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Comparative micromorphological study of wild and micropropagated *Dioscorea bulbifera* Linn.

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PEER REVIEW

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Comments

This is a good paper elucidating the importance of micro characters in the confirmation of the identity of both micro propagated and wild samples of *D. bulbifera*. The paper also provided information on the best conditions for obtaining a high level of genetic materials for the conservation of the plant.

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ABSTRACT

Objective: To study the leaf epidermis of wild and micropropagated *Dioscorea bulbifera* Linn. (*D. bulbifera*) in order to document useful diagnostic features that may be employed for correct crude drug identification and to clear any taxonomic uncertainties in the micropropagated medicinal plant.

Methods: Growth responses of micropropagated *D. bulbifera* were observed on Murashige Skoog medium supplemented with 6-benzylamino purine (1.0 mg/L)+α-naphthaleneacetic acid (0.2 mg/L)+cysteine (20 mg/L) using nodal segments as explants. Leaves of the wild and micropropagated plants were studied microscopically.

Results: More than 80% shoot regeneration and formation of 10%-30% whitish-brown callus were observed within 3 weeks. The highest root proliferation was obtained from Murashige Skoog medium of 6-benzylamino purine (0.05 mg/L) and α-naphthaleneacetic acid (0.01 mg/L) with mean root length of (27.00±1.25) mm and elongated single shoot of mean length (38.00±11.09) mm. Leaf epidermal features that revealed similarities between the wild and micropropagated plants included amphistomatic condition, presence of mucilage, glandular unicellular trichome with multicellular head, polygonal cells with smooth walls, stomata type and shape. Slight variations included thick cuticular wall with closed stomata in wild plant compared to thin walled opened stomata in the *in vitro* plant. Opening of stomata accounted for larger average stomata sizes of (7.68±0.38) μm and (6.14±0.46) μm on the adaxial and abaxial surfaces, respectively of the micropropagated plant compared to the wild.

Conclusions: The diagnostic features obtained in the study could serve as a basis for proper identification for quality control for standardization of the medicinal plant.

KEYWORDS

Dioscorea bulbifera, Micropropagation, Microscopy, Stomata, Trichome, Standardization, Medicinal plant

1. Introduction

Dioscorea bulbifera Linn. (*D. bulbifera*) belongs to the family Dioscoreaceae assigned to the order Dioscorales. It is commonly called air potato, air yam or bulbil-bearing yam. It is a vigorous climber plant native of West Africa predominantly found in forest gaps and forest edges[1]. Clockwise twinning direction was reported in *D. bulbifera* and measures up to 12 m[2]. The leaves are shiny green, alternate with a long petiole. Just like every yam plant, *D. bulbifera* possesses an annual vegetative cycle. The aerial bulbil serves as the main storage organ rather than underground tubers or bulbils[3]. At the beginning of the growing season, tubers and new bulbils produce thick sphagetti-like root from the rhizomatous head. Vegetatively, *D. bulbifera* is cultivated by planting the whole bulbils,

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but it is not grown much commercially because of the unpalatable bitter taste[4].

In Southwest Nigeria, *D. bulbifera* grows under the shade that contains a substrate composed of high levels of organic material and often over the tops of low-lying vegetation and into tree canopies. Most farmers regard the plant as weed competing with their farm products and clear them from the farm. Consequently, the plants are destroyed and are not eaten. In spite of the great medicinal application of *D. bulbifera*, preference is largely given by local people to the flavor of other yam species. This has led to the near extinction status of this plant in the southeastern part of Nigeria[5]. It is therefore imperative to consider conserving this medicinally useful plant genetic resource. The use of micropropagation techniques for endangered plant genetic resources conservation was recognized late in the 70s[6]. At the moment, *in vitro* technique has found increasing significance as a complementary means to *in situ* plant conservation, especially for vegetatively propagated and recalcitrant seed-producing species[7]. Although the development of successful *in vitro* propagation and storage protocols are reliable methods that make possible the establishment of extensive germplasm collections, plants cultivated *in vitro* present certain anatomical and physiological characteristics intrinsic to culture environment[8]. Maintaining the genetic identity of donor plant and a completely viable acclimatized plant yield after *in vitro* storage are the essential prerequisites for using *in vitro* culture techniques for *ex-situ* conservation purpose. Morpho-anatomical, biochemical and molecular parameters have also been used to monitor integrity and stability of *in vitro* grown plants[9].

