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SUSTAINABLE PLANTLET REGENERATION FROM HYPOCOTYL SEGMENTS AND ZYGOTIC EMBRYO OF *SECURIDACA LONGIPEDUNCULATA* FRESEN. (POLYGALACEAE) - AN ENDANGERED MEDICINAL PLANT

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Abstract

Plant regeneration was achieved by culturing sterile hypocotyl segments, and mature zygotic embryos (MZE) of *S. longipedunculata* Fresen excised from sterile seeds. In this study, de-coated and sterilized seeds were first grown into seedlings *In vitro*, disjointed into root, hypocotyl, and leaf, segments. The hypocotyl segments (0.5cm) were cultured in root induction media containing MS basal medium + sucrose (30 g l^{-1}) fortified with 0-5 mg l^{-1} NAA and 0-5 mg l^{-1} IBA either alone or in combination to produce roots. Rooted hypocotyl segments were cultured in shoot induction media containing 0-1mg l^{-1} IAA, 0-1 mg l^{-1} KIN, and 0-1 mg l^{-1} GA for shoot induction. Second, mature zygotic embryos (MZEs) excised from seeds were cultured based on 3×4×5 factorial – Control, MS and B5 basal media, three carbon sources (sucrose, glucose, and fructose), and control at five concentrations (0-4%) in a completely randomized design at $p \leq 0.05$ and repeated two independent times for reproducibility of results. Results of the observed parameters revealed that after 45 days, the highest number of roots (11.66±0.23), most increased root length (3.43±0.23), and highest percent root generation (86.66±3.33) were produced in hypocotyl explants in 0 mg l^{-1} NAA+5.0 mg l^{-1} IBA. More so, the earliest induction (35.66±0.66) was seen in 0.5 mg l^{-1} NAA+0.5 mg l^{-1} IBA. After 72 days of culture, the highest number of new shoots (2.0 per explant), the highest number of leaves (5.0±1.00), the highest number of shoots (3.80±0.11), highest percent regeneration (88.33±1.60), and highest leaf area (2.06±0.23) were produced in rooted hypocotyl explants at 1mg l^{-1} each for IAA, KIN, and GA relative to other treatments. In MZE culture, 3% sucrose in MS medium was the most preferred for zygotic embryo culture and differed significantly from other treatments at $p \leq 0.05$. These young plantlets, *In vitro,* were hardened and raised in the field. These regenerated plantlets would ensure the conservation of this endangered plant as a primary source of medicine.

Key words: Mature zygotic embryos, Regeneration, hypocotyl segments, Engangered plant, *Securidaca longidunculata*.

Introduction

S*ecuridaca longipedunculata* Fresen of the family polygalaceae, commonly known as Violet tree is locally called *Chipvufanaor mufufu* in Swahili whereas in Nigeria, it is well known as "Ezeogwu" (king of herbs) by the Igbos (Okoli *et al*., 2005), "Uwar magunguna" (mother of medicines) by the Hausas (Tshisikhawe *et al*., 2012; Saidu *et al*., 2015) and the Fulanis call it *aalali*. It is widely used for almost every plausible illness (Kadiri *et al*., 2013). It is indigenous to North-Central Nigeria and cuts across tropical Africa (Chika *et al*., 2017) with white or violet flowers. It has long been employed in African traditional medicine (Mongalo *et al*., 2015). It is known to have an essential medicinal and ethnomedicinal importance (Mustapha *et al*., 2013) because of its wealth of therapeutic characteristics.

Recently, the side effects of synthetic medicine, expensive allopathic medicine, and inadequate medical facilities force most of the populace to rely or depend heavily on traditional systems, especially those of high antioxidant characteristics, thus considerably increasing demand (Karakas, 2020). Many researchers showed that the indigenous uses of *S. longipedunculata* can be summed up to include use as sexual booster and treatment of gastrointestinal ailments, sexually-related infections, skin infections, fever, pneumonia, toothache, liver disease, bronchitis, rheumatism, and snakebites (Mustapha *et al*., 2013). The extract of this plant is rich in anti-inflammatory, antiulcer, antianemia, and antiplasmodial properties (Ojewole *et al*., 2008), which has led to overexploitation and increased threats to the habitat of this plant, making it vulnerable (Tshisikhawe *et al*., 2012; Oni *et al*., 2014). Tavhare & Nishteswar (2014) revealed that overexploitation and collection of medicinal plants have led to vulnerability and degradation of the ecosystem, thus devastating the resource base and *S. longipedunculata* is not an exception. When the seeds are available, problems associated with seed dormancy, high seedling mortality, and slower growth of young plants have caused the plant to be on the verge of disappearing. Similarly, Zulu *et al*., (2011) reported that survival, transplanting and propagation of *S. longependuculata* is difficult because the seeds hardly germinate, and if they do, seedlings grow very slowly. This has caused some African countries like South Africa to protect it legislatively (Tabuti *et al*., 2012; Tshisikhawe *et al*., 2012); Ghana, reported as a rare plant (van Andel *et al*., 2012), very threatened in Bulilima District, Zimbabwe (Viol, 2009) and heavily relied on in Benin (Ouedraogo *et al*., 2003). This research triggered a need for alternative means of regeneration of *S. longipedunculata*.

