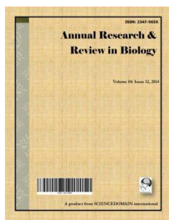

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Multiple Shoots Induction from Indigenous Nigerian Pumpkin (*Cucurbita pepo* L.)

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Authors' contributions

This work was carried out in collaboration between all authors. Author OOO designed the study. Author AOA performed the laboratory experiment. Author OAB assisted in the experimental set-up. Authors OOO, OAB and OSA carried out the statistical analysis. Authors OAB and OSA managed the literature searches and wrote the first draft of the manuscript. Author OOO read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to develop a protocol for *in vitro* regeneration of a Nigerian indigenous pumpkin (*C. pepo* L.) via seedling-derived cotelydon, cotyledonary node and hypocotyl explants.
Study Design: A combination of 0.00, 1.00, 2.00 and 3.00 mgl^{-1} of 6-Benzylaminopurine (BAP) and 0.00 or 0.05 mgl^{-1} of 2,4-Dichlorophenoxy acetic acid (2,4-D) for each explant type were set up in three replicates making a total of 36 culture vessels in the entire experimental set up. Five explants were cultured per combination.
Place and Duration of Study: The work was conducted in the Plant Tissue Culture Laboratory of the Department of Biological Sciences, College of Science and Technology, Covenant University, Ota, Ogun State, Nigeria, between January 2017 and July 2017.

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Methodology: Hypocotyl, cotyledonary node and cotyledon explants derived from 4-week old-seedlings were cultured on MS medium fortified with 0.00, 1.00, 2.00 and 3.00 mgl^{-1} of BAP in combination with 0.00 or 0.05 mgl^{-1} of 2,4-D and investigated for callus, shoot and root induction.
Results: A combination of 1.00 mgl^{-1} BAP with 0.05 mgl^{-1} 2,4-D was optimum for callus induction from hypocotyl and cotyledonary node explants, while for cotyledon explants, 2.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D was preferred. Cotyledonary node explants and cotyledonary node explant-derived callus responded with multiple shoots (4.50±0.042 and 4.07±0.067 shoots per explant, respectively) on full strength Murashige and Skoog (MS) medium (control) devoid of Plant Growth Regulators (PGRs). When the different explant types were cultured on MS media amended with the different concentrations of BAP in combination with 2,4-D, neither shoot nor root induction was observed. All the explants initiated roots when cultured on full strength PGR-free medium. Only cotyledon explant-derived callus formed roots (2.30±0.56cm) on PGR-free MS medium.
Conclusion: Regeneration of indigenous vegetables, such as *Cucurbita pepo* is achievable through hypocotyl, cotyledonary node and cotyledon explants with or without PGRs.

Keywords: BAP; 2,4-D; cotyledonary node; cotyledon; hypocotyl.

1. INTRODUCTION

The Cucurbitaceae family, also known as Cucurbits represents a large group of plants, which consists of approximately 130 genera and 800 species [1]. Many species in this group are of economic importance. Most commonly cultivated species are musk or sweet melon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.), watermelon (*Citrullus lanatus* (Thunb)) and summer or winter squash (*Cucurbita ficifolia* Bouche., *Cucurbita mixta* Pangalo., *Cucurbita moschata* Poir., *Cucurbita maxima* Duch. and *Cucurbita pepo* L.) [2]. The Cucurbita genus is regarded as a major vegetable crop in many regions of the world [3]. In 2005, it altogether accounted for about 20.4 million tonnes of crop production [4].

Cucurbita pepo consists of different range of vegetable cultivars including pumpkins, winter squash and the various types of summer squash including zucchini and cocozelle [5] China is the world's leading producer of *C. pepo*, contributing about 30% of the world's production [6]. In many parts of Nigeria, *C. pepo* is grown mainly for its fruits and leaves, which are consumed as a vegetable [7].