Leaf micro-characters can be used for distinguishing crude drugs even when they are fragmented[10]. A combination of different diagnostic features (quantitative and microscopic parameters such as stomata and trichome types with sizes, shapes and sizes of epidermal cell, thickness of cell and cell inclusions) has been used for taxonomic distinction and recognition in the angiosperm family[11-15]. Species mis-identification of herbal drugs due to numerous local names given to the same species or several species of crude drug with the same local name is common in Africa[16]. Therefore, foliar micromorphological description of crude drug may complement macroscopic description of medicinal plants which could be useful in preparing a monograph for its identification, thus providing a useful tool for collection and preservation of the species.

D. bulbifera, besides being important as an edible yam, is used in traditional medicine[17]. Among the many documented medicinal folk uses, the plant is used to paste produced from the tubers to treat swelling and as a cure for snakebite and scorpion stings in Africa and Asia, and the bulbils are used to cure thyroid diseases and cancer in traditional Chinese medicine[18-20]. The plant had been reported to possess analgesic, anti-inflammatory and antimicrobial properties as well as used for diseases such as leprosy, tumours, piles, dysentery, syphilis and ulcers[21-23].

In view of the biological and medicinal importance of *D. bulbifera*, and the report of its near extinction in southeast region of Nigeria, the present study was designed to compare qualitative and quantitative micro-morphological characteristics of the wild and micropropagated plants which may be employed for correcting crude drug identification.

2. Materials and methods

2.1. Plant material

Bulbils and leaves of *D. bulbifera* were collected in November, 2011 from the field at Alabata village in Moniya, Ibadan, Southwest Nigeria. The plant was identified by Mr. O. A. Osiyemi at the Forest Herbarium Ibadan where the voucher specimen with number "FHI 109529" was deposited.

2.2. Source of explant

Following dormancy break of the bulbils (after 16 weeks), they were dipped into black polythene bags with holes, filled with top soil and kept in the green house to minimize microbial contamination of plants. Healthy vines with active buds were collected from 12 weeks old plants of *D. bulbifera* raised and maintained in the green house. The vines were excised from the mother plant with single node intact (used as explants). Similarly, explants with single node were excised from underground bulbil (12 weeks old) sprouting after dormancy break but kept indoor away from light source (the sun).

2.3. Micropropagation

Healthy nodal segments (12 weeks old) obtained from the green house and underground bulbil grown indoor (Figure 1A and 1B) were disinfected using a drop of Tween 20 in distilled water in a sterilized bottle with constant shaking for 3 min.

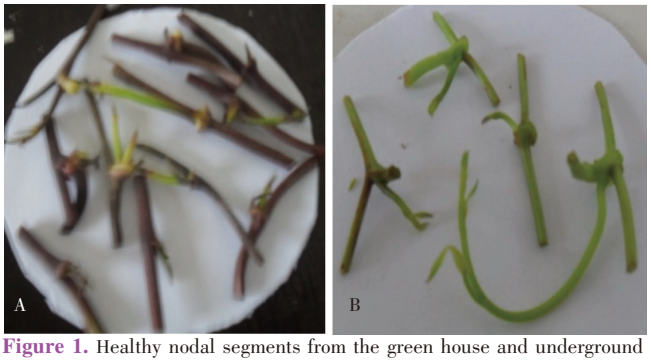

Figure 1. Healthy nodal segments from the green house and underground bulbil grown indoor.
A: Purple nodal segments obtained from underground bulbil following dormancy break; B: Greenish nodal segments from green house.

They were rinsed in distilled water until explants were

clean and free from Tween 20. Thereafter, they were soaked in 70% alcohol for 10 min and finally treated with 3% sodium hypochloride for 15 min and then rinsed thoroughly by intermittent shaking with sterilized distilled water (3×). The surface-decontaminated explants were trimmed on the edges and cut to the required length (1.0-1.5 cm) before vertically in Murashige Skoog (MS) medium-filled culture tubes (one explant per tube)[24].

2.4. Culture medium and conditions

MS medium was used as the basal medium for shoot and root proliferation. The medium was supplemented with 30 g/L sucrose and gelled with 5 g/L agar. The pH of the medium was adjusted to 5.8 prior to autoclaving. The basal medium was fortified with cysteine (20 mg/L), combination of α-naphthaleneacetic acid (NAA) (0.0-1.5 mg/L) and 6-benzylamino purine (BAP) (0.0-1.0 mg/L), NAA (2.0 mg/L) and NAA [2.0 mg/L+BAP (0.5 mg/L)]. Routinely, 10-15 mL each of molten medium was dispensed into sterile (20×150) mm culture tubes. For each treatment, 10 replicates were used and all the experiments were repeated three times. The cultures were incubated at (27±1) °C with a photoperiod of 16 h at an intensity of 10-20 μmol $m^{-2}s^{-1}$. After 3-6 weeks of culturing, cultures were subcultured in fresh basal medium depending on the experiments.

