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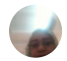
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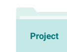
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Protocol Optimization for *in vitro* Micro Propagation of *Dioscorea bulbifera* Linn. Germplasm

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Abstract

Dioscorea bulbifera is a wild, underutilized yam species of nutritional and medicinal importance. It is known to treat ulcers, sores, wounds, spasms, dysentery, diabetes, and cancer and its bioactive component known as Diosgenin has exhibited anti-oxidant, anti-inflammatory, antibacterial, plasmid curing, antidiabetic anticancer and anti-fertility activities. The current world demand for diosgenin is approximately between 50,000 and 80,000 kg per annum and the present world requirement of steroidal drugs for pharmaceuticals in terms of diosgenin is huge equivalent to about 10,000 tons of Dioscorea tubers per annum of which *D.bulbifera* provides 8-10% in addition to its highest nutritional value. In Uganda, this species inhabits forests such as Bwindi Impenetrable forest and Mabira central forest reserve among others. However, these forests are rapidly being destroyed due to deforestation. Domestication of this plant species is a viable alternative to contributing to its conservation, which can be promoted through establishing a seed system for it. Therefore, this study aimed at developing an in-vitro micropropagation protocol for *Dioscorea bulbifera*. Shoots were induced through culturing nodal explants on full-strength Driver and Kuniyaki Walnut (DKW) media supplemented with varying concentrations of Kinetin (Kn) (0.00, 1.50, 1.75, 2.00, and 2.25) mg/l and fixed concentrations of Benzyl aminopurine (BAP) (1.00 mg/l) and Naphthalene acetic acid (NAA) (0.50 mg/l). The shoots were rooted on half-strength DKW media supplemented with varying concentrations of Naphthalene acetic acid (NAA) (0.0, 1.5, 2.0, 2.5) mg/l. The highest number of shoots were formed on DKW medium supplemented with 1.75mg/l Kn + 1mg/l BAP + 0.5mg/l NAA at 100% compared to other shoot induction treatments. However, the hormone combination of 1.5 mg/l Kn + 1mg/l BAP + 0.5 mg/l NAA produced the tallest plantlets with the broadest leaves at mean shoot height and leaf diameter of 1.5750 cm and 1.3969 cm, respectively. On the other hand, 2.5 mg/l NAA induced the longest roots at mean root length of 6.719 cm and 100% of the shoots rooted at this hormone concentration compared to the other treatments.

Keywords: Micro-propagation; DKW medium; Nodal explants; *Dioscorea bulbifera*; *in-vitro* propagation

Introduction

Yam is a multi-species crop that belongs to family Dioscoreaceae and genus Dioscorea [1-3]. All species are of tropical origin, cultivated in South East Asia, West Africa, and south and Central America [4] for their edible starchy tubers. The plant is characterized by growth of aerial tubers or bulbils. In Africa, the west African region contributes 95% of the world's yam produce with considerable varietal and genetic diversity [5]. Over ten different species are cultivated as staple food for millions of people [6], with *D. cayenensis* and *D. rotundata* complex as the most important species representing more than 97% of the total yam production in West Africa [7]. Guinea yam (*D. cayenensis* and *D. rotundata* complex) is the most important species and represents more than 97% of the total yam production in West Africa [7]. In Uganda, the commonly grown yam landraces include Kyetutumula (*D. cayenensis*), Kisebe (*D. alata*), Nakasoma (*D. alata*), Kaama (*D. abyssinica*), Ndaggu Nganda (*D. alata*) and Ndaggu Nziba (*D. alata*), and Balugu (*D. cayenensis*) and *D.bulbifera* [8]. *D. bulbifera* is of great medicinal importance, among others; the numerous therapeutic applications in various pathophysiological conditions like ulcers, sores, wound, spasms, dysentery, diabetes, and cancer [9]. Also, Spectacular success in extensive research has proved that these activities are attributed to its unique phytochemistry. Bioactive

