritari and Lahde, 1978), temperature (Kubin and Kemppainen, 1994; Fleming *et al.*, 1994), and water (Winsa, 1995) for plant growth.

Decreased germination with increasing temperature (Fig. 1) shows the effects of elevated temperature on the viability of *E. kebericho* seeds. It is reported that, in germinating seeds of other many plant species, high temperature causes greater membrane and embryo damages through lipid peroxidation (Erko, 2006). A number of studies also indicated that different temperature optima for different plant species (Negash, 1992; 2003).

E. kebericho seeds are characterized by a short viability period when stored at room temperature (Fig. 2), thus loosing their capacity for germination relatively quickly. Similar studies also confirm that seeds of other tropical plant species such as *Podocarpus falcatus, Prunus africana* stored at room temperature for a long time gradually lose their germination potential (Negash, 2003; 2004).

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. Furthermore, a rapid and efficient method of plant multiplication is of particular importance in perennial species such *as E. kebericho*.

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