2.5. Estimation of growth of D. bulbifera in cultures

Growth of plant was assessed 4-6 weeks. Shoot and root proliferation were scored and measured after 6 weeks of culturing. The growth factors used were number of leaves, callus formation, shoot number, number of nodal segments (buds) on shoots and shoot length.

2.6. Epidermal surface tissue preparation

Fresh leaves of wild and *in vitro* cultivated plant of *D. bulbifera* cut in the standard median position and then soaked in concentrated nitric acid for 3-5 h in a covered Petri dish were used for epidermal study. The Petri dish was placed on a hot water bath until bubbles were noticed on leaves which signified readiness of epidermal peel removal. The epidermal peel was rinsed (5×) to remove concentrated nitric acid and thereafter cleared with soft Carmel hair brush to dissociate tissue remains from the surfaces. The abaxial and adaxial surfaces were carefully mounted on a clean glass slides and dehydrated by graded series 50%, 70%, 80% and 100% ethyl alcohol and later stained with safaranin O for 2 min; excess stain was removed by adding few drops of 70% ethyl alcohol. Glycerin was used as mountant, and epidermises were covered with coverslips and the edges of the slide sealed with nail varnish to prevent dehydration. Slides were examined for assessment of the qualitative diagnostic features such as stomata type, cuticular striation on walls, mucilage and shape of epidermal cell with quantitative parameters such as epidermal cell size, stomata and epidermal wall dimension were recorded.

The mean±SE, minimum and maximum values were calculated for all variables. Photomicrograph (Ziess standard 25) of the epidermises was obtained using Motic camera mounted on a compound light microscope and attached to a Pentium IV computer. The techniques utilized in this study were in accordance with methodology previously reported in literature[25-27].

3. Results

3.1. Growth responses of micropropagated plant

In the present study, MS medium+BAP (0.05 mg/L)+NAA (0.01 mg/L) produced the highest mean root length of (27.00±1.25) mm with an elongated single shoot of mean length (38.00± 11.09) mm (Figure 2A, Table 1) representing 70% explants response after 8 weeks of culturing.

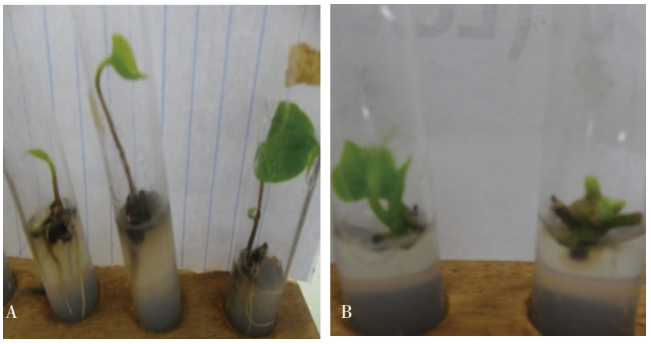

Figure 2. Multiple root formation with single elongated shoot and no shoot and root formation.

A: Multiple root formation with single elongated shoot; B: Bud break with single leaf (no shoot and root formation).

Table 1

Callus, shoot, leaf and root formation from nodal segments of *D. bulbifera* (parameters of growth scored after 6 weeks).

Media treatment	Disinfection treatment of nodal segment	Cysteine (mg/L)	Explant response (%)	Callus (%)	Bud break (d)	Mean No. of leaves	Mean No. of shoot	Mean shoot length (mm)	Mean No. nodes	Mean root length (mm)
0.05mg/L BAP+0.01mg/L NAA	70% alcohol (10 min), 3%NaOCl (15 min)	-	70	20	18-21	1.00±0.00	1.00±0.00	38.00±11.09	0.00±0.00	27.00±1.25
1.5mg/L BAP+0.30mg/L NAA	70% alcohol (10 min), 3% NaOCl (10 min)	-	20	20	18-20	1.05±0.05	0.12±0.01	0.67±0.02	0.00±0.00	0.00±0.00
1.0mg/L BAP+0.2mg/L NAA	70% alcohol (10 min), 3%NaOCl (15 min) *N1	20	90	10	14-16	3.00±0.31	1.33±0.17	30.89±5.86	2.86±0.34	0.00±0.00
1.0mg/L BAP+0.2mg/L NAA	70% alcohol (10 min), 3%NaOCl (15 min) **N2	20	80	30	10-13	2.67±0.54	1.17±0.17	53.33±3.50	4.17±0.70	25.00±10.61

Values are expressed as mean±SEM; 10 replicates per treatment.

