THE POTENTIAL FOR DEVELOPING AN *IN VITRO* METHOD FOR PROPAGATING *Brachylaena huillensis* (SILVER OAK)

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Abstract

B. huillensis (Silver oak) is an important multipurpose timber tree species in the family Asteraceae which is both economically and socially imperative. Nevertheless, it is facing over-exploitation, poor natural regeneration and seed bank is lacking. Establishing natural regenreation trend of the threatened *B. huillensis* is important as it may provide bench mark for utilization trend and conservation strategies. Also, *in vitro* propagation can become an important alternative to conventional propagation of *B. huillensis*. Through the reviewed studies tissue culture has been found to be a potential method for rapid mass propagation of many endangered and threatened plants species. In this review we outlined the status of natural regeneration and the possibility of developing tissue culture methods for propagating *B. huillensis*.

Key words: Asteraceae, Natural regeneration trend, Threatened, Tissue culture methods

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INTRODUCTION

Background Information

Silver oak is the common name for *Brachylaena huillensis* in the Family Asteraceae, also known as Muhuhu/ Mkarambati in Kiswahili (WCMC, 1998; Chonge, 2002). It is a threatened species in Tanzania (Ruffo and Maliondo, 1990; IUCN, 1998). It is native to

Central, East and Southern Africa (Kigomo *et al*., 1994). In Tanzania, *B.huillensis* is found both in coastal and highland forests to about 2000 m above sea level (Mbuya *et al*., 1994; WCMC, 1991). The species is dominant in ever-green bush, dry coastal forests and semi deciduous dry upland forests at 1500 m to 2000 m above the sea level (FAO, 1986; Mrema, 2006). Silver oak is a dark grey and smooth tree and grows up to 35m tall with a maximum diameter of at least 85 cm under favourable conditions (Kigomo *et al*., 1994).

Seed germination of *B. huillensis* under natural environment is usually poor, about 2-10 % and viability is lost after six months at room temperature. Seeds are difficult to collect because of the small size and many are eaten by insects (Mbuya *et al*., 1994). Also the tree is dioecious that require balanced sex ratio between male and female plants to attain sustained regeneration.

Besides that, its natural regeneration can successfully occur only if a sufficient amount of growing space is available for seed germination and subsequent growth of seedlings (Panna and Sundriyal, 2008). Hence, adults and seedlings of *B. huillensis* are patchily distributed or over dispersed (Marshall and Jenkins, 1994). This is because of reproduction, dispersal, regeneration and survival of the tree species (Kigomo *et al*., 1991).

Essentially, the species is multipurpose. It is used for timber and carving artefacts (Mbuya *et al*., 1994), charcoal, essential oil, (Mbuya *et al*., 1994; Cunningham, 1998; Bryce and Chihongo, 1999), sleepers, flooring blocks, furniture, and turnery (Marshall and Jenkins, 1994). Moreover, the species is used as fence posts, building poles, transmission poles, and ornamental and medicine roots for schistosomiasis and leaves for diabetes (Cunningham, 1998). Basically, it is clearly apparent that *B. huillensis* is indispensable not only to the communities' livelihoods but also to the economic and ecological development. Apart from all these potentials of *B. huillensis*, reports regarding its natural regeneration trend are not in place. Similarly, there is no reliable alternative method of regeneration other than seeds and that widen its danger of extinction.

In this view, there is a need to have in place the regeneration trend information for *B. huillensis*. Also, alternative means for its regeneration preferably tissue culture is requisite.

Natural regeneration and reproduction and of *B. huillensis*

B. huillensis is normally propagated by seedlings and prefers high rainfall (900m-1,200m) and red soil and there is no evidence of vegetative propagation (Kigomo *et al*., 1994. The

germination is usually poor, about 2-10 % and viability is lost after six months at room temperature while growth rate is slow to medium and grows well with other trees but poorly in the open areas (Mbuya *et al*., 1994). *B. huillensis* regenerate on patches and extending in one direction from the parent and occur up between 5m and 17m from the female parent (Kigomo *et al.,* 1991). The distribution in the patches appears to rule out allelopathy possibly due to distribution of female tree, poor fruits dispersal and non random fruits dispersal (Mrema, 2006).