The use of explants is emerging as a tool for increased numbers and crop productivity (Chandler *et al*., 2011). Through direct or indirect morphogenesis and somatic embryogenesis, new whole plants can be grown from different types of explants (Tileye *et al*., 2005). Nearly every part of the plant can be used to initiate tissue culture,

including the embryo. Hypocotyl segments have been successfully used to culture and regenerate plants (Ebrahimie *et al.*, 2006). Embryo culture is of two categories: those of relatively mature and differentiated embryos and those of immature, early division phase proembryos. Developmental processes especially that of an embryo can be experimentally studied through *In vitro* culture of a zygotic embryo (Haslam & Yeung, 2011). Embryo culture was first achieved in 1904 by Hanning from the excision and growth of mature embryo seeds of Raphurzus and Cochlearia in mineral salt and sucrose media into plantlets. Since accomplishing this feat, excised embryos from ovules and seeds have been grown under a controlled environment and aseptic conditions. The protocol for the nutritional requirements, growth conditions and differentiation of some plant species has been successfully developed and documented (Haslam & Yeung, 2011).

In vitro plant cell cultures are not fully autotrophic - hence carbohydrates are needed in culture media to sustain osmotic potential. This carbohydrate also serves as an energy source for physiological and developmental processes, including shoot morphogenesis and proliferation, induction of roots, embryogenesis and organogenesis, and an anosmatic agent's role (Amhad, 2007; Novero *et al.*, 2010; Smith, 2013). *In vitro,* culture systems in polygalaceae are greatly manipulated by varying the levels and types of carbon sources to achieve maximum plantlet growth as reported by Fang *et al.*, (2012) on *Polygala fallax* and Nery *et al.*, (2021) on *Polygala paniculata.*

Before this study, there have been insufficient or no published studies on the regeneration of *S. longipedunculata* through zygotic embryos and few studies on regeneration through hypocotyl explants. The objective was to achieve maximal plant regeneration from hypocotyl segments as well as mature zygotic embryos from excised seeds of *S. longipedunculata* while the other parts of the seeds such as the endosperm can be utilized for its pharmaceutical properties and at the same time enhancing the population of this threatened species.

Materials and Methods

Sample collection and media composition: Fresh *S. longipedunculata* fruits were collected from a wild-growing population of Eziani, Nsukka Local Government Area, Enugu State, Nigeria ($6^0$51’24’N$7^0$23’45’E). The basal media used for MZEs were those of Murashige and Skoog (MS) (1962) and Gamborg *et al*., (B5) (1968) consisting of 3 × 4 × 5 (basal media, carbon sources and carbon source concentrations) factorials. The basal media used for root induction from hypocotyl culture consisted of MS fortified with 0-5 mg l^{-1} IBA, 0-5 mg l^{-1} NAA, and 3% sucrose. Those of shoot induction consisted of MS enhanced with 3% sucrose, 0-1 mg l^{-1} IAA, 0-1 mg l^{-1} KIN and 0-1 mg l^{-1}GA.

Sample preparation: Seeds of *S. longipedunculata* were separated from the fruits (Fig. 4a) and husks were removed. The seeds were de-coated and thoroughly and surface sterilized using standard procedures and placed in a shaker (Iwashiya Bioscience Multi Lay-up shaker, Japan) at 120 rpm for 20 mins to ensure that the sterilant touched all parts of the seeds. Sterile distilled water was used to rewash the seeds three times to completely remove the sterilant. The de-coated seeds, in an aseptic condition, were opened exposing the embryo. Sterile seedlings of *S. longipedunculata* were raised from seeds grown invitro under sterile conditions. The seedlings were aseptically dismembered into hypocotyl, leaf, and root segments. The Hypocotyl segments were selected and cut into about 0.5 cm long segments.

Mature zygotic embryo culture: The experiment comprised 30 treatments with 10 cultures per treatment for each basal medium. Under asepsis, inoculation of the sterile mature embryo into the sterilized media in the clean hood transferred into tubes and set up for growth under standard protocols.

Root organogenesis and shoot formation from hypocotyl segments of *S. longipedunculata*: Hypocotyl segments were cultured on MS basal medium fortified with 3% sucrose, NAA (0 -5 mg l^{-1}) and IBA (0 -5 mg l^{-1}) either alone or made up 15 treatments with 3 replicate flasks each. Each flask contained 5 hypocotyl segments. The rooted-hypocotyl explants were further cultured on MS medium consisting of 3% sucrose, supplemented with 0-1 mg l^{-1} IAA, 0-1 mg l^{-1} KIN and 0-1 mg l^{-1} GA either alone or in a combination of all. This gave rise to 12 treatments with three replicate flasks each. Each flask contained five rooted-hypocotyl explants.

Observation and data collection: Regeneration of embryo explants of *S. longipedunculata* was examined daily from inoculation day. When maximum growth was achieved, plantlets were removed from the culture tubes and scored for the following growth parameters: root lengths, shoot lengths, number of leaves, number of roots, leaf area, sprout rate and percentage regeneration. The number of roots and leaves were visually counted.

Statistical analysis

Data collected were analyzed by subjecting them to one-way analysis of variance (ANOVA) using SPSS software (22.0 versions). With Duncan’s New Multiple Range test at 0.5% level, means were separated. The operations were repeated two independent times.

Results

The time course of the percent sprouting of zygotic embryos (Fig. 4b) as shown in (Figs. 1, 2 & 3) revealed the effects of the various types and concentrations of carbon sources in the basal media. Sucrose at 3% and 4% in MS media had the highest percent sprouting at 7, 14, 21 and 28 days. They significantly differed from other carbon source types and concentrations at $p \leq 0.05$. Similarly, sucrose at 3% and 4% in B5 media, had the highest per cent sprouting at 7, 14, 21 and 28 days. They were statistically significant from other carbon source types and concentrations at $p \leq 0.05$. The inclusion of carbon sources without basal medium showed drastic reduction in percent sprouting when compared to cultures with basal media (MS and B5). In cultures containing 3 % and 4% sucrose without basal medium, the highest percent sprouting (60%) was recorded (Fig. 3).