A previous study on the nutrient composition of the leaves of *C. pepo* by Duke and Ayensu [8], showed that the leaves contain 43.8% protein, which is comparable to that of soybean [9]. The fruits are characterized by low fat content (2.3%), carbohydrates (66%) and proteins (3%) and are very high in carotenoids [10]. Seeds of *C. pepo* are eaten either raw or roasted and are utilized in cooking and baking as an ingredient in cereals, breads, cakes and salads [1]. The use of the seeds as an antidiabetic, antihypertensive, antitumor, antimutagenic, immunomodulatory, antibacterial, anti-hypercholesterolemic, intestinal antiparasitic, antalgic, and anti-inflammatory agent [11] has been well reported.

In Africa, plants form an essential part of life in several indigenous communities [12]. Cultivation and utilization of indigenous pumpkin in Nigeria is declining. Therefore, development of an efficient regeneration method for the mass production of indigenous *C. pepo* species is necessary, as this will address the problem of availability of propagules and scarcity in the market.

The evolution of plant biotechnologies has been fast-paced in recent times [13]. Plant biotechnology has proven to be a suitable option for the improvement of *Cucurbita* species through plant tissue culture and genetic transformation [3]. Development of regeneration technology for *in vitro* culture is an important step in crop improvement [14] Plant regeneration via shoot organogenesis is a more suitable and rapid approach [15] in comparison to traditional *in situ* cultivation. Previous studies have documented regeneration in *Cucurbita* genus. Ananthakrishnan et al. [14] reported the regeneration of *C. pepo* from seedling-derived cotyledon explant through direct organogenesis. Carol et al. [16] in their work reported the initiation of somatic embryos via cotyledon explant in six squash cultivars (*C. pepo*). Schroeder [17] documented the regeneration of zucchini squash (*C. pepo*) from flesh pericarp wall-derived callus through somatic embryogenesis.

Several studies have reported the importance of factors such as plant growth regulator (PGR)

balance, culture conditions, genotype and explant type on successful plant regeneration [18]. In addition, the original hormonal content of explants plays an important role in directing *in vitro* responses [19]. A careful review through existing literatures shows that there are no reports on the micropropagation of indigenous Nigerian cultivars of *C. pepo*. Therefore, the aim of the present study was to develop a highly repetitive protocol for the *in vitro* regeneration of an indigenous Nigerian pumpkin from seedling-derived cotelydon, cotyledonary node and hypocotyl explants.

2. MATERIALS AND METHODS

2.1 Source of Primary Biological Material

Fruits of indigenous Nigerian pumpkin were purchased from Lucada market, Igbesa (Latitude 6.533602 and Longitude 3.134161), located in Ado-Odo local government area of Ogun State, Nigeria.

2.2 Media Preparation and Sterilization of Instruments

The study was carried out in the Plant Tissue Culture Laboratory of Covenant University, Ota, Ogun State. MS [20] basal medium containing 0.8% agar (w/v) (PhytoTechnology laboratories, USA) was used. PGRs were added to the medium and pH was adjusted to 5.7-5.8, prior to autoclaving at 121℃, 15 psi for 15 min.

2.3 Seed Collection and Preparation

Seeds were removed from the fruit and washed under running tap water for 10 min prior to disinfection. One drop of Tween 20 was added and seeds were rinsed 4-5 times with sterile distilled water before transferring to a laminar airflow cabinet (Cleatech, USA), where the seeds were treated with 70% ethanol for 1 min. Seeds were surface disinfected with 30% (v/v) sodium hypochlorite for 20 min. After disinfection, seeds were rinsed with sterile distilled water six times and blotted dry with sterile blotting paper (Whatmann No. 1). The sterilized seeds were placed in culture vessels containing MS medium and allowed to germinate into seedlings for 4 weeks.

2.4 Explant Source

In vitro seedlings were used as explant source. Small pieces of hypocotyl, cotyledonary node and cotyledon excised from 4 week old seedlings were used as explants. Sterilized hypocotyl, cotyledonary node and cotyledon explants were inoculated on to the surface of the semi-solid MS media in culture vessels with their abaxial surface making contact with the medium.