Natural regeneration of *B. huillensis* can successfully occurs only if a sufficient amount of growing space is available for seed germination and subsequent growth of seedlings (Panna and Sundriyal, 2008). Canopy trees strongly determine the under storey light regime and tend to reduce the growing space for the recruitment of young trees into the canopy layer, thus consolidating their dominance (Borghetti and Giannini, 2004). In natural forests, the onset of regeneration processes depend on the creation of open patches (gaps) as a result of death and felling of mature trees. Gaps become preferential sites for natural regeneration, and the characteristics of mature forest ecosystems largely depend on gap recruitment dynamics (Borghetti and Giannini, 2004).

Generally, in managing resources like trees and natural forest one need to be familiar with the available stock and the rate at which the stock is regenerating for sustaining the respective resource. Regeneration status of all plant species in a given stand is considered to be good if numbers of seedlings are greater than saplings and saplings are greater than adult trees. While, regeneration status of all species is considered to be fair if seedlings are greater than saplings and saplings are less or equal to adult trees (Sukumar *et al*., 1992). So in order to ensure *B. huillensis* is managed sustainably as well as preventing it from going extinct its reliable information on the trend of natural regeneration is of great importance.

Tissue culture as an alternative method of propagation in plants / trees

In view of the fact that the world population is increasing rapidly, there is extreme pressure on the available trees and forests. Following these pressures has rendered some plant species endangered, vulnerable, and or threatened (IUCN, 1998). Therefore, for continuous flow of wood and its products as well as sustainable utilization of forest resources application of biotechnology methods for propagation of some trees / plants species is vital.

Traditionally, plants are propagated from seeds. The disadvantage of this method is that it is difficult to control seed quality, which may greatly affect the property of the adult plant. Conversely, plant and tissue cultures have been enabled to increase the knowledge in many areas including differentiation, cell division, cell nutrition and cell preservation but now, cells are cultivated *in vitro* in bulk or as clone from single cells to grow whole plants from isolated cells, then induce callus and develop complete plantlets by organogenesis or by embryogenesis (Pande and Gupt, 2013). Therefore, tissue culture can replace traditional methods of cultivation. It is possible to raise the quality plant by means of propagating large quantities of superior somatic seedlings throughout the year without seasonal constraints Nalawade, (2004).

In vitro propagations are novel methods of conserving the natural populations of plants, reducing the risk of their extinction Satish, (2003). *In vitro* propagation techniques impart vigor for the conservation process of the medicinal and other plants and also maintain the clonal uniformity not achieved by using seeds. The same Author concluded that using tissue culture protocols for the propagation of superior and/or endangered genotypes of plants, it is possible to produce healthy and disease-free plants which could be released to their natural habitat or cultivated on a large scale for the plant/tree product of interest. Currently, a substantial number of endangered and threatened species have been successfully regenerated using *in vitro* culture methods through use of shoot tips, leaves, and leaf bases (Seeni and Latha, 2000).

Plant tissue culture is the science or art of growing plant cells, tissues or organs on artificial media by isolating them from the mother plant (George, 1993). By using plant tissue culture techniques, complete new plants can be obtained from different explants through direct or indirect morphogenesis and through somatic embryogenesis. It is an alternative method of propagation of plant (George and Sherrington, 1984) and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants.

Based on the plant species, media composition and culture conditions, *in vitro* propagation could be achieved by direct and/or indirect shoot organogenesis and/or somatic embryogenesis. Therefore, it is important to employ tissue culture techniques to develop micropropagation methods for threatened tree species like *B. huillensis.*

Micropropagation in the family Asteraceae

Brachylaena huillensis is a timber tree species in the family Asteraceae. It is a threatened species in Tanzania (Ruffo and Maliondo, 1990; IUCN, 1998; WCMC, 1998; Chonge, 2002). Micropropagation in this family has been employed in some species including; *Eclipta alba, Hymenoxys acaulis Lychnophora pinaster, Gerbera jamesonii* (De Souza, *et al*., 2007; (Paduchuri *et al.*, 2010; James, 2002; Paskaran *et al.*, 2005) among others. However, currently micropropagation in *B. huillensis* tree species is yet to be done. This creates the need for developing alternative means of propagation of *B. huillensis* through biotechnological techniques to rescue it from extinction.