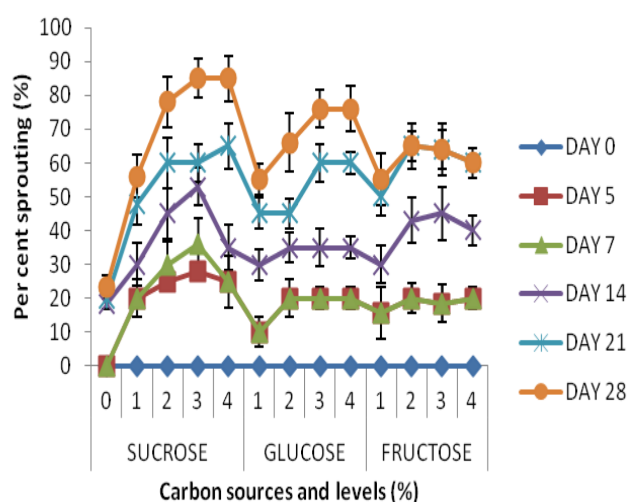

Fig. 1. Time course in percentage sprouting of *S. longipedunculata* mature zygotic embryo in varied concentration and types of carbon sources in Murashige and Skoog basal medium.

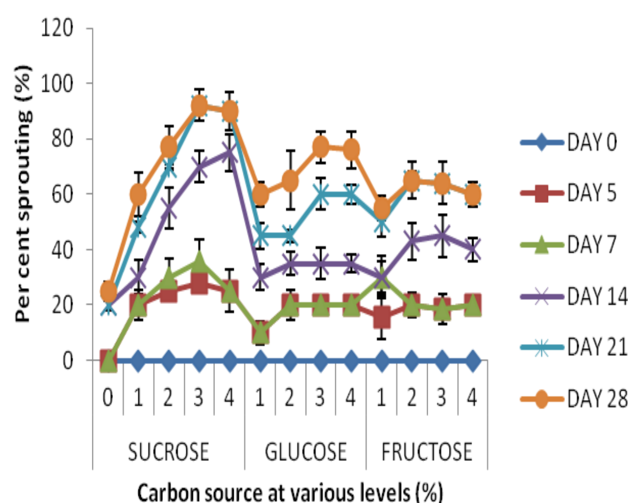

Fig. 2: Time course in percentage sprouting of *S. longipedunculata* mature zygotic embryo in varied concentration and types of carbon sources in Gamborg's (B5) basal medium

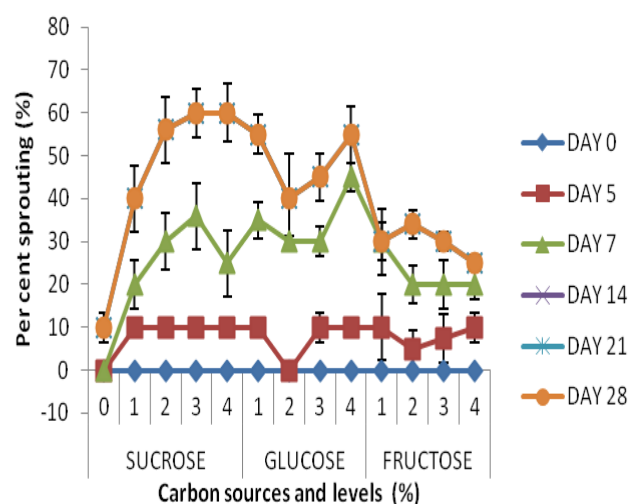

Fig. 3. Time course in percentage sprouting of *S. longipedunculata* mature zygotic embryo in varied type and concentration of carbon sources without basal medium.

Interestingly, Table 2 showed that sucrose at 3% and 4%, glucose at 1% and 4%, and fructose at 1% did not differ significantly in terms of plantlets' length of the shoot when B5 was used as the basal medium. Glucose at 2% and 4% supported maximum root elongation relative to other treatments. Sucrose at 3% had the highest leaf area, although, not differed significantly from sucrose at 4%, glucose at 1, 2, 3, 4%, and fructose at 1%. However, glucose at 2% had the highest number of roots while sucrose at 2, 3, 4% had the highest fresh weight relative to other treatments.

It was shown in Table 3 that in the absence of basal medium, sucrose at 3% supported the highest length of shoot relative to other treatments. On the other hand, sucrose at 4% had the longest root.

S. longipedunculata plantlets were successfully regenerated from zygotic embryos cultured in all concentrations of glucose and fructose (1-4%) studied. However, maximum morphological parameters (shoot length, fresh weight, root length and leaf area) were not achieved.

The increase in the concentration of carbon source (sucrose, glucose and fructose) in the present investigation did not increase the values of the dependent variables tested.

Results of direct root organogenesis and de-differentiation from hypocotyl segments of *S. longependunculata* (Fig. 4c) are presented in Table 4. Hypocotyl segments (0.5 cm) in MS medium with only sucrose at 3 per cent did not differentiate into callus, or regenerate into roots. At 5.0 mg l^{-1} NAA, roots number (6.66±0.88), with length of root (1.20±0.11) was regenerated from hypocotyl segments. NAA at 0.5 mg l^{-1} differed significantly from other treatments in terms of induction per cent, even though roots were not produced. The sole inclusion of IBA at 0.5 mg l^{-1}, and 1.0 mg l^{-1} neither induced callus nor produced roots. Roots were however, produced when IBA was increased to 3.0 mg l^{-1}. At 5.0 mg l^{-1}IBA, callus was induced from hypocotyl with an induction per cent (83.33(3.33), and the number and length of roots were the highest. These attributes differed significantly from those of other treatments.

