Development of embryogenic callus protocol for *Brachylaena huillensis* (SILVER OAK)

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Abstract

The present investigation was conducted to explore embryogenic potential of *Brachylaena huillensis* using leaves as explants. The second pair of juvenile leaves of tips of *B. huillensis* naturally growing seedlings were collected, sterilized and cultured on woody plant medium (WPM) containing various concentrations levels of 2, 4-D, BAP and a blend of the two phytohormones to induce embryogenic callus. For the first time, embryogenic callus was explored from leaf explants of *B. huillensis* in Tanzania using tissue culture techniques. The hormonal combination of BAP (5µM) and 2, 4-D (5µM) was recorded as most appropriate for high percentage (8.3 ± 3.3) of viable and healthy callus. Concentration of 5µM was the best in callus induction in the current study. Actually, the study of *in vitro* propagation focusing on callus induction using leaves in *B. huillensis* is novel; it is the corner stone for *in vitro* propagation for the tree species.

Key words: Asteraceae, threatened species, callus induction, *In vitro* propagation, growth regulators.

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Introduction

Brachylaena huillensis common name Silver Oak is a multipurpose timber tree species in the family Asteraceae (WCMC, 1998; Choge, 2002). There has been a very high demand for *B. huillensis* wood and its products leading to overexploitation. It is a threatened tree species (Ruffo and Maliondo 1990; IUCN, 1998). *B. huillensis* is suitable for timber and carving artefacts, charcoal, essential oil, (Mbuya *et al.,* 1994; Cunningham, 1998; Bryce and Chihongo, 1999), sleepers, flooring blocks, furniture, and turnery (Marshall and Jenkins, 1994). Moreover, due to its durability, the species is used as fence posts, building poles, transmission poles, ornamental and medicine for schistosomiasis and leaves for diabetes (Cunningham, 1998). Even so, *B. huillensis* is illegally exploited for timber, charcoal, transmission poles, carving, building poles, fencing posts, ornamental, medicine, perfumery and toilet preparations, sleepers, flooring blocks, furniture, and turnery (Chonge, 2002; Bryce and Chihongo, 1999; Marshall and Jenkins, 1994). Nonetheless, currently *B.huillensis* regenerates only through seedlings and produces seeds with poor germination, seeds are also difficult to collect because of small size, many are eaten by insects and at present there is lack of seed bank (Mbuya *et al.*, 1994). These reasons have hindered and rendered the natural regeneration of the tree species unsure.

A good 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). So far, *B. huillensis* has no callus induction protocol in place. In view of its inherent qualities and restricted distribution, its propagation and multiplication through tissue culture is urgently required. Plant tissue culture offer advantages over conventional methods for multiplication and large-scale production of woody 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* (Gautherest, 1943), many gymnosperms have been successfully. 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. 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). The objective of the present study was to develop efficient callus induction protocol for *B. huillensis* for *in vitro* propagation.

Materials and methods

Plant material

Plant materials were obtained from the healthy naturally growing seedlings in Bombo West Forest Reserve (BWFR) in Tanzania. The BWFR is located in Korogwe district, Tanga region. The reserve lies between latitude 4 ^{o} 52' and longitude 4 ^{o} 47' S and 38 ^{o} 39' and 38 ^{o} 43' E. It is situated 60 kilometres from Korogwe town on Lwengera valley about 380 m to 680 m above sea level (Lovett and Pocs, 1993). Alternatively, it is 83 kilometres from Tanga town through Tanga -Mashewa road via Maramba. The reserve is owned by the central government; it was gazetted in 1959 with a Government Notice (GN) 1 of 1959 and has an area of 3,523.5 ha. (Lovett and Pocs, 1993).

Explants source

The second pair of leaves of Silver Oak (*B. huillensis*) seedlings tips was the source of explants material that was collected from the healthy naturally growing seedlings in Bombo West Forest Reserve in early May 2013. The tip leaves were chopped from the naturally growing seedlings and then preserved in a cool box with ice blocks and transported to Mikocheni Agriculture Research Institute (MARI) laboratory in Dar es Salaam where this research was carried out. The leaves spent twenty four hours on transit before culture initiation.

Surface sterilization of the explants

In the laboratory the leaves were placed in a bottle containing distilled water. The water contained two detergents, namely liquid soap and tween -20, which enhance the effectiveness of the disinfectant by breaking the surface tension between water and the plant tissues. For effectiveness, the explants in the solution were agitated continuously for 5 minutes, later the leaves were rinsed four times with distilled water. The bottles containing the already washed leaves were transferred to the transfer room (lamina flow) and immersed in sodium hypochlorite (NaOCl) 1.5%, v/v) with two drops of tween-20 for 10 minutes. Subsequently, the leaves were rinsed four times with sterile distilled water and later dipped in 70% ethanol for 10 seconds. Thereafter, the leaves were rinsed four times using sterile distilled water to remove the traces of the sterilizing agent before culturing for callus formation. The sterilized explants were trimmed suitably to remove sterilizing agent affected parts. Leaf discs of about 1 square centimeter with and without midrib were sliced from the sterile leaves and cultured (Fig. 1 A).