components from *D. bulbifera* known as Diosgenin has exhibited anti-oxidant, anti-inflammatory, antibacterial, plasmid curing, antidiabetic and anticancer activities [10-13] and anti-fertility [14] making exploitation of its roots and bulbils high especially in regions where it grows like Kibale forest National park in Western Uganda [15]. Despite the fact that *D. bulbifera* species has vast medicinal values and curative results on several diseases, previous studies indicate that *D. bulbifera* has high toxicity properties and should be used with care as indicated in [16] where the ethanol extracts of *D. bulbifera* rhizome indicated toxic ingredients that led to hepatotoxicity. Hepatotoxicity is the most common form of toxicity in this plant and is evidenced as jaundice, vomiting, nausea and liver dysfunction [17]. The results of another study showed that the chloroform fraction of the methanolic extract of *D. bulbifera* was toxic when used on SD rats [18]. Additionally, fractions (saponins and flavonoids) had the effects on liver function by significant changes of liver index, liver weight, biochemical indexes, appearance and diet. Interestingly, the degree of *D. bulbifera* toxicity was related to the dose and time of drug administration [19]. Generally, the adverse or toxic effects are mainly speculated to be associated with the phytochemicals present in the plant specifically diosbulbins B and D [20,21]. However, diosgenin shows high biocompatibility and low toxicity. For instance, in an acute study, the oral administration of a single dose of 112.5–9000 mg/kg ethanol extracts of *Dioscorea* sp. containing 28.34% Diosgenin (31.7-2550.6 mg/kg) did not result in any signs of acute toxicity in rats [22]. This makes diosgenin a safe compound to use in pharmaceuticals. Diosgenin, a raw material for oral contraceptive is of high demand in the world [23] and is usually obtained from Dioscorea species since they contain it in higher yields compared to other sources like *Trigonella foenum* [24]. Among the Dioscorea species, *Dioscorea bulbifera* has emerged with the highest saponins [25] an indicator that it's a potential raw material for Diosgenin.. The current world demand for diosgenin is approximately between 50,000 and 80,000 kg/annum and the present world requirement of steroidal drugs for pharmaceuticals in terms of diosgenin is huge equivalent to about 10,000 tons of Dioscorea tubers per annum of which *D.bulbifera* provides 8-10% [26] of the raw material for oral contraceptive and there's an increasing need for oral contraceptives in the world [23]. Moreover, the plant also carries a higher nutritional value compared to other *Dioscorea species* [27], with the highest amounts of proteins, calcium, magnesium, sodium and zinc, highest values of vitamins B1, B3 and C. This high nutritional value justifies its huge consumption as food in the Eastern part [28], the Northern part [29], and the Central parts of Uganda [4,18]. Indeed, over exploitation as food and medicine coupled with the fact that it's a wild plant with its cultivation affiliated to the deforested wild, has greatly impacted on its conservation and sustainability and thus sending it to a near extinction status according to IUCN [29]. Trimanto *et al.*, suggested that promoting forest conservation, in addition to conducting germplasm collection back up and domestication can improve their conservation status [31]. Likewise, traditional conservation strategy involving whole plant through field gene banks have been fronted. However, such are subject to risks of losses because of human error, climatic and biological factors hazards [32]. Furthermore, other traditional methods of propagation via tuber cuttings are limited by very low multiplication ratio, difficulties to transport due to its bulkiness, extended dormancy period and transfer of pests and diseases among others [33].To solve these problems, alternative methods of rapid propagation like *in vitro* propagation method are inevitable if large scale multiplication of high-quality planting materials are to be ensured [34]. In fact, *in vitro* propagation offers many advantages over conventional methods like mass propagation, produces pathogen free planting materials, enables clonal propagation and enables year-round nursery production [35]. Protocols for *in vitro* propagation of several Dioscorea species have been developed and these include *D.hirtiflora* [35], *D.opposita* [36], *D.oppositifilia* [36], *D alata* [37], *D.bulbifera* [38] and *D. wightii* [39]. All these studies used Kinetin (Kn), benzyl amino purine (BAP), Benzyl adenine (BA), indole-3-acetic acid (IAA) and Naphthalene acetic acid (NAA) either in singular or in combination as plant growth regulators. The combination of BAP, Kinetin and NAA in shoot induction was selected comparing with the studies of [37] in *Dioscorea alata* since it gave out the highest percentage (98.8%) of microtubers that differentiated into shoots . This same combination of plant growth regulators has been used in the micro propagation of *Rosa hybrida* L. [40] from nodal segment with 86% shoot formation obtained in 8 days. All these protocols used these plant Growth regulators with Murashige and Skoog medium (MS) and no studies have used Driver and Kuniyaki Walnut (DKW) media. *Dioscorea spps* have been propagated from nodal segments [41] in comparison to other explant sources like Cryopreserved embryogenic tissue [42] because the nodal segments has proved to be the best source of explant for the purpose of commercial micropropagation since it gives a rapid initiation stage resulting in clean cultures [43]. The aim of this work was thus to multiply *D.bulbifera* species *in vitro*, especially to produce microtubers and use them as explants for commercial micropropagation. Many factors such as the presence or the absence of growth regulators, the concentration of sugars in the medium, the mineral composition of the medium and the photoperiod are known to influence the tuberization *in vitro* [44]. Therefore, the effects of growth regulators (BAP, Kin and NAA; in combination) and sucrose, on micro tuberization and micropropagation of *D. bulbifera* were studied. *In vitro* protocol for micropropagation through nodal segment is not available for Ugandan aerial yam germplasm. Therefore, there is need to develop a protocol for *in vitro* propagation of this germplasm for a sustainable exploitation of the plant *D.bulbifera* in Uganda.

