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PLANT TISSUE CULTURE

Shoot organogenesis from leaf discs of the African ginger (*Mondia whitei* (Hook.f.) Skeels), an endangered medicinal plant

Driciru Patricia[1] · Buah Stephen[1] · Adriko John[1]

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Abstract

The African ginger (*Mondia whitei*) is an endangered, endemic African medicinal plant, widely used as an aphrodisiac and to manage gastrointestinal problems and anorexia. Due to high demand, it is over-utilized from the wild without replenishment and destroyed by inappropriate harvesting methods. Therefore, there is a need to develop an efficient conservation system for the African ginger. This study focused on developing an *in vitro* propagation procedure for the African ginger through indirect shoot organogenesis. Optimal callus formation was induced from leaf explants cultured on Murashige and Skoog (MS) basal medium fortified with 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The 1.5 mg L^{-1} 6-benzylaminopurine (BAP) concentration was optimal with 50% of the callus-forming buds. For shoot formation, media with 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA resulted in 60% of the buds developing shoots. Regenerated shoots were rooted on woody plant medium (WPM) fortified with 0.1 mg L^{-1} BAP and no rooting hormone included. The survival rate of weaned and acclimatized plants was 70% and up to 80% respectively. The study demonstrates an *in vitro* propagation route for the African ginger which could support its mass production for medicinal use, availing of planting materials, conservation, and commercialization purposes.

Keywords Regeneration · *In vitro* propagation · Conservation · Organogenesis

Introduction

Traditional medicine has been part of Africa's cultural heritage with medicinal plants being commonly used in health care in both the urban and rural African communities (Afolayan and Adebola 2004). It is estimated that 80% of people in the developing world use medicinal plants for their primary health care (Vines 2004). The African ginger (*Mondia whitei* (Hook.f.) Skeels) is a perennial woody climber medicinal plant widely distributed in moist tropical and subtropical forests of Africa (Venter *et al.* 2009). It has been used as an aphrodisiac throughout the regions of its distribution (Agea *et al.* 2008). A study conducted by Lampiao *et al.* (2008) showed that an aqueous extract of *M. whitei* enhances human sperm motility. Other recorded medicinal values of *M. whitei* include use in the treatment of gastrointestinal problems (Gundidza *et al.* 2009), asthma, and anorexia (Koorbanally *et al.* 2000) and as an anti-depressant (Watcho *et al.* 2006). The African ginger is also used for nutrition (Aremu *et al.* 2011) due to its rich thiamine, potassium, and sodium contents (Chinonyerem and Maryjane 2017) and for food flavoring (Gbadamosi and Aboaba 2016).

Mondia whitei use and collection from the wild is continuously increasing due to its demand (Cunningham 1989). Several African countries including South Africa, Kenya, and Uganda have undertaken some initiatives to conserve *M. whitei* owing to its medicinal value (CTA 2007, Eilu *et al.* 2007). However overall, there is minimal cultivation of these species classified as endangered in Uganda (McGeoch 2004; Agea *et al.* 2008). Over-exploitation of the African ginger is due to demand for its medicinal attributes, poor land use systems, and harvesting techniques (Agea *et al.* 2008). However, proper replenishment and conservation systems have not yet been set up for *M. whitei* in most of its distribution areas including Uganda.

Several conventional methods have been used to conserve this plant including protecting its natural habitats (Eilu *et al.* 2007) and monitoring its cultivation (CTA 2007). However, such methods have been limited by the requirements of vast

✉ Adriko John
adrikoj@yahoo.com

[1] Biodiversity and Biotechnology Programme, National Agricultural Research Laboratories (NARL), National Agricultural Research Organization (NARO), P.O. Box 7065, Kampala, Uganda

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land and limited planting material, necessitating alternative conservation approaches using biotechnological tools. Biotechnology provides a non-conventional method of plant propagation, which can be used to intensively conserve plant biodiversity through *in vitro* conservation or cryopreservation and mass production of planting materials using tissue culture (Panis *et al.* 2001).