2.5 Experimental Design

A combination of 0.00, 1.00, 2.00 and 3.00 mgl^{-1} of 6-Benzylaminopurine (BAP) and 0.00 or 0.05 mgl^{-1} of 2,4-Dichlorophenoxy acetic acid (2,4-D) for each explant type were set up in three replicates to give twelve combinations of BAP × 2,4-D (Table 1) each for cotyledon, cotyledonary node and hypocotyl explants, making a total of 36 culture vessels in the entire experimental set up. Five explants were inoculated per combination. Culture vessels were kept in an incubator (ThermoSCIENTIFIC, USA). Cultures were maintained at a temperature of 25±2°C, 8 h dark and 16 h photoperiod provided by cold fluorescent lamps of 120 $\mu molm^{-2}s^{-1}$ intensity for five weeks.

2.5.1 Callus induction

MS medium was used. Growth regulators, BAP (1.00, 2.00 and 3.00 mgl^{-1}) in combination with 2,4-D (0.05 mgl^{-1}) were investigated for organogenic callus induction from all the explant types. Callus cultures were maintained at a temperature of 25±2°C, 70% humidity and 16 h photoperiod provided by cold fluorescent lamps of 120 $\mu molm^{-2}s^{-1}$ intensity for 5 weeks. Induced calluses were also transferred to full strength MS media without PGRs (0.00 mgl^{-1} BAP and 2,4-D) after 5 weeks to investigate indirect organogenesis.

2.5.2 Direct multiple shoots and root induction from explants

Hypocotyl, cotyledonary node and cotyledon explants were inoculated into MS media amended with BAP (1.00, 2.00 and 3.00 mgl^{-1}) in combination with 2, 4-D (0.05 mgl^{-1}) for root induction. Explants were also transferred to full strength MS without PGRs. Cultures were incubated under 16 h photoperiod at a temperature of 25±2°C.

2.5.3 Indirect multiple shoots and roots induction from calluses

Five-week old organogenic calluses induced from hypocotyl, cotyledonary node and cotyledon explants were investigated for shoot and root initiation. Cream coloured and friable calluses

obtained from hypocotyl, cotyledonary node and cotyledon explants were transferred to full strength MS medium devoid of PGRs. Cultures were maintained at a temperature of 25±2°C and 16 h photoperiod.

2.5.4 Rooting of harvested shoots

Regenerated shoots were harvested and cultured on full strength PGR-free MS medium for rooting.

2.6 Statistical Analyses

Cultures were evaluated by visual observation on a weekly basis. Data were recorded on callus induction (diameter), multiple shoot formation (no of shoots), length and number of roots per explants. The data were analyzed statistically using IBM SPSS version 23. The significant differences among the means were calculated using Duncan's Multiple Range Test (DMRT) at P=.05. The results are expressed as a mean ± standard error of three repeated experiments.

3. RESULTS

3.1 Effect of BAP and 2,4-D on Callus Induction

Callus induction was observed when hypocotyl, cotyledonary node and cotyledon explants were cultured on MS medium containing 1.00, 2.00 and 3.00 mgl^{-1} of BAP in combination with 0.05 mgl^{-1} 2,4-D. As for hypocotyl-derived callus, the largest diameter was observed in the medium amended with 1.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D (P= .05, Table 1). However, hypocotyl callus diameter decreased in medium fortified with 2.00 mgl^{-1} BAP in combination with 0.05 2,4-D and 3.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D. Cotyledonary node explants initiated the largest callus diameter in medium supplemented with 1.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D. A significant decrease in cotyledonary node callus diameter was recorded in medium amended with 2.00 mgl^{-1} and 3.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D. However, the decrease in callus diameter recorded for medium supplemented with 2.00 and 3.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D was not statistically different (P= .05, Table 1). Cotyledon explant-derived callus diameters varied significantly, with callus diameter in medium amended with 2.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} greater than those in medium amended with 1.00 and 3.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D (p= .05). There was no significant difference in the diameter of calluses obtained in the medium augmented with 1.00 and 3.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D. All induced calluses were cream coloured and friable (Fig. 1). No callus induction was observed on the PGR-free control medium.