Effects of different sterilants' and antifungal/antibacterial in controlling microbial contamination in Woody Plant Media (WPM)

Plant tissue culture is a system of growing plant cells, tissue or organs that have been separated from the mother plant in artificial medium under aseptic condition (Omamor *et al*., 2007). Plant *in vitro* propagation is an aseptic technique for rapid multiplication of pest-free plant materials from organ and cell of desirable plant (Vuylsteke *et al*., 1985). Despite, microbial contamination is a constant problem, which often compromises development of *in vitro* cultures (Webster *et al*., 2003). Contamination is caused by microbes and which can be present in the explants (endophytic) or can be reintroduced from poor aseptic handling, unhygienic conditions in the laboratory or from laboratory instruments. Also, the growth media in which the plant tissue is cultivated is also a good source of nutrients for microbial growth. These microbes compete adversely with plant tissue cultures for nutrients and their presence often results in increased culture mortality or can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Oyebanji *et al*., 2009; Kane, 2003). In fact, the problem is exacerbated when explants are sourced directly from field grown plants (Rout *et al*., 2000; Odutayo *et al*., 2007). Besides that, explants contamination depends on several plant and environmental related factors such as species, age, explant source and prevailing weather condition (Rout *et al*., 2000).

Certainly, contamination in *in vitro* plant culture has economic impact because is considered to be the single most important reason for losses during *in vitro* culture of plants. In fact, losses due to contamination under *in-vitro* conditions average between 3-15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories (Leifert *et al*., 1989), consequently leading to the waste of time, effort and material. If not

mitigated, contamination can have serious economic problems. Despite the best timing and selection efforts, it is almost impossible to eliminate contamination from *in vitro* grown plants and losses due to contamination *in vitro* (Leifert *et al*., 1994).

The problem of microbial contamination can be combated through effective surface sterilization of explants for *in vitro* culture initiation. Basically, sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate plant tissues for *in vitro* cultures. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explants to various sterilants, the sequences of using these sterilants has to be standardized to minimize explants injury and achieve better survival (CPRI, 1992). In essence, requirements on the type concentration and time of exposure differ from one plant to another and for different parts of plants depending on their morphological characters like softness /hardness of the tissue (Srivastava, *et al*. (2010). Mathias, (1987) established that the incidence of bacterial and fungal contamination was higher in explants taken from the nodal region than in those taken from the stem internode as the bacteria may be associated with hairs present at the nodes. Hence, microbial contamination is one of the major challenges facing plant *in vitro* propagation during different stages of culture processes. So rigorous screening of the stock cultures for microbial contamination is highly essential. Thus, it is suggested that prudent selection of explants from the healthy parent plants coupled with an effective surface sterilization method should be the goal in avoiding culture contamination.

The disinfectants widely used are sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide, silver nitrate and bromine water ((Balandreau, 2001). Among these, hypochlorite is known to be a very effective killer of bacteria; even micromolar concentrations are enough to reduce bacterial populations significantly. When diluted in water the hypochlorite salts (NaOCl, lead to the formation of HoCl whose concentration is correlated with bactericidal activity (Nakagarwara *et al*., 1998).

Oyebanji, *et al*. (2009); Srivastava, *et al*. (2010); Rout *et al*. (2000) showed that sodium hypochlorite produced the highest reduction in bacterial and fungal contamination at time intervals between 20 - 45 minutes. Sodium hypochlorite usually purchased as laundry bleach is the most frequent choice for surface sterilization. It is readily available and can be diluted to proper concentrations. Consequently, its usage is recommended because of its simplicity and economy.

Ethanol is a powerful sterilizing agent but also extremely phyto-toxic. Therefore, the explant is typically exposed to it for only a few seconds or minutes. Though, to enhance effectiveness in sterilization procedure, a surfactant like Tween 20 is frequently added to the sterilizing solution. In addition, the sterilizing solutions containing the explants are continuously stirred during the sterilization period (Beaulieu, 2013). Following surface sterilization, explants are either submerged in an antifungal solution, or these agents are added to the culture medium to prevent the growth of microorganisms on the explants (Hatice, *et al*., 2009; Woo *et al*., 2008). Application of systemic fungicides such as benomyl before the collection of plant material also suppresses microbial contamination in plant *in vitro* cultures (Mng'omba, *et al*., 2007). Besides that incorporation of antibiotic and antifungal agents into the growth media of plant culture has been reported to eliminate microbial contaminants (Hazwani, *et al*., 2012; Hatice, *et al*., 2009; Woo *et al*., 2008; Reed, *et al*., 1995). Above all, prior to the establishment of aseptic culture, meticulous selection, identification, and maintenance of stock plants used as the source of explants is necessary. Similarly, maintaining the donor plants in clean and controlled environmental conditions delivers healthy and sterile explants (Sagare *et al*., 2001).