Plants of the family Apocynaceae have been successfully regenerated *in vitro* through somatic embryogenesis and organogenesis (Upadhyay *et al.* 1992; Kim *et al.* 1994; Kiranmai *et al.* 2015). Use of minimal auxins and cytokinins are required to regenerate plants without somaclonal variation (Edson *et al.* 1996). Previous studies on regeneration of *M. whitei* involved its micropropagation using nodal explants (McCartan and Crouch 1998) and somatic embryogenesis (Baskaran *et al.* 2015). The current study focused on developing an *in vitro* propagation system for the endangered medicinal plant *Mondia whitei* through indirect organogenesis from leaf discs and evaluation of its survival rate in the field. The findings of the study will be useful as an alternative route for mass regeneration of *M. whitei*, thereby aiding its conservation.

Materials and methods

Explant sourcing and disinfection *Mondia whitei* (Hook. f.) Skeels explants consisted of fresh immature leaves excised from 4-mo-old plantlets sourced from Plant Genetic Resources Centre (Entebbe Botanic Gardens), Entebbe, Uganda. The leaves were thoroughly washed with a mild detergent (2% liquid soap) under running tap water for 30 min and soaked in a fungicide (0.3% (w/v) Goldazim 500 SC (Amiran K Limited, Nairobi, Kenya); active ingredient carbendazim) for 25 min and rinsed three times with sterile distilled water. Explants were then surface sterilized with 1.9% sodium hypochlorite (Orbit Chemical Industries Limited, Nairobi, Kenya) for 15 min and rinsed three times with sterile distilled water.

Callus induction Leaf discs (approximately 1 cm^2) were sliced from the sterilized leaves using a scalpel blade and cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (Sigma-Aldrich, Darmstadt, Germany) fortified with 0.5, 1.0, 1.5, 2.0, or 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich), 20 mg L^{-1} ascorbic acid (Sigma-Aldrich), 30 g L^{-1} of sucrose (LOBA Chemie, Mumbai, India), and 3 g L^{-1} gelrite (Sigma-Aldrich) for callus induction. The media combinations were sterilized by autoclave set to 121°C for 15 min. Two leaf discs were cultured in each jar containing 30 mL of medium, and there were five jars per replicate for each treatment. The cultures were maintained at 25 ± 2°C with 16-h light/8-h dark photoperiods, 80 to 85% relative humidity, and light intensity of 50 μmol m^{-2} s^{-1} for a period of 4 wk.

Bud induction Induced callus was sub-cultured on MS medium supplemented with 0.25 mg L^{-1} 2,4-D and 0.5, 1.0, 1.5, 2.0, or 2.5 mg L^{-1} 6-benzylaminopurine (BAP) (Sigma-Aldrich). The cultures were maintained on MS medium containing these different plant growth regulator (PGR) combinations, 20 mg L^{-1} ascorbic acid, 30 g L^{-1} of sucrose, and 3 g L^{-1} gelrite (Sigma-Aldrich) at 25 ± 2°C, at 16-h light/8-h dark photoperiods, 80 to 85% relative humidity, and light intensity of 50 μmol m^{-2} s^{-1} for 4 wk.

Shoot formation The buds were transferred to sterilized MS medium fortified with BAP and 1-naphthalene acetic acid (NAA) (Sigma-Aldrich) combined in the following concentrations: 2.0 mg L^{-1} BAP plus 1.0 NAA mg L^{-1}, 0.5 mg L^{-1} BAP plus 0.25 mg L^{-1} NAA, and 1.0 mg L^{-1} BAP plus 0.5 mg L^{-1} NAA, 20 mg L^{-1} ascorbic acid, 30 g L^{-1} of sucrose, and 3 g L^{-1} gelrite. Two buds were placed in a single jar and each treatment had five jars per replication. The cultures were maintained on medium at 25 ± 2°C with 16-h light/8-h dark photoperiods, 80 to 85% relative humidity, and light intensity of 50 μmol m^{-2} s^{-1} for 6 wk. Shoot formation data was captured weekly for 4 wk.

Rooting and plantlet acclimatization Induced shoots were rooted on half-strength McCown and Lloyd's woody plant medium (Sigma-Aldrich) supplemented with 0.1 mg L^{-1} BAP to induce roots. After 8 wk on rooting medium, the rooted plantlets were removed from the culture medium and washed in distilled water. The plantlets were then transferred to plastic cups containing steam-sterilized garden soil mixed with organic manure and sand (2:1:1) in the screenhouse with the following conditions: 12-h of light, 12-h dark, 25 ± 5°C, and a relative humidity of 80 to 100%. Two wk later, the plants were transferred to bigger buckets and maintained in the screenhouse under the same conditions.