Materials and Methods

Plant material and explant source

Arial tubers collected from Mabira central forest reserve (0°37.026'S 30°39.376'E, 0°24.112'N33°1.228'E, 0°24.108'N33°1.224'E, 0°23.687'N33°.899'E, 0°24.025'N33°.277'E, 0°24.123'N33°.369'E, 0°24.123'N33°.364'E) were harvested and stored in a dry cabinet over 16 weeks until they produced buds. Following dormancy break of the bulbils, they were planted at National Agricultural Research Laboratories (NARL) in poly bags of 30 cm x 30cm (Diameter x Height) filled sterilized top soil mixed with sand and

manure in the ratio of 1:1:1 and kept in a greenhouse at 70% shade and humidity of over 80% to mimic conditions similar to the forest environment. The planted tubers were watered once daily for 4weeks and allowed to sprout. The shoots were staked using 4 m-long bamboo sticks and allowed to climb to the top of the stakes after which nodal explants were collected from these shoots. These aerial buds from Mabira central forest reserve were selected because they contain high quantities of Diosgenin (unpublished data) compared to other accessions from different regions of Uganda.

Explant Preparation and Surface Sterilization

Tender stems of the plant were harvested and gently washed using tap water and a mild detergent (1% liquid soap) and cotton wool. The leaves were removed from the stems and cut into about 5cm-long pieces. These explants were then soaked in 0.25 % (v/v) chloroxylenol active (Reckitt Benckiser Arabia, U.A.E) for 30 minutes and thereafter rinsed three times with sterile distilled water. These were further immersed in 1.9% (w/v) sodium hypochlorite (Orbit Products Africa Ltd, Kenya) for 15 minutes and rinsed in 3 changes of sterile distilled water and then air-dried on sterile blotting paper.