3.2 Effect of BAP and 2,4-D on Direct Shoot Induction

When the different explant types were cultured on MS medium amended with 1.00, 2.00 and 3.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D, no shoots induction were observed. Cotyledonary node explants responded with multiple shoots (4.50±0.428 shoots/explant) (Table 2) after five weeks on MS medium without PGRs (0.00 mgl^{-1} BAP and 0.00 mgl^{-1} 2,4-D) (Fig. 2). However, neither hypocotyl nor cotyledon explants formed shoot on full strength PGR-free medium.

3.3 Effect of BAP and 2,4-D on Direct Root Induction

No root induction was recorded when hypocotyl, cotyledonary node and cotyledon explants were cultured on MS medium supplemented with 1.00, 2.00 and 3.00 mgl^{-1} of BAP in combination with 0.05 mgl^{-1} of 2,4-D (Table 3). However, all the explant types responded with root initiation on full strength PGR-free MS medium (Fig. 3). The longest root length (13.33±0.882 cm) was observed when cotyledon explants were cultured in PGR-free medium. Hypocotyl explants had the shortest root length (0.56±0.125 cm).

3.4 Indirect Shoots and Roots Induction from Calluses

When hypocotyl, cotyledonary node and cotyledon explant-derived calluses were cultured on full strength MS medium without PGRs after 3 weeks, cotyledonary node explant-derived callus responded with multiple shoots (4.07±0.067 shoots/explant) (Fig. 4) whereas, cotyledon explant-derived callus responded with roots (2.30±0.56 cm) on PGR-free MS medium. Neither shoots nor roots were induced, when hypocotyl explant-derived callus were cultured on full strength MS medium devoid of PGRs. The result of the effect of PGR-free MS on indirect shoots and roots induction from the various explants-derived calluses is presented in Table 4.

Table 1. Effect of BAP and 2,4-D on callus induction

2,4-D (mgl^{-1})	BAP (mgl^{-1})	Hypocotyl explant-derived callus diameter (cm)	Cotyledonary node explant- derived callus diameter (cm)	Cotyledon explant-derived callus diameter (cm)
0.00	0.00	0.00 ± 0.000^{d}	0.00 ± 0.000^{c}	0.00 ± 0.000^{c}
0.05	1.00	2.40 ± 0.058^{a}	2.40 ± 0.058^{a}	1.23 ± 0.145^{b}
0.05	2.00	1.30 ± 0.153^{b}	1.63 ± 0.186^{b}	2.07 ± 0.968^{a}
0.05	3.00	1.00 ± 0.000^{c}	1.27 ± 0.393^{b}	0.77 ± 0.145^{bc}

Mean value ± S.E.M = Mean values ± Standard error of means for each of the three replicates. Means followed by the same letter within columns are not significantly different (P= .05) using Duncan's Multiple Range Test (DMTR)