One year later, the plants were distributed and planted in farmer fields in the Jinja, Kaliro, Rakai, and Dokolo districts of Uganda. Seedlings planted at each site varied depending on available land for establishment of the medicinal gardens. To determine survival, the fields were visited 6 mo after planting and surviving plants were captured as a percentage of seedlings planted. These districts were selected because *M. whitei* is known to naturally inhabit these locations.

Statistical analysis All experiments were repeated three times, and each treatment had four to five replicates. Data was collected on various parameters and analyzed using the R statistical software (RStudio-0.98.1102). The means were separated using the Welch two-sample *t* test ($p = 0.05$).

Table 1. Effect of different 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations on callus induction from leaf explants of *Mondia whitei* (Hook.f.) Skeels

2,4-D (mg L^{-1})	*Percentage of callus formed per treatment	Callus texture	Color
0.0	0.0 ± 0.0	-	-
0.5	80 ± 0.3	Compact	Cream
1.0	100 ± 0.0	Compact	Cream
1.5	100 ± 0.0	Compact	Cream
2.0	100 ± 0.0	Compact	Brown
2.5	100 ± 0.0	Compact	Brown
	$p = 0.31$		

*Values represent mean ± SE of three experiments with five replicates

Results

Callus induction Calluses were visible 3 wk after explant inoculation on callus induction media. Callus induction reached 100% from all the leaf discs cultured on media supplemented with 2,4-D except media with 0.5 mg L^{-1} 2,4-D, where only 80% explants induced callus. The callus cultures all had a compact texture irrespective of the treatment. However, calluses induced on MS medium with low concentrations of 2,4-D (0.5, 1.0, and 1.5 mg L^{-1}) were cream in color whereas those with higher 2,4-D concentrations (2.0 and 2.5 mg L^{-1}) were brown, and later became necrotic. There was no significant difference in the percentage callus induction among the treatments ($p = 0.31$). High 2,4-D concentrations result in callus death (necrosis) thus not appropriate for regeneration processes (Table 1).

Shoot bud induction Shoot buds were seen 4 wk after callus inoculation on bud induction media. Shoot bud induction was successful at BAP concentrations of 0.5 mg L^{-1} (30% of the callus formed buds), 1.0 mg L^{-1} (40% of the callus formed buds), and 1.5 mg L^{-1} (50% of the callus formed buds). BAP concentration of 1.5 mg L^{-1} was the most efficient for shoot bud induction, but at higher BAP concentrations (2.0 and 2.5 mg L^{-1}), no buds were formed, but rather the calluses turned brown. The percentage of callus that developed buds did not significantly differ ($p = 0.19$) with varying concentrations of BAP (Fig. 1).

Shoot formation Shoots formed from all treatments 6 wk after bud inoculation on shoot induction media. The highest percentage of the buds (60%) formed shoots on 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA, followed by 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA where 40% of the buds developed shoots, and 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA, which induced shoots in 20% of the buds. The remaining buds that did not form shoots became vitrified in all the three treatments. There was no significant difference in the percentage of buds that formed shoots with varying the BAP concentrations in media ($p = 0.1$) (Table 2).

Rooting Eighty percent (80%) of the shoots successfully formed roots when transferred onto half-strength woody plant medium with 0.1 mg L^{-1} BAP for 4 to 6 wk. Roots reached 1.5 to 2 cm in length by the eighth wk. The shoots were well developed with an average shoot height of 3.0 cm and leaf diameter of 0.5 cm.