Culture conditions

The callus induction media were composed of basal woody plant medium (WPM) (Lloyd and McCown 1981) with full strength supplemented with dichlorophenoxy acetic acid (2, 4-D) and Benzyl Aminopurine (BAP) both at three concentration levels (0, 1, 5 & 10µM) and (0, 1, 5, & 10 µM) respectively. In addition, cefotaxime (antifungal) 0.03mg/liter was added in the medium after cooling to about 38 degrees centigrade. The pH of the medium was adjusted to 5.6 before autoclaving at 121 degrees centigrade. The surface sterilized explants (leaf discs) were inoculated on the WPM medium abaxial side in contact with the medium and labeled properly. Each petri dish (sterile) with 20ml of the WPM medium contained five explants. Four replications with 5 explants in each were maintained for each treatment and 20 explants in each treatment were evaluated. The culture without growth regulators served as a control. The cultures containing the explants (leaf discs) in a petri- dish were kept in a growth room at a temperature of 25 ± 2 degrees centigrade, 60 – 70% relative humidity and white fluorescent light with a 16-h photoperiod. The number of explants which formed callus was record after 37 days after culture initiation.

Statistical analysis

The statistical analysis was carried out using the 2-way analysis of variance (ANOVA) with the computations being performed with the software program STATISTICA. The means are reported with standard errors. The fisher least significance difference (L.S.D.) was used to compare treatment means at $p = 0.05$ level of significance (Steel and Torrie, 1980).

Results

The present investigation was carried out to explore the morphogenic potential of the multipurpose timber tree, *B. huillensis*. The leaf explants of *B. huillensis* formed callus on WPM medium containing full strength supplemented with BAP and 2, 4-D at various concentrations (1, 5, &10 µM). The explants showed swelling within 14-16th day of inoculation; however callus formation started on 17th day at the margins of the explants and subsequently spread over the entire leaf discs. Among the two growth regulators employed in the present study with different concentration, a cock tail of 5µM BAP and 5µM 2, 4 D gave the best result in induction of light green fast growing embryogenic callus (8.3 ± 3.3) (Table 1; Fig. 2 A & B). Numerically, the lowest callus number was obtained in the medium containing BAP alone (Table 1). In the medium without plant growth regulators all explants turned black and died. It was observed that orientation of the explants (leaf discs) on the medium had effect on callus formation. Callus development occurred only to the explants cultured right side up Figure 1 (abaxial side in contact with the medium). The explants cultured upside down (adaxial side in contact with the medium turned brown and died (Fig. 1b). The results indicated that there was a significant ($p = 0.05$) difference between growth regulators whereby BAP & 2, 4-D alone were inferior to a mixture of BAP and 2, 4-D in inducing callus (Table 1). Moreover, the result showed that there was a significant ($p = 0.05$) different between concentration levels of the growth regulators. The overall performance indicated that the best result was obtained at concentration of 5µM. This was closely followed by 10 and 1µM.

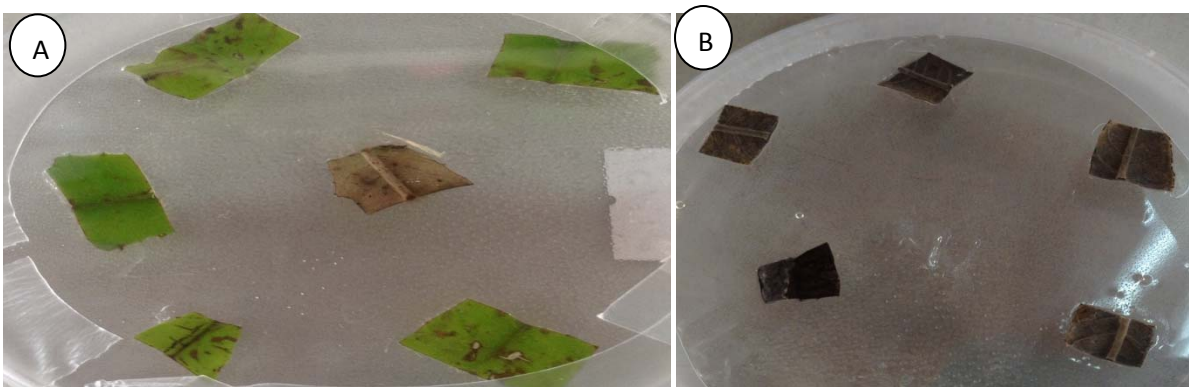

Fig. 1: A: = Six leaf discs of *Brachylaena huillensis* cultured on WPM medium supplemented with a bend of BAP and 2, 4-D, -five green leaf discs cultured right side up (abaxial) and one at the middle upside down (adaxial); B: = *Brachylaena huillensis* leaf discs culture adaxially (upside down) on WPM medium supplemented with a bend of BAP and 2, 4-D.