*N1: 12 weeks old nodal segment of *D. bulbifera* obtained from green house; **N2: 12 weeks old nodal segment obtained from underground bulbils of *D. bulbifera* following dormancy break.

Responses of nodal segments of *D. bulbifera* in MS media supplemented with BAP (1.5 mg/L) and NAA (0.3 mg/L) were poor as 20% of growth was recorded with no shoot elongation and root formation after culturing for more than 6 weeks (Figure 3B). MS medium supplemented with BAP (1.0 mg/L)+NAA (0.2 mg/mL)+cysteine (20 mg/L) cysteine showed significant response of nodal segments of *D. bulbifera* with more than 80% growth response in which bud break was achieved within 2 weeks (Figure 3A and 3B). The quality of shoots and overall growth response in terms of average shoot length and number of leaves was best in this combination in which an average number of leaves (3.00±0.31), average number of shoots (1.33±0.17), average shoot length (53.33±3.50) mm and average root length (25.00±10.61) mm were recorded.

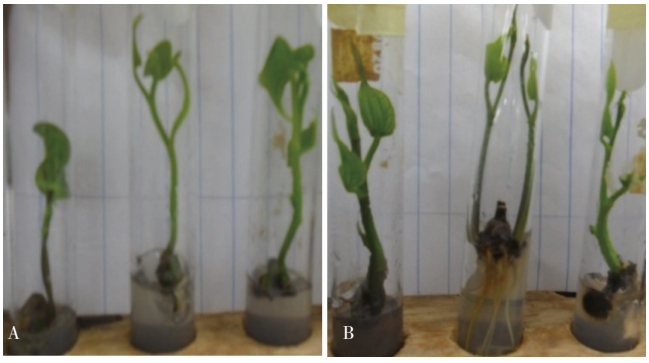

Figure 3. Nodal segments of *D. bulbifera* with elongated shoots.
A: Nodal segments from green house with elongated shoot and leaves; B: Nodal segments from underground bulbil with elongated shoots and root proliferation.

Subculturing of nodal segments of *D. bulbifera* obtained from Figure 3A and 3B after 6 weeks of growth in the same media resulted in formation of multiple shoots, 3.60±0.87 [mean length (49.20±5.71) mm] within 6 weeks of culturing, which was higher compared to multiple shoots, 2.40±0.40 [mean length of (33.60±7.48) mm] (Table 2) obtained from excised nodal segments from green house (Figure 4A and 4B).

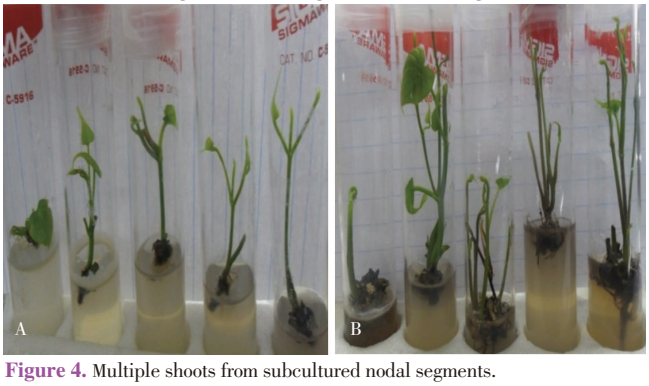

Figure 4. Multiple shoots from subcultured nodal segments.
A: Multiple shoots obtained from green house; B: Multiple shoots obtained from underground bulbil.

Generally, parameters of growth, such as number of leaves, mean multiple shoots and mean length showed optimal and better growth response in nodal segments obtained from underground bulbils (100% free of contamination) compared with nodal segments obtained from the green house (70% free of contamination) (Table 2). Introduction of excised nodal segments from 6 weeks old plant in fresh MS media+BAP (1.0 mg/L)+NAA (0.2 mg/L)+cysteine (2.0 mg/L) resulted in formation of a single bulbil with aerial root after 4 weeks of culturing (Figure 5A). Similarly, introduction of 6 weeks old cultured plant from MS media+BAP (1.0 mg/L)+NAA (0.2 mg/L)+cysteine (20 mg/L) in fresh MS media supplemented with NAA (2.0 mg/L)+cysteine (20 mg/L) resulted in 50% production of single nodal bulbil with aerial root attached to the bulbil and profuse whitish callusing at the base of the nodal vine after 8 weeks of culturing (Figure 5B).