The synergistic effects of both NAA and IBA at the same concentration (0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA, 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} IBA, 3.0 mg l^{-1} NAA + 3.0 mg l^{-1} IBA, 5.0 mg l^{-1} NAA + 5.0 mg l^{-1} IBA) induced callus and regenerated roots simultaneously except for NAA 0.5 mg l^{-1} + IBA 0.5 mg l^{-1} and NAA 1.0 mg l^{-1} + IBA 1.0 mg l^{-1} where only callus induction was observed. On the other hand, hypocotyl explants in NAA 5.0 mg l^{-1} + IBA 5.0 mg l^{-1} did not produce callus but regenerated into roots. NAA 5.0 mg l^{-1} + IBA 3.0 mg l^{-1} produced both calli and roots, and NAA 3.0 mg l^{-1} + IBA 5.0 mg l^{-1} equally produced similar results. The latter differed significantly from the former in terms of induction per cent, the number of roots produced, root number, days to induction and percentage root regeneration.

Adventitious root formation in MS+ NAA (3.0 mg l^{-1}) + IBA (5.0 mg l^{-1}) produced the best result for number of roots, root length, fewer days to induction and per cent root regeneration.

Table 1. Growth indices of plantlets regenerated from mature embryo culture of *S. longipedunculata* in MS medium after four weeks.

Treatments	Length of the shoot (cm)	Length of root (cm)	Number of leaves	Leaf area (cm^2)	Number of roots	Fresh weight (g)
Control	2.73 ± 0.33^{ef}	2.50 ± 0.28^{b}	2.00 ± 0.11^{bc}	2.31 ± 0.09^{ab}	3.00 ± 0.57^{de}	0.12 ± 0.00^{h}
Sucrose 1%	4.96 ± 0.43^{bc}	2.16 ± 0.17^{b}	3.33 ± 0.33^{a}	1.98 ± 0.24^{bc}	4.00 ± 0.57^{cde}	0.16 ± 0.01^{efg}
Sucrose 2%	5.70 ± 0.64^{bc}	2.33 ± 0.23^{b}	2.66 ± 0.33^{ab}	2.10 ± 0.11^{abc}	5.00 ± 0.57^{c}	0.23 ± 0.01^{a}
Sucrose 3%	7.53 ± 0.67^{a}	2.63 ± 0.38^{b}	3.66 ± 0.33^{a}	2.76 ± 0.06^{a}	8.66 ± 0.88^{a}	0.23 ± 0.01^{a}
Sucrose 4%	4.70 ± 0.15^{cd}	5.13 ± 0.72^{a}	3.00 ± 0.57^{ab}	2.16 ± 0.06^{abc}	7.00 ± 0.57^{b}	0.22 ± 0.02^{ab}
Glucose 1%	3.56 ± 0.53^{de}	2.03 ± 0.29^{bc}	2.00 ± 0.00^{bc}	2.20 ± 0.06^{abc}	4.66 ± 0.33^{cd}	0.17 ± 0.02^{def}
Glucose 2%	4.83 ± 0.24^{cd}	3.06 ± 0.28^{b}	2.00 ± 0.50^{bc}	1.91 ± 0.06^{bc}	3.66 ± 0.33^{cde}	0.19 ± 0.01^{cde}
Glucose 3%	6.23 ± 0.29^{b}	3.10 ± 0.05^{b}	3.00 ± 0.57^{ab}	1.88 ± 0.05^{bc}	4.66 ± 0.33^{cd}	0.21 ± 0.04^{abc}
Glucose 4%	4.50 ± 0.51^{cd}	4.86 ± 0.46^{a}	3.00 ± 0.57^{ab}	1.84 ± 0.06^{bc}	5.00 ± 0.57^{c}	0.20 ± 0.00^{bcd}
Fructose1%	1.30 ± 0.40^{g}	0.26 ± 0.14^{d}	0.33 ± 0.33^{d}	0.46 ± 0.46^{e}	1.00 ± 0.57^{f}	0.14 ± 0.01^{gh}
Fructose2%	1.90 ± 0.45^{fg}	1.10 ± 0.20^{cd}	1.00 ± 0.57^{cd}	1.11 ± 0.56^{de}	2.66 ± 0.33^{e}	0.13 ± 0.01^{h}
Fructose3%	2.80 ± 0.30^{ef}	0.80 ± 0.36^{d}	1.33 ± 0.33^{cd}	1.65 ± 0.06^{bcd}	3.66 ± 0.33^{cde}	0.15 ± 0.02^{fgh}
Fructose4%	2.73 ± 0.20^{ef}	0.70 ± 0.11^{d}	1.00 ± 0.03^{cd}	1.48 ± 0.07^{cd}	3.66 ± 0.33^{cde}	0.14 ± 0.01^{gh}

Data are represented as mean ± S.E, and significant means are separated with dissimilar alphabets on the same column using the DMRT at $p \leq 0.05$

Table 2. Growth indices of plantlets regenerated through mature embryo culture of *S. longipedunculata* in B5 medium after four weeks.