Plantlet acclimatization The rooted shoots were weaned in sterilized matrix consisting of soil, manure, and sand in the

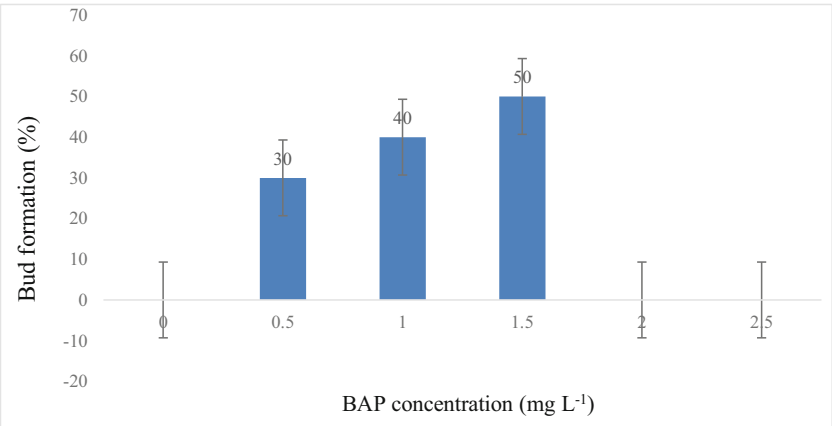

Figure 1. Effect of 6-benzylaminopurine (BAP) on organogenesis (shoot bud formation) from calluses of *Mondia whitei* (Hook.f.) Skeels.

Table 2. Effect of combinations of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) on shoot formation of *Mondia whitei* (Hook.f.) Skeels

BAP and NAA (mg L^{-1}) combination	Percentage shoot formation (%)
0.0 + 0.0	0 ± 0.0
0.5 + 0.25	20 ± 0.1
1.0 + 0.5	40 ± 0.3
2.0 + 1.0	60 ± 0.3
	$p = 0.1$

ratio 1:1:1, respectively. The plantlets were kept in the screenhouse for 2 mo at humidity of 80 to 85%. The survival of the weaned plants was 70%.

Eighteen wk were required to regenerate, multiply, and wean *M. whitei* by this method, which includes 3 wk for callus induction, 4 wk for bud induction, 4 wk for shoot induction, 4 wk for shoot proliferation and rooting, and 3 wk for acclimatization in the screenhouse. Each leaf explant generated 30 shoots from the period of initiation of the leaf explant on callus induction medium to its culture on the shoot regeneration medium, and this increased with frequency of sub-culturing before transfer to the rooting medium (Fig. 2).

Field planting Seedlings were planted at four field sites in four districts with varying responses. Rakai had the highest seedling survival rate (80%), followed by Jinja (60%) and Kaliro (20%). No seedling survived in the Dokolo site due to prolonged drought and lack of irrigation.

The survival rate was higher where the amount of rainfall received was adequate and the medicinal gardens were well maintained (Table 3).

Discussion

This study focused on developing an *in vitro* regeneration system for the African ginger as one of the strategies for contributing to its conservation. Successful optimization of plant regeneration protocol from African ginger leaf discs within 18-wk time period was reported.

In the present study, no somatic embryos were formed from the calli unlike studies done by Baskaran *et al.* (2015) and Dharmendra *et al.* (2010). Callus formation occurred continuously from explants cultured on MS medium supplemented with 0.5 mg L^{-1}, 1.0 mg L^{-1}, and 1.5 mg L^{-1} 2,4-D. However, 1.5 mg L^{-1} 2,4-D concentration induced callus of the best quality and greatest mass. These results are in line with that of Dharmendra *et al.* (2010) where optimum callus formation was achieved with 1.5 mg L^{-1} 2,4-D. Browning and non-viable callus from media with 2,4-D concentrations above 1.5 mg L^{-1} indicated that high concentrations induced synthesis of toxic secondary metabolites (Bernat *et al.* 2018), leading to inhibition of cell growth.

Although there was no significant effect of BAP concentration on percentage bud formation, 1.5 mg L^{-1} BAP induced more buds than 0.5 and 1.0 mg L^{-1}. Above 2.0 mg L^{-1} BAP, no buds were induced concurring with previous studies that exceeding optimal BAP levels inhibits bud proliferation (Arab *et al.* 2014; Akbaş *et al.* 2009; Thorpe *et al.* 2008). The highest percentage of shoot development was obtained from medium containing 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA. The buds that did not form shoots became vitrified, probably because the concentration of BAP in the medium was too high (Pâques 1991). Bud vitrification was observed in all the three different combinations of BAP and NAA. These results agree with those obtained by Singh *et al.* (2009) and Dharmendra