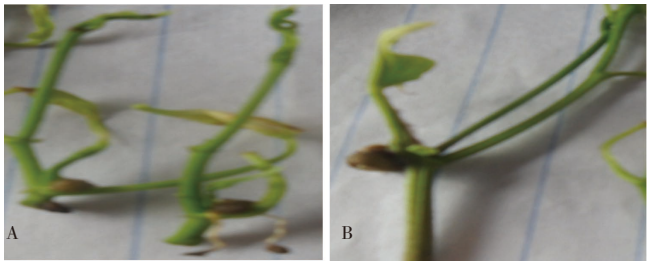

Figure 5. Single bulbil with aerial root and single bulbil at the nodal base.
A: Formation of single bulbil with aerial root; B: Single bulbil at the nodal base.

In all media treatments, 10%-30% callus was generated (Figure 6A). Also, subculturing of 6 weeks old *in vitro* plants from MS media+BAP (1.0 mg/L)+NAA (0.2 mg/L)+cysteine (20 mg/L) in MS media+NAA (1.0 mg/L)+BAP (0.2 mg/L) resulted in profuse white callus at the base after 8 weeks of subculturing with suppressed shoot growth (Figure 6B).

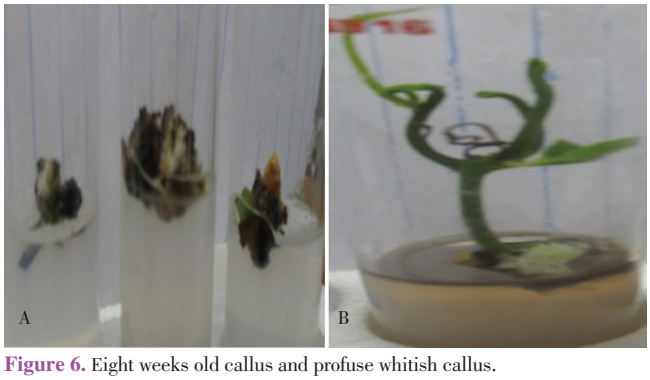

Figure 6. Eight weeks old callus and profuse whitish callus.
A: Eight weeks old callus from nodal explants of *D. bulbifera*; B: Profuse whitish callus with shoot suppression.

Table 2
Subcultured nodal segments from N1 and N2 in same media composition (parameters of growth scored after 6 weeks).

Media treatment	Mean No. of leaves	Mean No. of shoots	Mean shoot length (mm)	Mean No. of nodes
1.0 mg/L BAP+0.2 mg/L NAA+20 mg/L cysteine (*N1)	4.20±0.80	2.40±0.40	33.60±7.48	2.75±0.29
1.0 mg/L BAP+0.2 mg/L NAA+20 mg/L cysteine (**N2)	5.80±1.46	3.60±0.87	49.20±5.71	3.00±0.00

Values are expressed as mean±SEM; 10 replicates per treatment.
*N1: 12 weeks old nodal segment of *D. bulbifera* obtained from green house; **N2: 12 weeks old nodal segment obtained from underground bulbils of *D. bulbifera* following dormancy break. Comparison of mean of parameters of growth using independent student *t*-test for each column showed no statistical significance at $P>0.05$.

3.2. Macroscopy characteristics

Morphologically, the leaf description of the wild and micropropagated plants were similar in terms of leaf venation which was parallel and vein convergence at the base, leaf shape in both was cordate with long petiole and alternate arrangement, leaf margin was entire with aerial bulbils produced from the node in both wild and micropropagated plants.

3.3. Microscopy of wild and micropropagated plants

Comparative qualitative and quantitative features of foliar epidermis of wild and micropropagated *D. bulbifera* are presented in Table 3. The useful diagnostic features included amphistomatic leaves, anomocytic and anisocytic stomata and smooth epidermal wall as shown in Figures 7 and 8.