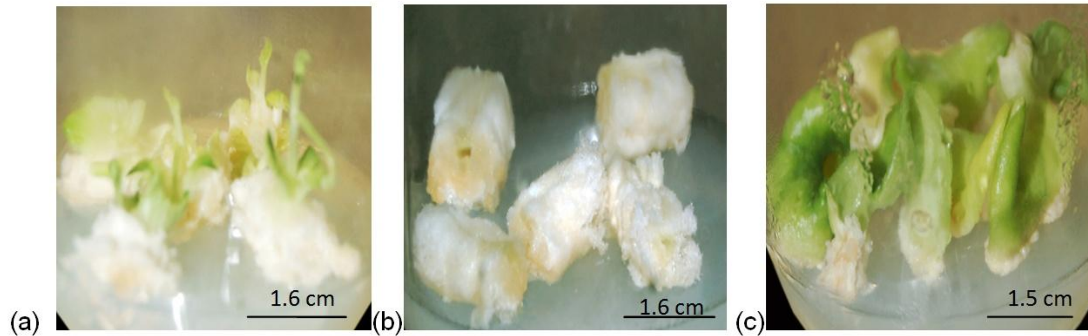

Fig. 1. Callus induction by explants on MS medium amended with BAP in combination with 2,4-D (a) Cotyledonary node explant-derived callus induced on MS + BAP (1.00 mgl^{-1}) + 2,4-D (0.05 mgl^{-1}) (b) Hypocotyl explant-derived callus induced on MS + BAP (1.00 mgl^{-1}) + 2,4-D (0.05 mgl^{-1}) (c) Cotyledon explant-derived calus on MS + BAP (2.00 mgl^{-1}) + 2,4-D (0.05 mgl^{-1})

Table 2. Effect of BAP and 2,4-D on shoot induction after 5 weeks

2,4-D (mgl^{-1})	BAP (mgl^{-1})	Hypocotyl (number of shoots/explants)	Cotyledonary node (number of shoots/explants)	Cotyledon (number of shoots/explants)
0.00	0.00	0.00±0.000	4.50±0.428	0.00±0.000
0.05	1.00	0.00±0.000	0.00±0.000	0.00±0.000
0.05	2.00	0.00±0.000	0.00±0.000	0.00±0.000
0.05	3.00	0.00±0.000	0.00±0.000	0.00±0.000

Mean value ± S.E.M = Mean values ± Standard error of means for each of the three replicates

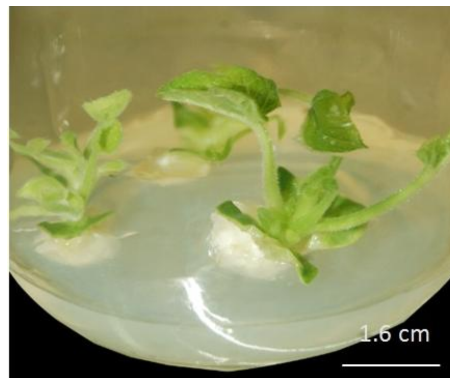

Fig. 2. Cotyledonary node explants with direct multiple shoots induced on medium without plant growth regulators after 5 weeks

Table 3. Effect of BAP and 2,4-D on root length

2,4-D (mgl^{-1})	BAP (mgl^{-1})	Hypocotyl (cm)	Cotyledonary node (cm)	Cotyledon (cm)
0.00	0.00	0.56±0.125	2.75±0.250	13.33±0.882
0.05	1.00	0.00±0.000	0.00±0.000	0.00±0.000
0.05	2.00	0.00±0.000	0.00±0.000	0.00±0.000
0.05	3.00	0.00±0.000	0.00±0.000	0.00±0.000

Mean value ± S.E.M = Mean values ± Standard error of means for each of the three 3 replicates

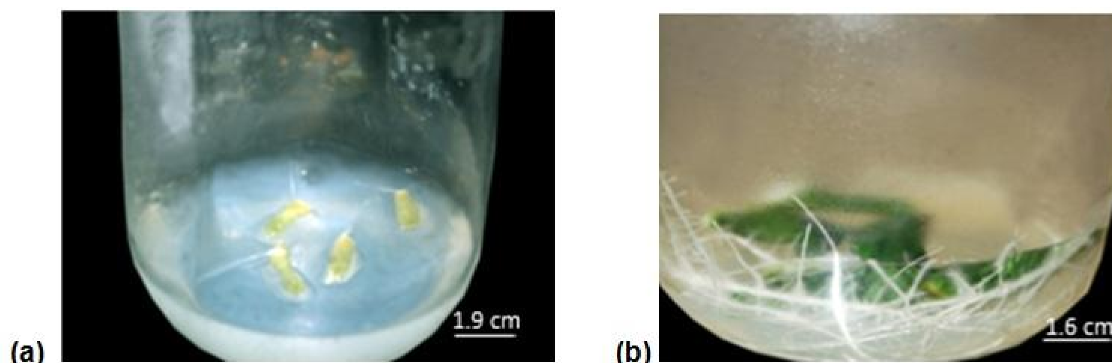

Fig. 3a and b. Effect of BAP and 2,4-D on root induction and growth length

Table 4. Indirect multiple shoots and roots induction from calluses subcultured on full strength PGR-free medium after 3 weeks of incubation