Therefore, testing of different concentrations of sterilants' and new antifungal/antibacterial in controlling microbial contamination is necessary in developing an in vitro protocol for the propagation of new candidates such as *B.huillensis*.

Secretion of phenolic compounds on *in vitro* culture in plants

In vitro micropropagation has proved in the recent past a means for supply of planting material for forestry (Ahuja 1993; Laximisita and Raghavaswamy 1998). However, *in vitro* tree propagation has been a difficult proposition compared with other plants due various factors including; genotype, age, part of the plant, phenolic compounds and physiological state and time of the year when the explants are collected and cultured tissue (Chakrabarty *et al*., 2007). One of the most common problems associated with the *in vitro* establishment of many monocotyledonous and woody species is the deleterious effects of oxidized phenols (Munne´-Bosch and Pen˜ uelas 2003; Chakrabarty *et al*., 2007). Many plants produce dark phenolic substances after wounding. Accumulation of such compounds in medium adversely affects the growth and survival of *in vitro* explants. Roussos and Pontikis (2001) showed that accumulation of these compounds leads to necrosis and death of olive explants.

The phenols are synthesized by the plants and in many cases excreted and then oxidized (Ozyigit, 2008). In tissue culture studies, phenolic substances, especially oxidized phenols generally affect *in vitro* development negatively (Arnaldos *et al*., 2001). Oxidized phenolic compounds may inhibit enzyme activity and result in the darkening of the culture medium and subsequent lethal browning of explants (North *et al*., 2013; Compton and Preece, 1986; Laukkanen *et al*., 1999). Phenolic secretions and other exudates in woody tissue culture systems lessen explant initiation, growth, and development (Kerns and Meyer, 1986). Therefore, pre-conditioning of explants with media supplements such as ascorbic acid and activated charcoal is necessary to limit production of these substances (Welsh *et al*., 1979).

The antioxidant, ascorbic acid, has been used successfully in the past to inhibit the exudation of phenols (Strosse *et al*., 2004; Titov *et al*., 2006) and to reduce oxidative browning in various plant species (Arditti and Ernst, 1993; George, 1996; Abdelwahd *et al.*, 2008). Thus, ascorbic acid is able to scavenge oxygen radicals produced when the plant tissue is wounded, by protecting the cells from oxidative injury. Ascorbic acid is a powerful reducing agent found usually in millimolar concentrations in plants, and is proposed to play an important role in scavenging reactive oxygen species generated during stress conditions in plants and animals (Smirnoff, 2005; Chawla, 2002; Foyer, 1993). It also plays multiple roles in plant growth, such as cell division, cell enlargement, acting as co-factor for many enzymes and stomatal regulation (Conklin 2001; Lee and Kader, 2000; Asada, 1999; Barth *et al*., 2006). Moreover, ascorbic acid is also considered as an important molecule that regulates the peroxidase activity in actively dividing cells (Stasolla and Yeung, 2007). Its addition to the medium has reduced blackening to an acceptable level (Almaz *et al*., 2001). Thus, Ascorbic acid is useful and effective in managing the problem of phenolics and improving plant growth *in vitro* (Abdelwahd *et al*., 2008). Therefore, testing of different concentrations of ascorbic acid in controlling lethal browning in *in vitro* cultures is necessary.

Callus induction in *in vitro* culture in plants

Callus initiation is a first step to achieve tree/ plant regeneration. Since 1934 when the first callus was obtained from cambial explants of *Pinus pinaster* (Gauheret, 1943), many gymnosperms have been successfully regenerated through callus formation. Callus is basically a more or less non-organized tumor like tissue, which usually arises on wounds of differentiated tissue and organs. Callus is formed through three developmental stage viz. induction, cell division and proliferation. During induction metabolic rate of cell is

stimulated, duration of which depends on the physiological status, nutritional and environmental factors (Arora and Chawla, 2005). Owing to increased metabolic rate, cells synthesize more cellular contents and finally divide to form many cells.