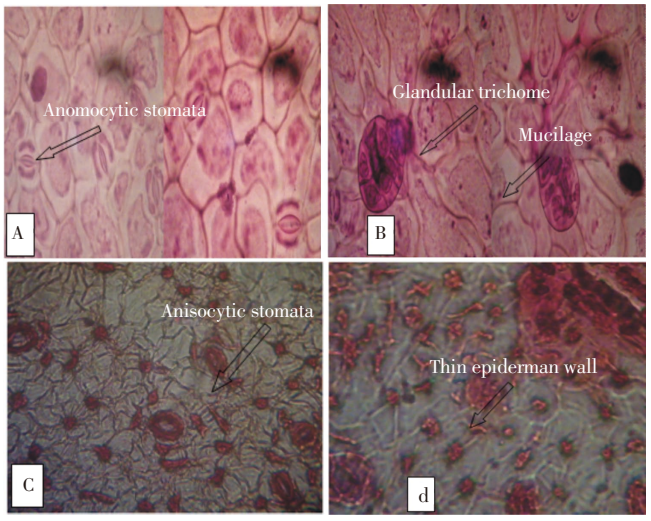

Figure 7. Foliar microscopic features of abaxial epidermis of *D. bulbifera*.
A & B: Wild plant; C & D: *In vitro* plant (400×).

Slight variations included closed stomata pore in the wild and open in the *in vitro* cultivated plants, cuticularization of epidermal walls was observed in the adaxial and abaxial surfaces of the wild whereas it was noticed only on the abaxial surfaces of the *in vitro* plant. Wild plant had thicker lignified walls than the micropropagated plant and mass of mucilages were evenly spread in the epidermal cell of the wild but condensed and scattered in the *in vitro* propagated plant (Figures 7 and 8).

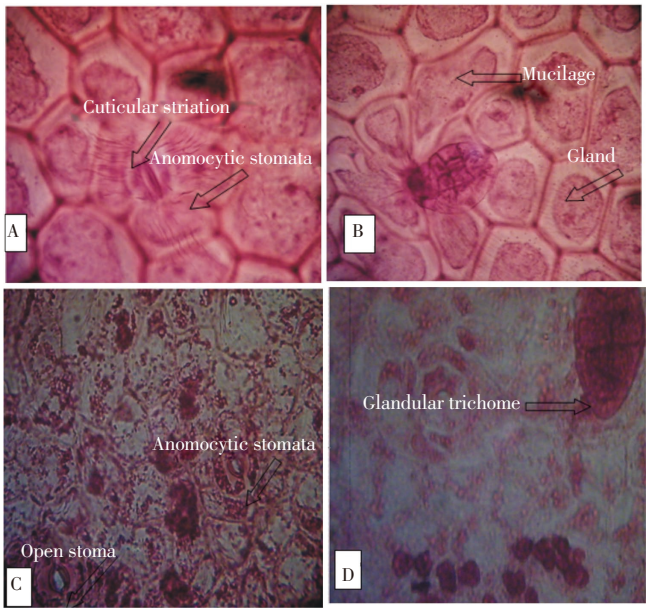

Figure 8. Foliar microscopic features of adaxial epidermis of *D. bulbifera*.
A & B: Wild plant; C & D: *In vitro* plant (400×).

4. Discussion

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated

Table 3
Comparative qualitative and quantitative features of foliar epidermis of wild and micropropagated *D. bulbifera*.

Parameters		Wild cultivated plant (μm)	Micropropagated plant (μm)
Adaxial surface	Epidermal cell length	27.5 (49.0±5.25)* 82.5	20.48 (30.21±2.95)* 51.2
	Epidermal cell width	27.5 (40.5±2.73)* 55.0	10.24 (17.15±1.58)* 25.6
	Stomata length	15.0 (19.0±0.67)* 22.5	15.36 (18.18±0.70)* 20.48
	Stomata width	5.0 (6.0±0.41) 7.50	5.12 (7.68±0.38) 10.24
	Trichome length	52.5 (56.0±1.19)* 60.0	43.53 (49.15±1.14)* 53.76
	Trichome width	32.5 (37.5±1.29)* 45.0	28.16 (29.7±0.78)* 33.28
Abaxial surface	Epidermal cell length	25.0 (50.5±5.93) 22.5	23.04 (42.75±6.13) 76.8
	Epidermal cell width	22.5 (36.25±2.51)* 47.5	10.24 (19.20±2.30)* 35.84
	Stomata length	15.0 (17.50±0.83) 22.5	12.8 (17.15±0.86) 20.48
	Stomata width	5.0 (5.75±0.38) 7.50	5.12 (6.14±0.46) 7.68
	Trichome length	40.0 (47.25±1.56) 57.5	38.4 (44.03±2.04) 61.44
	Trichome width	27.5 (31.5±0.85) 35.0	30.72 (35.07±1.62) 48.64
Diagnostic features	Cell wall pattern	Smooth	Smooth
	Epidermal cell shape	Polygonal	Polygonal
	Trichome type	Glandular unicellular trichome with multicellular head	Glandular unicellular trichome with multicellular head
	Stomata type	More of anomocytic than anisocytic	More of anomocytic than anisocytic
	Mass of mucilages	Evenly and wide spread within epidermal cell	Condensed as a single spot