Shoot Induction

The 5 cm-long surface-sterilized stems were cut in-between internodes to obtain single nodal explants which were cultured on full-strength Driver and Kuniyaki Walnut (DKW) media (PhytoTechnology Laboratories, United States) that contained full strength macro and micronutrients, 20 mg/l Ascorbic acid (Duchefa Biochemie, The Netherlands), 30g/l sucrose (Loba Chemie, India) and 3 g/l Gelrite (PhytoTechnology Laboratories, United States). supplemented with varying concentrations of Kinetin (Kn) (Duchefa Biochemie, The Netherlands) (0.00, 1.50, 1.75, 2.00, and 2.25) mg/l and fixed concentrations of Benzyl aminopurine (BAP) (Duchefa Biochemie, The Netherlands) (1.00 mg/l) and Naphthalene acetic acid (NAA) (0.50 mg/l) (PhytoTechnology Laboratories, United States). The cultures were maintained at 16-h light / 8-h dark photoperiods, 80-85 % relative humidity and light intensity of 50 µmol m^{-2} s^{-1} for 4w and the pH of the medium was 5.8 and autoclaved at 121 ^{o}C for 15 min. During the period of culture incubation, data was taken on explant response (presence or absence of shoot(s)), inititation date (date on which explants were inoculated on the media for the first time) response date (date on which shoots were visible), number of shoots per node, shoot height (cm) and shoot diameter (cm) (distance from the left leaf margin to the right leaf margin (in the middle position) of the first leaf to form). From the data collected, the percentage explant response (%) was calculated by dividing the number of explants that responded by the total number of explants and expressing it as a percentage; days to response was calculated by counting the number of days between initiation date and response date and the mean days to response was obtained by dividing the sum of days to response per treatment by the total number of replicates in the respective treatment. Mean number of shoots (cm) per node was calculated by dividing the number of shoots formed per node by the total number of explants per respective treatment; the mean shoot height (cm) was obtained by summing the readings for shoot heights per treatment and dividing it by the total number of shoots in the respective treatment. On the other hand, the mean leaf diameter leaf diameter (cm) was calculated by dividing the sum of the readings of the diameter of the first leaf developed by the total number of leaf diameter measurements per respective treatment.

Root Induction

The shoots generated were maintained on half-strength DKW for 4w after which were transferred to half-strength DKW medium fortified with different concentrations of NAA (0.0, 1.5, 2.0, 2.5) mg/l and maintained at 16-h light / 8-h dark photoperiods, 80-85 % relative humidity and 50 µmol m^{-2} s^{-1} light intensity for another 4w. Data was collected on response (presence or absence of roots), number of roots formed per shoot and length of roots per treatment. Percentage response was then calculated by dividing the number of shoots that rooted by the total number of shoots in the respective treatment and multiplying the resultant figure by 100; as in [44] mean number of roots per treatment was calculated by summing the number of roots per treatment and dividing it by the number of replicates/shoots per treatment while the mean root length was calculated through summing the measurements for root length per treatment and dividing it by the number of root length measurements in the respective treatment.

Weaning and acclimatisation

Rooted micro propagules were removed from culture jars and roots were washed under running tap water to remove agar. The plantlets were transferred to black perforated polythene bags of 10cmx 18cm (diameter by height) in a medium consisting of loam soil, sand and manure in the ratio of 2:1:1 [33]. The plants were covered with a transparent bag to create a humid chamber. After 7 days, the plants were removed from the humid chamber and watered whenever required. Plants were weaned in a screen house that could provide 70% shade. The survival rate of plantlets was recorded after 3 weeks [38]. Plants reached 8 - 10 cm height within 6 weeks (Figure 1).

Data Analysis

The experiments were completely randomized with 16 replicates per treatment and the experiments were repeated 2 times. Comparison of means of 5 independent groups was done in order to determine whether there is statistical evidence that the associated population means are significantly different and also the smallest significant difference between two means (amongst the total number of means being investigated) was computed using Fisher's Least Significant Difference test. Parameters of mean days to response, mean number of shoots, mean shoot height and mean leaf diameter, mean number of roots and mean root length were analysed using Minitab version 16 at the $P < 0.05$ level of significance.