Table 1: Effects of growth regulators and their concentration (BAP, 2,4D, and a blend of BAP) in callus induction in *B. huillensis*

Treatments	No. of leaf explants formed callus
Growth regulators	
BAP	2±1.1 b
2, 4-D	2.8±1.5b
BAP & 2, 4-D	8.3 ± 3.3 a
Concentrations (µM)	
0	0 ± c
1	3.7 ± 2.2 b
5	9.3 ± 3.4 a
10	4.3 ± 2.4 ab
2 -Way ANOVA F. Statistic	
Growth regulator	7.2 *
Concentration	6.8*
Growth regulators * Concentration	ns

Values represent means ± standard error. Means followed by the same later(s) within column are not significantly different at p=0.05 according to Fisher's Least Significance difference. BAP = Benzyl Aminopurine, 2, 4-D = Dichlorophenoxy acetic acid, * = Significant at $p \leq 0.05$; ns= Not significant.

Discussion

The protocol for embryogenic callus induction of *B. huillensis* was developed using juvenile leaves (second air of the tips) of seedlings cultured on a WPM medium supplemented with a mixture of 5µM 2, 4-D and BAP 5 µM (Fig. 3 A & B). Principally, the study of *in vitro* propagation focusing on callus induction through leaves in *B huillensis* is novel so it is the beginning of *in vitro* propagation in *B. huillensis*. Callus formation started at the margins of the explants and subsequently spread over the entire leaf discs (Fig. 2 B). This is because the cut ends of leaf explants provided a way for the nutrients and growth regulators to be absorbed efficiently from the medium and start functioning according to the nature of the growth regulator (Reynoired *et al.,* 1993). The results concur with the following authors in studies carried out in woody plants (Sarin *et al.,* 2011; Yadav *et al.,* 2011; Oluwaseun *et al.,* 2004). Similar to our study, 2, 4-D and BAP in combination were reported to induce callus formation in a variety of plant culture systems with a rate of cell proliferation and intrinsic activity higher than that obtained with other growth regulators (Murthy *et al.,* 1999); Capelle *et al.,* 1983;). also established that the best callus induction was induced in *Gebera* when leaf explants were cultured on woody plant media (WPM) supplemented with a mixture of BAP and (2, 4-D).

The principle of Skoog and Miller (1957) stated that both auxin and cytokinin are necessary for regenerative callus induction which also holds good for *Adhatoda vasica* as organogenic callus from leaf discs explant was observed in a combination of cytokinin- BAP (5µMl) and auxin-NAA (5 µMl). The results of the present study showed that there is a significant difference between BAP & 2, 4-D and a mixture of the two growth regulators in callus induction. The difference in the performance may be due to the role of a particular growth regulator in callus induction. The type of plant growth regulators used varies according to cell culture, purpose and plant type. Koroch *et al.,* 2003 found that auxins stimulate RNA metabolism and induce the transcription of messenger RNA which code the proteins that are required for the chaotic cell proliferation and ultimately the callus formation. Nahid *et al.,* 2007 reported that 2,4-D induce cell division and enlargement at optimum concentrations which is associated with increase in the activities of autolytic and synthetic enzymes by effecting cell wall plasticity and by synthesizing new cell wall materials. It was noted that the auxin 2, 4-D generally have the efficiency of initiation of high frequency of callus (>50%) from the leaf explant in many plant species

(Marsolais, *et al.,* 1991; Mok, *et al.,* 1985; Ozias-Akins, *et al*., 1989; Mok, *et al*., 1982; Murashige *et al.,* 1962). Modh *et al.* (2002) reported that BAP stimulate RNA and protein synthesis which activate enzyme activity for cell division and cell wall loosening. Basically, considerable variability of growth regulators exists among genera, species, and even cultivars in the type and amount of auxin and cytokinin required for induction of morphogenesis. Moreover, application of growth regulators in combination has a profound effect in callus induction.