Treatments	Length of shoot (cm)	Length of root (cm)	Number of leaves	Leaf area (cm^2)	Number of roots	Fresh weight (g)
Control	3.33 ± 0.46^{cde}	2.73 ± 0.32abc	2.00 ± 0.01bc	2.17 ± 0.25ab	3.33 ± 0.88cdef	0.13 ± 0.01ef
Sucrose 1%	2.60 ± 0.41^{def}	2.16 ± 0.16bc	2.00 ± 0.02bc	1.58 ± 0.15abc	4.66 ± 0.33bcde	0.17 ± 0.02bcde
Sucrose 2%	3.20 ± 0.47^{cde}	2.73 ± 0.32abc	2.00 ± 0.11bc	1.25 ± 0.04bcd	5.33 ± 0.33abcd	0.22 ± 0.01a
Sucrose 3%	5.22 ± 0.11^{ab}	3.30 ± 0.40ab	3.00 ± 0.40ab	2.49 ± 0.36a	6.00 ± 1.47abc	0.19 ± 0.09abc
Sucrose 4%	5.43 ± 0.24^{ab}	2.26 ± 0.23bc	3.33 ± 0.33ab	1.80 ± 0.11ab	5.33 ± 0.66abcd	0.21 ± 0.02ab
Glucose 1%	5.60 ± 1.05a	3.16 ± 0.44ab	3.66 ± 0.33a	2.06 ± 0.15ab	5.33 ± 1.33abcd	0.18 ± 0.01bcd
Glucose 2%	3.66 ± 0.65bcd	4.56 ± 1.26a	2.33 ± 0.33bc	2.13 ± 0.34ab	8.00 ± 1.00a	0.17 ± 0.02bcde
Glucose 3%	3.63 ± 0.24bcd	2.16 ± 0.33bc	2.00 ± 0.14bc	2.15 ± 0.02ab	6.66 ± 1.33ab	0.18 ± 0.01bcd
Glucose 4%	4.66 ± 0.38abc	4.30 ± 0.05a	2.00 ± 0.07bc	2.14 ± 0.08ab	3.66 ± 0.33bcdef	0.16 ± 0.05cdef
Fructose1%	4.90 ± 0.23abc	3.36 ± 0.31ab	3.33 ± 0.33ab	1.83 ± 0.07ab	4.66 ± 0.88bcde	0.16 ± 0.01cdef
Fructose2%	3.30 ± 1.30cde	3.53 ± 1.77ab	2.00 ± 1.15bc	1.25 ± 0.62bcd	2.66 ± 1.33def	0.15 ± 0.07def
Fructose3%	0.93 ± 0.29f	0.76 ± 0.37c	0.33 ± 0.33d	0.66 ± 0.66cd	0.66 ± 0.33f	0.13 ± 0.01ef
Fructose4%	1.63 ± 0.31ef	0.70 ± 0.20c	1.33 ± 0.33cd	0.46 ± 0.46d	2.00 ± 0.57ef	0.12 ± 0.01f

Data are represented as mean ± S.E and significant means are separated with dissimilar alphabets on the same column using the DMRT at $p \leq 0.05$

Table 3. Growth indices of plantlets regenerated through mature embryo culture of *S. longipedunculata* without basal medium after four weeks.

Treatments	Length of shoot (cm)	Length of root (cm)	Number of leaves	Leaf area (cm^2)	Number of roots	Fresh weight (g)
Control	0.34 ± 0.08^{e}	0.33 ± 0.14c	-	-	1.33 ± 0.33de	0.09 ± 0.02d
Sucrose 1%	2.63 ± 0.18^{bcd}	1.00 ± 0.26bc	1.33 ± 0.33b	1.62 ± 0.07a	2.66 ± 0.88bcd	0.14 ± 0.01ab
Sucrose 2%	3.10 ± 0.23bc	1.13 ± 0.53abc	1.33 ± 0.33b	1.66 ± 0.05a	2.33 ± 0.33cd	0.12 ± 0.01abc
Sucrose 3%	4.16 ± 0.35a	2.46 ± 1.02ab	2.33 ± 0.33a	1.47 ± 0.06ab	4.33 ± 0.33b	0.12 ± 0.04abc
Sucrose 4%	2.76 ± 0.42bcd	2.96 ± 1.18a	2.00 ± 0.00ab	1.51 ± 0.17ab	4.00 ± 0.57bc	0.14 ± 0.02ab
Glucose 1%	1.96 ± 0.26d	1.03 ± 0.24ab	1.66 ± 0.33ab	1.20 ± 0.00c	4.00 ± 0.57bc	0.11 ± 0.01bcd
Glucose 2%	3.23 ± 0.18b	2.33 ± 1.09ab	1.66 ± 0.33ab	1.36 ± 0.14bc	7.00 ± 1.15a	0.13 ± 0.02ab
Glucose 3%	2.30 ± 0.15cd	1.00 ± 0.26bc	2.00 ± 0.00ab	1.46 ± 0.03ab	3.66 ± 0.33ab	0.14 ± 0.01ab
Glucose 4%	2.30 ± 0.80cd	0.60 ± 0.10c	1.50 ± 0.50b	1.30 ± 0.10bc	3.50 ± 0.50bc	0.12 ± 0.01abc
Fructose1%	0.10 ± 0.10e	0.06 ± 0.06c	-	-	0.33 ± 0.33e	0.12 ± 0.01abc
Fructose2%	0.43 ± 0.03e	0.30 ± 0.05c	-	-	1.33 ± 0.33de	0.14 ± 0.01ab
Fructose3%	0.30 ± 0.15e	0.36 ± 0.03c	-	-	1.66 ± 0.33de	0.12 ± 0.00abc
Fructose4%	0.16 ± 0.08e	0.30 ± 0.05c	-	-	1.33 ± 0.33de	0.10 ± 0.00cd

Data are represented as mean ± S.E and significant means are separated with dissimilar alphabets on the same column using the DMRT at $p \leq 0.05$

Table 4. Effects of concentrations of NAA and IBA on callus induction and adventitious root formation from hypocotyl explants after 45 days in culture.