The unique feature of callus is that it is biologically potent to develop normal roots and shoots i.e. ultimately it can form a complete plant. Callus culture is considered potential tool to achieve tree regeneration via embryogenesis or organogenesis (Ramage *et al.,* 2002). Aboel-Nil, (1987) estimated that 247,100 hectares could be reforested with plantlets produced from 100L of callus only in three months. Callus formation is governed by the source of explants, nutritional composition of the medium and environmental factors (Arora and Chawla, 2005).

Basically, a nutrient medium consists of all the essential major and minor plant nutrient elements, vitamins, plant growth regulators, and a carbohydrate as carbon source with other organic substances as optional additives. The basis of all nutrient media is a composition of essential nutrients (Ramage *et al.,* 2002). Media is one of the important components in the propagation of plants *in vitro* as there is no common media for all plant. The composition of the media used for establishment of aseptic cultures is important (CPRI, 1992). Although more than 50 different devised media formulations have been used for the *in vitro* culture of tissues of various plant species (Gamborg *et al.,* 1976; Huang and Murashige, 1977) the formulation described by Murashige and Skoog (1962) (MS) medium is most commonly used, often with relatively minor changes (Chand *et al*., 1997; Jha *et al*., 1985; Koblitz *et al.*, 1983a,b; Rout *et al.,* 1999, in press c; Saxena *et al*., 1998; Uduebo, 1971; Wakhlu *et al.,* 1990; Zhou *et al.*, 1994). However, most woody plants perform better in Woody Plant Media (WPM) than Murashige and Skoog (Mc Cown, 2000). Lloyd and Mc Cown, (1980) found that the medium widely used for micro culture of woody plants is Woody Plant Medium (WPM). The formulation was developed after it was discovered that many woody plants could not tolerate the relatively high salt, high chloride levels encountered in a medium formulations like MS (Smith, 2000).

On the other hand, type and concentrations of growth regulators have different response on callus induction in different plats cultures. Singh *et al*. (2013) established that the best callus induction was induced in *Gebera* when leaf explants were cultured on WPM supplemented with dichlorophenoxyacetic acid (2, 4-D). Rout *et al.* (2000) found that induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential

application of growth regulators and the control of conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced. The levels and kinds of plant growth regulators included in the culture medium largely determine the success of tissue culture work. The auxin:cytokinin ratios yield rapid growth of undifferentiated callus (Ammirato, 1983; Bajaj *et al*., 1988; Rout *et al*., 1997; Skoog *et al.*, 1957).

Regarding to incubation conditions; light, temperature, and relative humidity are important parameters in culture incubation. Both quality and intensity of light as well as the photoperiod are very critical to the success of *in vitro* culture experiments (Murashige, 1977). Exposure to light for 12–16 h per day provided by cool, white fluorescent lamps is usually recommended. The temperature usually employed in the culture incubation room is approximately 25- 2 8^{o}C. Tropical species usually require higher temperature (27–30^{o}C) (Tisserart, 1981).

Chang *et al*. (2001) found that only few tree species with explants from mature trees have been propagated by tissue culture methods. In general juvenile tissues from forest trees are more responsive to *in vitro* manipulation than that of mature tissues. The longer life span of trees may add to the problem of contamination *in vitro* by the symbiotic association of microorganisms. It is evident that there are few micropropagation protocols from mature tree explants. Also, many woody plant species and particularly tree legumes are known for their *recalcitrant* nature of regeneration (Jha *et al*., 2004; Anis *et al*., 2005; McCown, 2000). In many woody plants, it is much easier to establish juvenile than adult explants *in vitro*.

Generally, preconditioning of explants with media supplements such as growth regulators, culture condition and media composition are necessary conditions for callus induction in new candidates when establishing tissue culture protocol.

In conclusion, establishing natural regenreation trend of threatened species such as *B. huillensis* is important as it may provide bench mark for utilization trend and conservation strategies. Further, for effective propagation there is a need for developing alternative *in vitro* methods for the regeneration of the tree species in the red list.

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