Data are expressed as minimum, mean± SEM, and maximum, n=10. Comparison of diagnostic features of wild and *in vitro* foliar epidermis of abaxial and adaxial were carried out using independent student t-test. *: P<0.05.

plants in soil[28]. The combination and interaction of BAP and NAA had been reported to play an important role in *in vitro* propagation of nodal explants for multiple shoot induction[29]. NAA, an auxin, can effectively induce shoot emergence when combined with an adenine derivative (BAP). It is possible that the combination of BAP (1.0 mg/L)+NAA (0.20 mg/L)1+cysteine (20 mg/L) in this study was responsible for shoot regeneration as control without these plant growth regulators failed to grow after observation in culture medium for more than 6 weeks. Earlier workers reported shoot regeneration of 42%-75% in *Dioscorea rotundata* when cultured in MS medium supplemented with NAA (0.1 μmol/L)+BAP (0.20 μmol/L) and shoot plantlet regeneration of 60%-82% obtained in media made of NAA (0.05 μmol/L)+BAP (0.20 μmol/L) or BAP (0.46 μmol/L)+kinetin (0.50 μmol/L) in *Dioscorea alata*[29]. Similarly, MS medium supplemented with NAA (1.0 mg/L) and BAP (0.5-1.0 mg/L) was reported as the best concentration for multiple shoot bud induction in *Dioscorea opposita*. In *Dioscorea hispida*, BAP (2.0 mg/L)+NAA (0.50 mg/L) with ascorbic acid 100 mg/L, elicited optimal response in shoot in which an average of (6.00±0.18) shootlets with a mean shoot length of (5.00±0.28) cm per explant was recorded[30].

In vitro bulbil induction in this study agrees with a previous study in which incorporation of BAP (2.0 mg/L) in MS medium resulted in the formation of a single oval bulbil from the lowest node of *D. bulbifera*[31]. In the same report, NAA (0.50 mg/L) along with BAP (2.0 mg/L) in MS medium induced formation of a maximum of eight spherical bulbils. However, in the present study, induction of bulbil with aerial root (whitish) in MS media+NAA (2.0 mg/L)+cysteine (20 mg/L) is suggested rather than the use of BAP (2.0 mg/L) or NAA (0.50 mg/L)+BAP (2.0 mg/L) in MS medium.

The observation of whitish callus at the nodal base of *D. bulbifera* with resultant effect in suppression of shoot growth may be attributed to the presence of NAA at 1.0 mg/L in the MS media. Naphthalene acetic acid had been reported to inhibit root elongation in a wide range of concentrations from 0.01 μmol/L to 1 μmol/L[32]. The presence of mass of mucilage within the epidermal cell and the presence of glandular unicellular trichomes with multicellular head are indicative of the medicinal lodgments of bioactive principles in the plant and in a way may justify its use in traditional medicine[33]. A combination of these features had been found useful for taxonomic distinction and recognition in angiosperm families.

Mucilages are generally normal products of metabolism formed within the cell (intracellular formation) and or produced without injury to the plant[34]. Mucilages are evenly and widely spread within the epidermal cell of the wild plant than the micropropagated plant. Previous workers have reported that phytochemicals including saponins, polyphenols and mucilage are the likely active components of *Dioscorea* species[35-37]. Also, mucilages have been reported in the epidermis of senna (*Cassia acutifolia* Delile), Alexandrian senna (*Cassia augustifolia* Vahl), *Cassia auriculata* Linn. and *Thespia populnea* Linn.[38,39].

Anomocytic stomata type reported in this work agrees with a previous report of anomocytic stomata in the order Dioscorales as in the case of *Dioscorea hispida*[40]. Similarly, the foliar epidermal anatomy of *Dioscorea rotundata*, *Dioscorea cayenensis*, *Dioscorea dumetorum*, and *Dioscorea alata* was reported to consist of anomocytic stomata only for the four species of *Dioscorea* studied[41]. However, the present anatomical studies of the foliar epidermises of *D. bulbifera* revealed not only anomocytic stomata type but anisocytic. The presence of anisocytic stomata in *D. bulbifera* together with anomocytic stomata may serve as distinguishing character for its identification from other *Dioscorea* species.