Plant growth regulators (mg l^{-1})		Dependent variables				
NAA	IBA	Callus induction (%)	Number of roots	Length of root (cm)	Earliest induction	Root regeneration (%)
0	0	-	-	-	-	-
0.5	0	96.66 ± 3.33^{a}	-	-	32.33 ± 1.45^{bcd}	-
1.0	0	83.33 ± 3.33^{b}	-	-	31.33 ± 0.66^{cd}	-
3.0	0	25.00 ± 8.66^{d}	-	-	40.00 ± 2.88^{a}	-
5.0	0.	-	6.66 ± 0.88^{b}	1.20 ± 0.11^{b}	-	63.33 ± 3.33^{b}
0	0.5	-	-	-	-	-
0	1.0	-	-	-	-	-
0	3.0	-	1.66 ± 0.33^{d}	0.23 ± 0.03^{de}	-	65.33 ± 0.88^{b}
0	5.0	83.33 ± 3.33^{b}	11.66 ± 1.20^{a}	3.43 ± 0.23^{a}	28.33 ± 0.33^{d}	86.66 ± 3.33^{a}
0.5	0.5	43.33 ± 3.33^{c}	-	-	35.66 ± 0.66^{b}	-
1.0	1.0	43.33 ± 3.33^{c}	-	-	34.33 ± 1.45^{bc}	-
3.0	3.0	36.66 ± 3.33^{c}	0.66 ± 0.33^{de}	0.20 ± 0.11^{de}	32.00 ± 1.15^{bcd}	23.33 ± 3.33^{d}
5.0	5.0	-	3.66 ± 0.33^{c}	0.43 ± 0.03^{cd}	-	36.66 ± 3.33^{c}
5.0	3.0	13.33 ± 3.33^{e}	3.33 ± 0.33^{c}	0.56 ± 0.08^{c}	30.66 ± 1.76^{cd}	81.66 ± 4.40^{a}
3.0	5.0	36.66 ± 3.33^{c}	7.00 ± 0.53^{b}	0.63 ± 0.08^{c}	32.00 ± 1.73^{bcd}	86.66 ± 3.33^{a}

Data are represented as mean ± S.E and significant means are separated with dissimilar alphabets on the same column using the DMRT at $p \leq 0.05$