Callus	No of shoots/explant	Root length (cm)
Hypocotyl	0.00±0.000	0.00±0.000
Cotyledonary node	4.07±0.067	0.00±0.000
Cotyledon	0.00±0.000	2.33±0.560

Mean value ± S.E.M = Mean values ± Standard error of means for each of the three replicates

3.5 Rooting of Harvested Shoots on PGRs-free Medium

Micro-shoots regenerated from cotelydonary node explants and cotelydonary node-derived callus were harvested and transferred to full strength PGR-free medium for adventitious rooting and whole plant regeneration (Fig. 5).

4. DISCUSSION

Under suitable culture conditions, plant cells possess a capacity to regenerate organs from specialized somatic tissues through a process known as *de novo* organogenesis [21]. Studies have revealed that direct *in vitro* organogenesis is a rapid technique for the multiplication of true to elite plant cultivars, and is preferred for producing transgenic plants to circumvent somaclonal variation [22]. From our study, cotyledonary node explants initiated direct multiple shoots on full strength MS medium without any PGRs. This is in agreement with the work of Gulati and Jaiwal [23] who documented the production of direct multiple shoots from cotyledonary node explants of mungbean [(*Vigna radiata* (L.) Wilczek] cultured on PGR-free basal media. Induction of direct multiple shoots from cotyledonary node is one of the most reliable method of micropropagation in plants because buds emerging from meristematic organs and tissues possess great potentials for healthy development [24]. This is the first time that direct multiple shoots induction from cotyledonary node explants of any indigenous Nigerian pumpkin cultured on full strength PGR-free MS medium would be reported. Basically, a higher cytokinin to auxin ratio is required in a medium to initiate shoot proliferation and multiplication [25,26]. In the present study, the different concentrations of BAP in combination with 2,4-D was ineffective for inducing direct multiple shoots from all the

explant types investigated. Our findings substantiate the report of Hu and Wang [27] that higher concentrations of cytokinin reduced the number of micropropagated shoots. This outcome, however, disagrees with the report of Krug et al. [28] who documented that BAP is highly effective for cucurbit organogenesis and induction of multiple adventitious shoot bud differentiation. Neither hypocotyl nor cotyledon explants produced multiple shoots on PGR-free media. The absence of shoot formation observed may have resulted from the explant type used. The type of explant used, play a significant role in morphogenetic induction, as competent cells for adventitious shoot development in cucurbits appears to be limited to particular cotyledon regions [29].

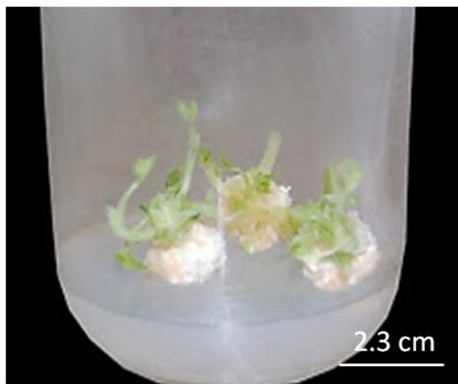

Fig. 4. Indirect multiple shoot induction from cotyledonary node-derived callus cultured on medium without plant growth regulators after 3 weeks

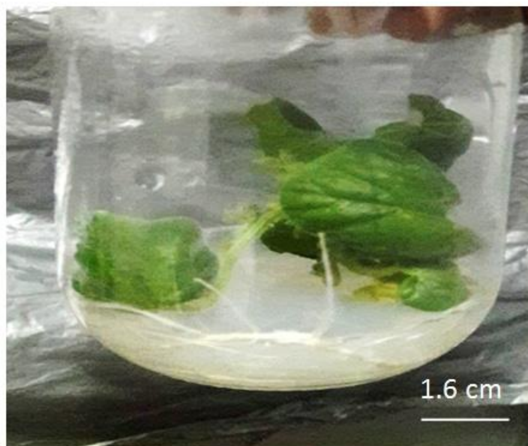

Fig. 5. Rooting and whole plant regeneration from cotelydonay node explant on full strength PGR-free medium after 7 days