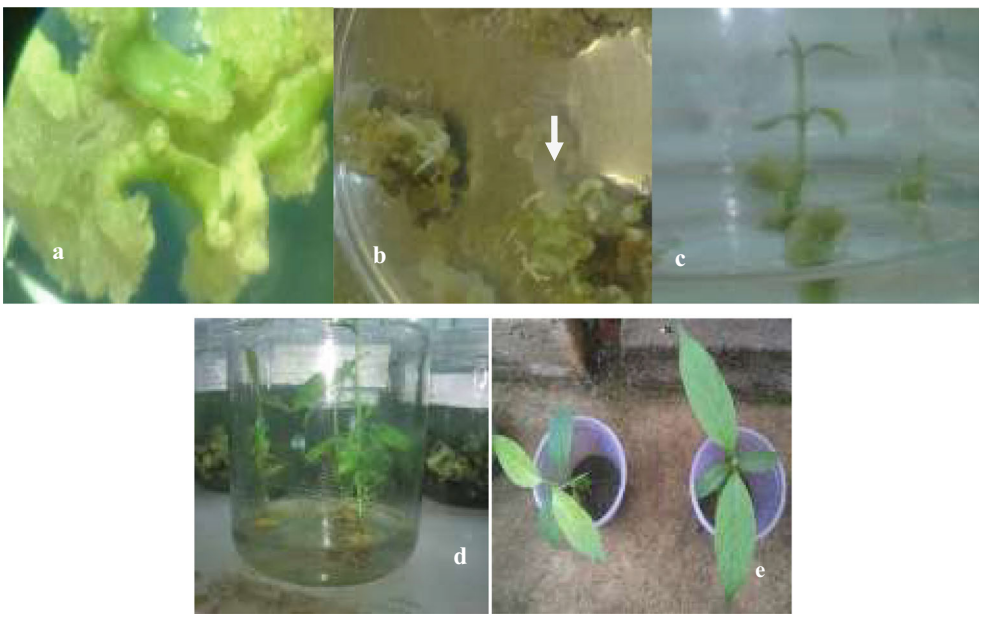

Figure 2. Regeneration process of *Mondia whitei* (Hook.f.) Skeels *Mondia*: (*a*) callus induced from leaf explant on Murashige and Skoog (MS) medium containing 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid; (*b*) buds (see *arrow*) developed from callus on MS medium containing 1.5 mg L^{-1} 6-benzylaminopurine (BAP); (*c*) plantlet formed from the buds on MS medium with 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} 1-naphthaleneacetic acid (NAA); (*d*) rooted plants on half-strength woody plant medium containing 0.1 mg L^{-1} BAP; (*e*) weaned plants of *M. whitei*.

Table 3 Survival of the tissue culture plantlets of *Mondia whitei* (Hook.f.) Skeels in selected districts in Uganda

District	Percentage survival
Dokolo	0
Kaliro	20
Jinja	60
Rakai	80

et al. (2010) who reported successful shoot regeneration with a combination of a higher cytokinin-to-auxin ratio in plant regeneration medium. The study achieved high frequency of rooting because the residual rooting hormone (NAA) in the shoot induction medium was able to induce roots in medium where rooting hormone (indole-3-butyric acid) had been excluded.

Survival of tissue culture plantlets of *Mondia whitei* was least in Dokolo and Kaliro districts because these areas faced severe drought unlike Jinja and Rakai districts. It is possible that the harsh conditions of drought would have had the same effect on non-tissue culture seedlings. Therefore, it cannot be ruled out that tissue culture plantlets of *Mondia whitei* cannot survive or have very low survival chances in Dokolo and Kaliro, given favorable weather conditions.

In conclusion, this study documents a complete protocol for regenerating *M. whitei in vitro* through callus-mediated shoot organogenesis, and possibility of survival of the regenerated plants in open fields given favorable weather conditions. These results can be used as a basis to regenerate and promote domestication of *Mondia whitei* by making available planting materials to medicinal plant farmers and plant genetic resources centers to enhance conservation. In addition, the callus route can be used for *in vitro* conservation of *Mondia whitei* cells through cryopreservation for years to enhance replenishment of this species. Field evaluation of *Mondia whitei* further indicates that it is possible to domesticate this plant species especially in areas where drought is not severe. However, more studies can be done to improve aspects of the protocol whereby bud regeneration and shoot regeneration frequencies are low to enhance mass production.

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