Results

Shoot Induction

All treatments induced shoot formation at different levels as illustrated in Table 1. The highest percentage response was observed in T2 followed by T0, then T3, T4 and T1, respectively. The shortest response time was seen in T1 followed by T4, T3, T0 and T2, respectively. The mean number of days to response was statistically different among all treatments except T1 and T3. On the other hand, the tallest shoots were formed by T1 followed by T2, T0, T4 and T3, respectively. Mean should height was statistically different in all treatments except T1 and T2, and T0 and T4. Leaf diameter was highest in T1, T0, T4, T3 and T2, respectively. Mean leaf diameter was statistically different among all treatments except T3 and T4

Treatment	PGR (mg/l)	Percentage response	Mean days to response	Mean shoot height (cm)	Mean leaf diameter (cm)
T0	0.00 Kn + 0.00 BAP + 0.00 NAA	93.3	7.438±5.400[a]	1.1438±0.7915[b]	1.0594±0.7006[b]
T1	1.50 Kn + 1.00 BAP + 0.50 NAA	85.7	5.063±2.257[c]	1.5750 ±1.2892[a]	1.3969±0.8042[a]
T2	1.75 Kn +1.00 BAP + 0.50NAA	100.0	7.031±3.441[ab]	1.4094±0.8906[a]	0.7125±0.4878[c]
T3	2 Kn + 1.00 BAP + 0.50 NAA	92.3	5.375±2.075[c]	0.6187±0.3805[c]	0.9187±0.6347[bc]
T4	2.25Kn+1.00 BAP + 0.50 NAA	86.7	5.781±2.121[bc]	1.1094±0.4980[b]	0.9250±0.6128[bc]
			P = 0.015	P<0.01	P<0.01

* Means that do not share a letter are significantly different according to Fischer's L.S.D test at P=0.05
Note: Kn: Kinetin, BAP: Benzyl aminopurine, NAA: naphthalene acetic acid, PGR: plant growth regulator
Table 1: Effect of different PGR combinations on shoot proliferation of *D. bulbifera*

Rooting

Treatment	NAA (mg/l)	Percentage Response	Mean number of roots	Mean root length (cm)
T0	0.0	10	0.906± 1.711[c]	0.2438±0.4852[c]
T1	1.5	70	4.313±1.635[b]	1.1625±0.4757[a]
T2	2.0	80	4.250±3.464[b]	0.7344±0.4397[b]
T3	2.5	100	6.719±5.201[a]	0.9094±0.5245[b]
			P<0.01	P<0.01

*Means that do not share a letter are significantly different according to Fischer's L.S.D test at P=0.05
Note: NAA: naphthalene acetic acid.
Table 2: Effect of NAA on root proliferation of *D. bulbifera*

Figure 1: Regenerated *D. bulbifera* plantlets resulting from single node cutting **(A)** shooting cultured during 28 days on DKW medium supplemented with 1.50 Kn 1.00 BAP and 0.50NAA mg/l; **(B)** Rooted plantlet regenerated from nodal shoot cultured during 28 days in DKW medium supplemented with 2.5 NAA (mg/l); and **(C)** weaned plant in mixture of loam soil, sand and manure in the ratio of 2:1:1 (v/v) after 21 days of culture

All treatments induced roots (Table 2) with the highest percentage response observed in T3 followed by T2, T1 and T0, respectively. However, the mean number of roots formed was highest in T3 followed by T1, T2 and least in T0. The mean number of roots was statistically significant in all the treatments except T1 and T2. The longest roots were induced by T1 followed by T3, T2 and T0, respectively. The mean root length was statistically significant among all treatments a part from T2 and T3.

Rooted plantlets were transferred to the greenhouse within 3-4 weeks. Plants reached 8 - 10 cm height within 6 weeks (Figure 1). The acclimatized plants were established in field condition without any morphological variation.