Cuticular striated wall has been reported in the epidermal imprint of the lower surface of the leaf of *D. bulbifera* using non-toxic mucilage[42]. Our observation of cuticular striations on the adaxial and abaxial epidermises of both wild and micropropagated *D. bulbifera* is in line with this report and can therefore be used as a diagnostic feature for standardization of the crude drug.

It is well known that most histological traits of plant are under the control of environmental conditions. Organs of an individual plant living under stress undergo adaptive changes which determine their more effective function[43]. *In vitro* culture explants are exposed to specific artificial conditions which differ from those in the natural environment. These specific conditions are responsible for the structural changes occurring in micropropagated plants. The main determinant factors are high relative air humidity, air composition and culture media content. The air humidity in culture vessels is very high ranging from 95%-100% and plant response is to enhance water diffusion into the cells, with subsequent increase in both parenchymatic cell sizes and intercellular spaces. Futhermore, the continuing cell enlargement retards the development of secondary walls (lignification and cutinization) resulting in thinner cell wall, reduced deposition of epicuticular waxes and poor mechanical tissue formation[44]. This supports the thin cell wall observed in the micropropagated plant in this study and may explain the slight variation of anatomical features in the foliar epidermis when compared with the wild. Thin cuticle seems to be a common feature for all *in vitro* cultivated plant species. Johansson *et al.* demonstrated the developmental differences in the cuticle of rose plants which occur in various micropropagation stages[45]. Therefore, in the case of shifting from *in vitro* to *ex vitro* cultures, the cutine is gradually synthesized, with the decrease relative humidity of the medium in which the plant lives.

In water saturated flask atmosphere such as the one presented in tissue culture experiments, the accumulation of specific gases (ethylene) in the confined atmosphere causes a dramatic increase in the transpiration rate leading to the failure of stomata to close[46]. This indeed may explain the opening of stomata pore and possibly the reason for having larger average width of stomata sizes in the micropropagated plant [adaxial surface (7.68±0.38) μm and abaxial surface (6.14±0.46 μm)] compared to the wild. Generally, mean length

of stomata size, epidermal cell size and trichomes of the abaxial and adaxial surfaces in wild plant were larger than micropropagated *D. bulbifera* while the average epidermal trichome in abaxial surface was larger compared with the wild.

Morphological, anatomical and phytochemical investigations are the integral part of herbal science. Anatomical characters can help in the identification of plant when morphological features are not distinct. Microscopic evaluation is an indispensable tool for identification of medicinal herbs and is one of the essential parameter in modern monograph. In this regard, the important microscopic features of the leaf of *D. bulbifera* (an important medicinal plant) such as anomocytic and anisocytic stomata, glandular trichomes with unicellular stalk and multicellular head, cuticular striations, presence of mucilage within epidermal cell and polygonal epidermal wall with smooth surfaces may serve as useful diagnostic features.

In conclusion, the diagnostic features reported in the wild and micropropagated plants could serve as a basis for proper identification and authentication of *D. bulbifera*.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Micro morphological characters are indispensable in the identification and authentication of medicinal plants samples. These have been employed in this paper to confirm the identity of the wild and micro propagated samples of *D. bulbifera*.

Research frontiers

The near endangered status of *D. bulbifera* makes it imperative to find appropriate means of its conservation. However, we can only conserve what we know hence the need to be sure that what is propagated *in vitro* is the same plant in the wild. This study combined taxonomic skill with tissue culture.

Related reports

The use of micro-propagation techniques for endangered plant genetic resources conservation was recognized late in the seventies (Ivanova *et al.*, 2011). The techniques utilized in this study were in accordance with methodology previously reported in literature (Saheed and Illoh, 2010; Smillie and Khan, 2010; Santos *et al.*, 2008).

Innovations and breakthroughs

Though many papers have reported the importance of micro characters, this report focused on the comparison between wild and micro propagated relatives using such characters and thus introducing another research dimension in biodiversity conservation.

Applications

The study further confirms the taxonomic importance of micro morphological characters in plant taxa as well as the identification of crude drug samples even when fragmentary.

Peer review

This is a good paper elucidating the importance of micro characters in the confirmation of the identity of both micro propagated and wild samples of *D. bulbifera*. The paper also provided information on the best conditions for obtaining a high level of genetic materials for the conservation of the plant.

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