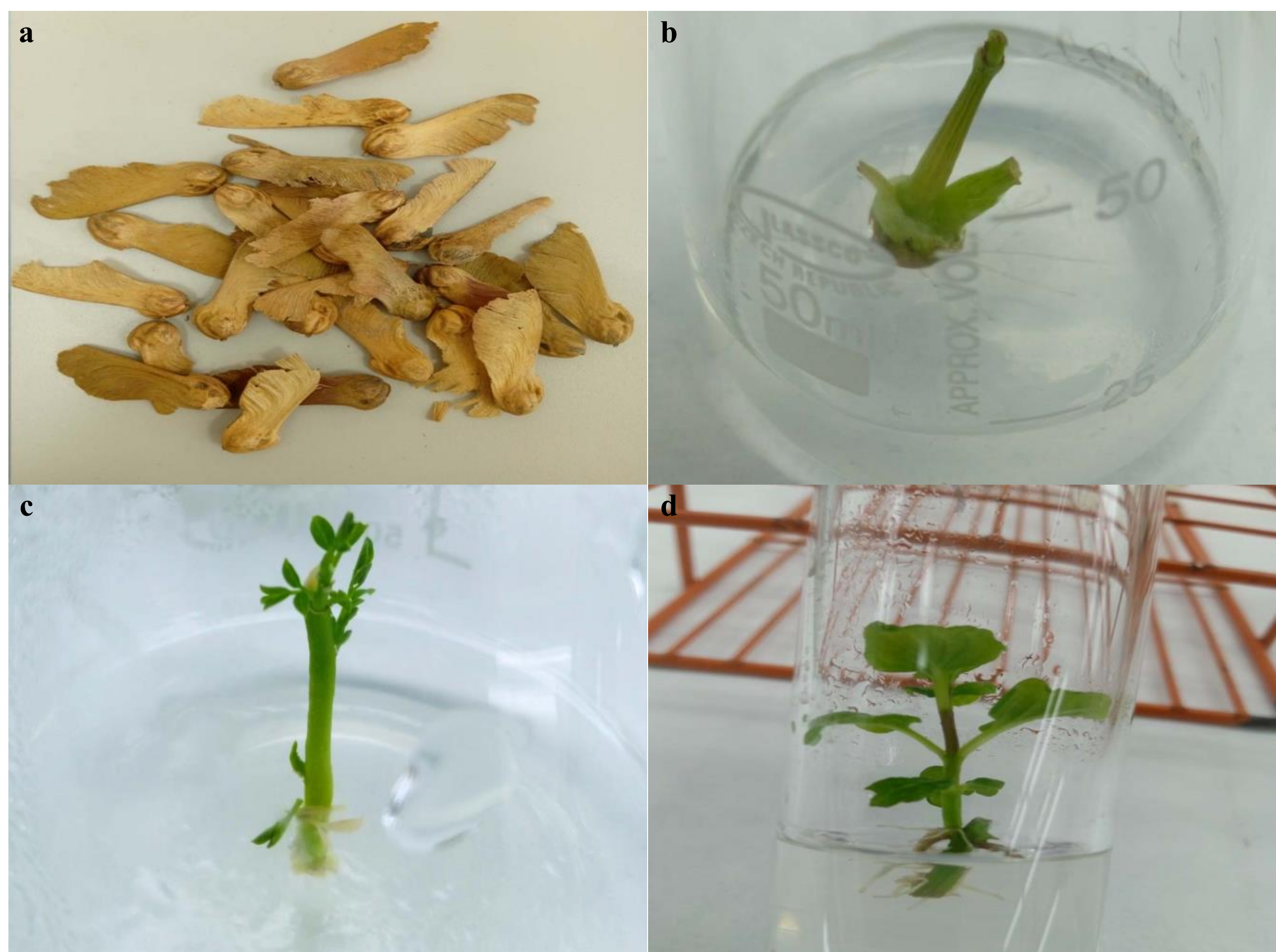

Fig. 4. Images from *Securidaca longipedunculata*. (a): Fruits of *S. longipedunculata* (b): Sprouting and swollen zygotic embryo of *S. longipedunculata* in MS medium with sucrose as carbon source (c): Plantlet emanating from hypocotyl segment with glucose as carbon source (d): Plantlet emanating from hypocotyl segment showing shoots.

Table 5 Effect of different concentrations of IAA, kinetin and GA on shoot formation from rooted-hypocotyl explants after 72 days in culture.

IAA (mg l^{-1})	kinetin (mg l^{-1})	GA (mg l^{-1})	No of shoots per explant	Number of leaves	Length of shoot	Regeneration (%)	Days to regeneration	Leaf area (cm^2)
0	0	0	-	-	-	-	-	-
0.1	0	0	-	-	-	-	-	-
0.5	0	0	-	-	-	-	-	-
1	0	0	-	-	-	-	-	-
0	0.1	0	-	-	-	-	-	-
0	0.5	0	-	-	-	-	-	-
0	1	0	-	-	-	-	-	-
0.5	0.5	0	1.33 ± 0.33^{b}	3.33 ± 0.57^{b}	2.43 ± 0.08^{c}	46.66 ± 3.33^{c}	68.33 ± 0.88^{a}	1.77 ± 0.06^{b}
1	1	0	1.66 ± 0.33^{ba}	3.33 ± 0.57^{b}	2.53 ± 0.14^{c}	63.33 ± 3.33^{b}	71.00 ± 2.64^{a}	1.26 ± 0.10^{c}
0.5	0.5	0.5	1.33 ± 0.33^{b}	3.66 ± 0.57^{b}	3.20 ± 0.15^{b}	65.00 ± 2.88^{b}	67.00 ± 1.15^{a}	1.36 ± 0.03^{c}
0.5	0.5	1.0	1.66 ± 0.33^{ba}	4.66 ± 0.57^{a}	3.70 ± 0.15^{a}	83.33 ± 3.33^{a}	60.33 ± 4.37^{b}	1.73 ± 0.15^{b}
1.0	1.0	1.0	2.00 ± 0.00^{a}	5.00 ± 1.00^{a}	3.80 ± 0.11^{a}	88.33 ± 1.66^{a}	53.33 ± 1.76^{c}	2.06 ± 0.23^{a}

Data are represented as mean ± S.E and significant means are separated with dissimilar alphabets on the same column using the DMRT at $p \leq 0.05$

*Days to regeneration at 0.00±0.00 implies there were no sprouting/differentiation

The combined effects of both auxins brought about the production of root. The synergistic effects of auxins on rooting were shown to sufficiently produce roots when both IAA and IBA at 1 mg l^{-1} each or IBA and NAA at the same concentration were utilized. Adventitious roots were produced by increasing the concentration to 5 mg l^{-1} and 10 mg l^{-1} respectively.

Similarly, results obtained from IBA at 1 mg l^{-1} and 3 mg l^{-1} did not produce adventitious roots with an increase in concentration like NAA (Table 4).

In table 5, the effects of IAA, kinetin, GA either singly or in combination showed that rooted-hypocotyl did not produce shoot under control treatment after 72 days in culture. Although, similar results were obtained when either IAA or kinetin was included to the media singly at 0.1 mg l^{-1}, 0.5 mg l^{-1} and 1.0 mg l^{-1}. There was an emergence of shoot when there was an addition of both IAA and kinetin at (0.5 mg l-1 and 1 mg l-1). Number of shoots per explant were (1.33±0.33, 1.66±0.33), number of leaves (3.33±0.57, 3.33±0.57), length of shoot (2.43±0.08 cm, 2.53±0.14 cm), and days to regeneration (68.33±0.88, 71.00±2.64) respectively and did not differ significantly at $p \geq 0.05$. However, there was a significant difference in the percentage regeneration and leaf area in the above PGRs combination.

The inclusion of the three PGRs simultaneously (IAA, kinetin, GA) had a significant effect at 1.0 mg l^{-1} each. All the dependent variables tested were the highest at these hormonal combinations. For example, the number of shoots per explants (2.00±0.00), number of leaves (5.00±1.00), shoots length (3.80±0.11 cm), least days to regeneration (53.33±1.76) and highest leaf area (2.06±0.23 cm^2). Although, they did not differ significantly with the combinations of IAA (0.5 mg l-1) + kinetin (0.5 mg l^{-1}) + GA (1.0 mg l^{-1}) in the number of shoots per explants produced, the number of leaves, length of shoot, and regeneration percentage (Fig. 4d).