Discussion

Micro-propagation of various plant species including many medicinal plants have been described by many authors during the last two decades [45]. In the present investigation micro-propagation of *D. bulbifera* was done and nodal segment was used as explants. DKW medium supplemented with NAA and kinetin was used for root and shoot growth. In this work, we cultured *D. bulbifera* in solid DKW medium; similarly *in vitro* propagation of other yam species is tried using solid medium [36]. In this study, different concentrations of Kn were found to affect the regeneration and development of *D. bulbifera* (Table 1). Application of Kn (1.5 mg/L) in combination with 1mg/L BAP+0.5 mg/L NAA to DKW medium stimulated higher *D. bulbifera* survival ratio if compared to DKW free medium. This study gives similar results as reported by [28,32] where 1.5 mg/L of Kn gave out increased number of leaves in *D. opposita* and *D. alata*. Infact, the capability of Kn to break bud dormancy and stimulate shoot multiplication has been reported in micro propagated *D. floribunda* [46] and Oppositifilia [38]. In connection to this, Potshangbam, [47] reported that combinations of Kn, BAP and NAA increased shoot and root induction of *Dendrobium chrysotoxum* Lindl. from 4.0 ± 1.4 leaves per explant. Furthermore, combination in the range of 1.5-2.0 mg/L Kn+1mg/L BAP+0.5 mg/L NAA has been reported to improve micro-tuber induction of *Chlorophytum borivilianum* [48]. In this respect, it can be concluded that interaction of Kn, BAP and NAA plays important role for *in vitro* propagation of nodal explant for multiple shoot induction. The fact that T0 without any plant growth regulators gave a shoot response of 93.3% with in 7.438±5.400[a] mean days, in addition to 1.1438±0.7915[b] cm mean height and 1.0594±0.7006[b] cm mean diameter proves that shoot induction in this protocol can be done with out the use of plant growth regulators. This is because the results of T0 do not vary much from T1 that where microtubers took 5.063±2.257[c] days to respond producing the largest mean shoot height of 1.5750 ±1.2892[a] cm and a mean leaf diameter of 1.3969±0.8042[a] cm yet even the percentage response of 85.7% is lower than that of T0. This actually makes this protocol a cheap, affordable and a cost effective one for commercial micropropagation of *D.bulbifera* in Uganda. Regenerating yam shoots with higher number of roots is an important factor that affects acclimatization stage and 2.5 mg/L NAA containing media gave shoots with the greatest number of roots of 6.719[a] and 0.9094[b] mean root length with 100% culture response. Behera *et al.*, [38] got similar results from rooting of *in vitro* Oppositifilia raised shoot-lets with ½ strength MS basal [41] of 6.5 ± 0.30 roots per plantlet and an average root length 4.5 ± 0.16 cm was recorded . T3 gave 100% root induction response and the most mean number of roots with 6.719±5.201[a] roots and the root mean length of 0.9094±0.5245[b] cm and similar results have been reported in [37] with kinetin and NAA. In this study, the result of acclimatization showed that the regenerated plantlets survived more in the mixture of loam soil, sand and manure in the ratio of 2:1:1 with (89.5%) survival rate. This result contrasts with acclimatization of many species where plantlets survived in vermiculite or in mixture of vermiculite and soil [49]. This can be partly explained by the fact that, naturally, *D. bulbifera* grows and develops well in the regions where the soil is mixed with sand and manure [37]. The current results are, however, valuable to the conservation of this wild medicinal plant, threatened by deforestation and overcollection [29] and to more sustainable foraging practices. The plant can be propagated successfully from nodal vines, and the seedlings of *D.bulbifera* can be used for ex situ conservation in living collections and the wider utilization of this germplasm.

Conclusion

This protocol can be used to multiply high Diosgenin yielding Dioscorea bulbifera germplasm from Mabira Central Forest reserve which consequently would promote its conservation and utilisation in Uganda because it is competent and economically viable.

Ethical consideration

Ethical approval and permission to conduct this study was obtained from the Mbarara University of Science and Technology Research Ethics Committee (MUST-REC) under the approval reference MUREC 1/7 and the Uganda National Council for Science and Technology (NS105ES).

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