In the present study, the concentration of growth regulators affected callus inductions. The lower (1µM) concentrations gave relatively poor response. Concentration of the plant growth regulator can vary for each plant species and can even depend on the source of explants or individual plant. 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. Essentially, growth regulators show their best performance at a certain level (optimal) and this level varies significantly from one plant to another. Too low or too high concentrations of growth regulators may be unfavorable on plant callus induction. Can *et al*., 2008 found that by increasing the concentration of growth regulators resulted into reduced callusing percentages. Conversely, Shirin *et al.* (2007) observed that meristematic cell division was blocked at supra optimal concentrations of auxins (2, 4-D) and cytokinins (BAP) which causes the inhibition of protein synthesis, leading to the browning and death of callus. Rout, *et al.,* 2000 found that the concentration levels of plant growth regulators included in the culture medium largely determined the success of tissue culture work. Thus as observed in this study, concentration levels of plant growth regulators was a major factor that controlled callus formation in the culture medium Figure 3 A & B.

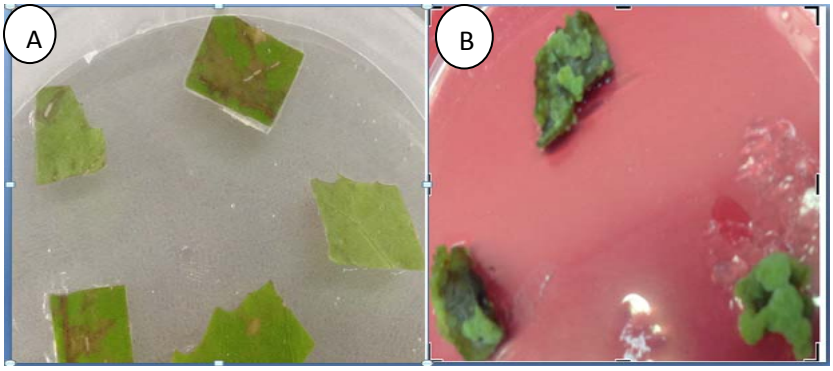

Figure 2: A: - *Brachylaena huillensis* leaf discs cultured on woody plant medium supplemented with a blend of 5µM Benzyl Aminopurine and 5µM Dichlorophenoxyacetic acid; B: - *B. huillensis* leaf discs cultured on woody plant medium supplemented with a blend of 5µM Benzyl Aminopurine and 5µM Dichlorophenoxyacetic acid commencing callus induction.

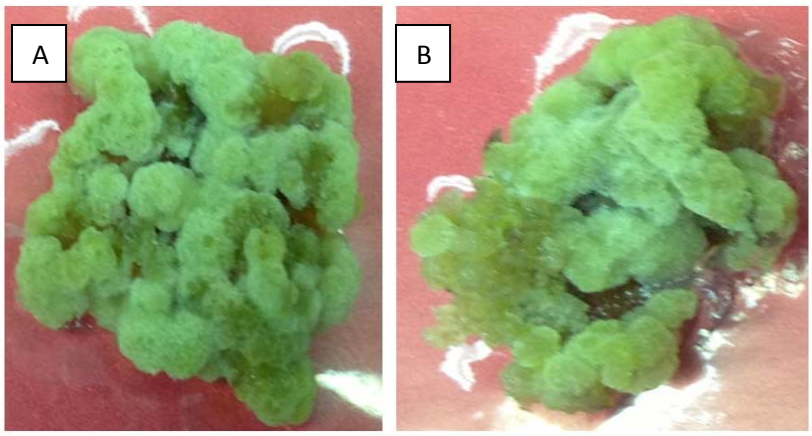

Figure 3:A & B - *Brachylaena huillensis* embryogenic callus induced on WPM medium supplemented with a blend of 5µM Benzyl Aminopurine and 5µM Dichlorophenoxyacetic acid.

In conclusion, the present study has explored for the first time the callogenic capacity of leaf explants of *B. huillensis.* The protocol for callus induction for *B. huillensis* is novel so it is the beginning for *in vitro* regeneration techniques through leaf explants. It is concluded that the use of juvenile leaf explants (approx. 2-3 months old) and WPM medium supplemented with a blend

of BAP and 2,4-D is the most suitable and reproducible protocol for induction of viable and good quality callus from *B. huillensis*. Regarding to growth regulators' concentration, 5µM had the best results in callus induction. Meanwhile, this protocol offers itself not only as a highly efficient method for mass clonal propagation of this species but also for its conservation. Based on these findings, we suggest further researches to advance the embryoids to plantlets and their establishment in the field and develop a protocol for *in vitro* propagation techniques using other parts of the tree species other than leaves. Further research on age and leaf orientation of leaf explant is also recommended.

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