Discussion

Results gathered revealed that 3% sucrose level was out-performed other carbon sources in all parameters tested, and the increase in sugar level to 4% (sucrose and glucose) resulted in increased root length. These findings agreed with Sumaryono *et al.*, (2012) who reported that at 3% concentration, the plantlet produced by sucrose was the most preferred plantlet in terms of stem height. This plantlet appeared stronger and more vigorous against other types of carbohydrate tested by the authors. The same outcome was obtained by Gubis *et al.*, (2005), who demonstrated that 3% sucrose basal medium produced more vigorous and healthier *Lycopersicon esculentum* plantlets than other types and levels of carbohydrate tested. Similarly, Buah *et al.*, (2000) reported that plantlets produced higher numbers of leaves on media containing sucrose compared to glucose and fructose. Singh (2014) also further showed that sucrose at 3% had nodes and shoot length while other carbon sources used (glucose and fructose) in MS medium did not induce shoots and nodes in *Bauhinia purpurea*.

Furthermore, Al-Khateeb (2001), on studying the culture of *Phoenix dactylifera* found that the moor roots were produced on media with 3% sucrose corroborating the results in *Metroxylon sagu* (Novero *et al.*, 2010). However, glucose at all levels supported the growth of zygotic embryos of *S. longipedunculata* even though they were significantly inferior to sucrose at 3%. Fructose, on the other hand, performed poorly relative to sucrose and glucose.

S. longipedunculata plantlets were successfully regenerated from zygotic embryos cultured in all concentrations of glucose and fructose (1-4%) studied. However, maximum morphological parameters (shoot length, fresh weight, root length and leaf area) were not achieved. Various authors reported that height of *Vaccinium vitisidaea* and *Eclipta alba* plantlets was better achieved on medium with 3% glucose (Baskaran & Jayabalan, 2005; Debnath, 2005). Sumaryono *et al.*, (2012) showed that Fructose produced a poisonous

substance (5-*hydroxymethyl*-2-*formaldehyde)* at high temperatures (e.g., during autoclaving). This substance induces vitrification and reduces the leaf's water potential, therefore affecting the expansion of plantlets' leaves.

The increase in the concentration of carbon source (sucrose, glucose and fructose) in the present investigation did not increase the values of the dependent variables tested. It is worthy to note that inadequate carbohydrate concentration can be toxic to the growth and development of the plantlets as reported by Tiexeira da Silva (2004) and Yaseen *et al.*, (2013). This report commemorates Kadota *et al.*, (2001) and Singh, (2014) findings which revealed that high concentrations of sucrose disrupted the growth of *Pyrus communis* and *Bauhinia purpurea* L., respectively, which might had caused a decrease in the plant's osmotic potential. An increase in sucrose concentration negatively affected the biomass compostion of *Triticum aestivum* and *Alocasia amazonica* plantlets (Javed & Ikram, 2008; Jo *et al*., 2009).

Adventitious root formation in MS+ NAA (3.0 mg l^{-1}) + IBA (5.0 mg l^{-1}) produced the best result for number of roots, root length, fewer days to induction and per cent root regeneration. This agrees with Tolera (2016) work on *Saccharum officinarum* L., micro shoot who reported a combined effect of NAA and IBA at 3 mg l^{-1} each for root formation. When auxins were used alone, it brought about the induction of brown calli with fewer roots. For example, both IBA and NAA at 1 mg l^{-1} and 3 mg l^{-1} respectively led to the induction of brown calli with or without roots. In agreement with the above observation, Spethmann (2000) opined that callus formation hinders root formation process due to increased concentration of exogenous auxin application. In the same light, Biradar *et al*., (2009) stated that "increased concentration of auxins, especially NAA, stimulated the natural production of ethylene inhibitory to root induction and elongation". In contrast to the study, Teklebrihan (2014) successfully used shoot tips of *S. longipedunculata* as explants in full strength MS with both IAA (2 mg l^{-1}) and either IBA or NAA for maximum root regeneration.

The combined effects of both auxins brought about the production of root. The synergistic effects of auxins on rooting according to Islam *et al*., (2005) were shown to sufficiently produce roots when both IAA and IBA at 1 mg l^{-1} each or IBA and NAA at the same concentration were utilized. Adventitious roots were produced by increasing the concentration to 5 mg l^{-1} and 10 mg l^{-1} respectively. This contrasts with the reports by Spethmann (2000) and Biradar *et al*., (2009). Their work showed that increased auxin application resulted to root inhibition.

Similarly, results obtained from IBA at 1 mg l^{-1} and 3 mg l^{-1} did not produce adventitious roots with an increase in concentration like NAA as explants required a higher level of Plant Growth Regulators to enhance the rooting process. Stefancic *et al*., (2005) reported in leaf cuttings of *Prunus* 'GiSelA5' that root formation was enhanced by the exogenous application of IBA because *prunus'* stability and insensitivity to auxin-degrading enzymes (Hartmann *et al*., 2002).

Conclusion

The root of *S. longipedunculata* bears suckers, which is its natural source of producing new propagules. However, this plant is disappearing at an alarming rate due to the high speed of human-induced pressure due to the unregulated harvesting/collection of subterranean plant parts from their natural stands. Problems associated with seed dormancy and high mortality rate during early seedling development affected the conventional propagation of this plant. Sustainable and efficient regeneration of *S. longipedunculata* is guaranteed by using the appropriate concentration of sugar in the Media for regeneration through Mature Zygotic culture and using a good combination of plant growth regulators in basal media for hypocotyl cultures.

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