The various combinations and concentrations of BAP and 2,4-D used in this study, induced calluses from all the explant types investigated. This outcome agrees with the work of Haque et al. [30] who reported that ninety percent of pumpkin explants initiated callus when cultured on media amended with 2,4-D and BAP. Albeit callus induction was recorded for all explant types, a combination of 1.00 mgl^{-1} BAP with 0.05 mgl^{-1} 2,4-D was optimum for callus induction from hypocotyl and cotyledonary node explants while for cotyledon explants, 2.00 mgl^{-1} BAP in combination with 0.05 mgl^{-1} 2,4-D was the best. This suggests that a combination of auxin and cytokinin plays a key role in initial callus proliferation in *C. pepo*. However, an increase in BAP concentration beyond the optimum concentration inhibits callus proliferation. From the results obtained, both hypocotyl and cotyledonary node derived-calluses had the largest diameter, whereas, cotyledon derived-callus had the smallest diameter. This is in agreement with the report of Pal et al. [6] who observed that calluses induced from hypocotyl explants of summer squash were larger in size than those from the cotyledon. Meanwhile, no callus formation was observed on PGR-free MS medium. Balogun et al. [31] observed same results when stem explants of *Telfaria occidentalis* were cultured on medium without PGRs. This indicates that *C. pepo* is highly sensitive to PGR for callus induction. All explants initiated creamy and friable calluses. This observation is similar to the report of Pal et al. [6] who documented the induction of creamy and friable callus from hypocotyl and cotyledon explants of summer squash.

Cotyledonary node explant-derived callus formed indirect multiple shoots on full strength PGR-free MS medium. This may be due to fact that the original nodal explant that produced the subcultured callus had pre-existing meristems. However, this result contradicts the report of Kumar and Singh [29] that without growth regulators, the rate of shoot regeneration was lower when compared with shoots regenerated from different media augmented with various concentrations of cytokinins.

Rhizogenesis is an essential step for *in vitro* plant propagation [32]. The combination of BAP and 2,4-D concentrations used were inefficient for root induction in all the explants types investigated. However, root formation was recorded when all explant types were transferred to full strength MS medium devoid of PGRs. This

disagrees with the report of Bhatt and Dhar [33] that growth regulator concentration has a significant influence on rooting percentage, root number and root length as compared to PGR-free control. Cotyledon explants responded with the longest root length suggesting that they are more amenable to root initiation in PGR-free medium compared to hypocotyl and cotyledonary node explants.

The harvested *in vitro* regenerated shoots produced long roots when rooted on full strength PGR-free MS medium. This result agrees with Lee et al. [34] who reported the successful rooting of elongated shoots of *C. maxima* on MS medium without plant growth regulators after 2 weeks of culture. Ananthakrishnan et al. [18] and Kulus [35] also reported rooting in plant growth regulator-free medium during organogenesis in *C. pepo and Kalanchoe tubiflora* (Harvey) Hamet.

5. CONCLUSION

The study investigated the *in vitro* regeneration of indigenous Nigerian pumpkin (*Cucurbita pepo)* via hypocotyl, cotyledonary node and cotyledon explants derived from *in vitro*-developed seedlings. The present research has shown that regeneration of indigenous vegetable such as *C. pepo* is possible through hypocotyls, cotyledonary node and cotyledon explants. The result of this study has also revealed that induction of direct morphogenesis (shoot and root) requires no PGR. This study developed a highly repetitive protocol on callus induction, multiple shoot formation and root formation. The study, therefore, forms a basis for somatic embryogenesis, production of secondary metabolites, regeneration and somaclonal variants suitable for genetic transformation and breeding of desirable economic traits in *C. pepo* as a vegetable, fruit and seed crop. Future studies could also focus on the reduction of plant tissue system costs by applying cheaper technologies [36].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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