Effect of low BAP Levels on Multiple Shoots Induction in Indigenous Nigerian Pumpkin (*Cucurbita pepo* Linn.)

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Effect of low BAP Levels on Multiple Shoots Induction in Indigenous Nigerian Pumpkin (*Cucurbita pepo* Linn.)

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Abstract. Indigenous Nigerian pumpkin is a cucurbitaceous plant primarily grown in Southwestern Nigeria for its young leaves, which are relished as pleasant-tasting vegetable. In spite of its nutritional value, the plant is scarcely available compared to other vegetables. As a result, development of a rapid *in vitro* regeneration procedure is imperative to ensure all year round availability. The influence of 6-Benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid on multiple shoots induction from cotyledonary node explant of indigenous Nigerian pumpkin has been previously reported. However, the concentrations of BAP used may have been high, hence the inhibition of shoots formation on medium amended with plant growth regulators (PGRs). Can low concentrations of BAP alone elicit multiple shoots from cotyledonary node explant of indigenous Nigerian pumpkin? To ascertain this, we cultured cotyledonary node explants derived from 3-week-old *in vitro* grown seedlings for 8 weeks on Murashige and Skoog (MS) basal medium fortified with 0.35, 0.45 and 0.55 mg/L BAP. Medium without BAP (0.00 mg/L) was used as control. Mean number of shoots per explant was not statistically significant ($P \leq 0.05$) among the BAP concentrations employed except for control and medium augmented with 0.45 mg/L BAP. Medium fortified with 0.45 mg/L of BAP gave 3.25±0.921 shoots per explant and was therefore most effective for multiple shoots induction. The result showed that low levels of BAP were capable of inducing shoots formation from cotyledonary node explant of indigenous Nigerian pumpkin. Nevertheless, much lower BAP concentration than used in the present study should be investigated for the likelihood to elicit higher shoot responses.

Keywords: BAP, cotyledonary node, *Cucurbita pepo*, pumpkin, multiple shoots induction

1. Introduction

Cucurbita pepo, a member of the cucurbitaceae family is one of the plant species cultivated since antiquity, with archaeological evidence from Mexico dating as far back as 7000 B. C. [1, 2]. The plant is indigenous to Northern Mexico as well as Southwestern and eastern USA, and was introduced to Europe in the 16th century along alongside other *Cucurbita* species [3, 2]. While *C. pepo* is less resistant to heat compared to *C. moschata*, and thus less suitable for tropical Africa, it is cultivated on a small scale in all countries of the continent [3]. China is the world's largest producer of *C. pepo* with an average production of roughly 5.3 million tonnes between 1994 and 2017 [4].

Generally known in English by the names pumpkin, squash, gourd and marrow, and locally as "Elegede" in Southwest Nigeria, *C. pepo* is cultivated mainly for its leaves which are utilized as vegetables [5, 6]. The young leaves (locally called "Gboro") are known to be amongst the most palatable vegetables consumed in Southwestern Nigeria. Unlike other leafy vegetables, the young leaves do not require any form of pre-treatment or processing prior to cooking [5]. According to Duke and Ayensu, [7] the leaves are very high in protein (43.8%) which is comparable with that of soybean.

Proximate, mineral and anti-nutrient analysis of the pulp shows that pumpkin is rich in fibres and carbohydrates, and low in anti-nutrients like phytates, oxalates and tannins [8]

In September 2015, at the United Nations Summit of world leaders, the 2030 Sustainable Development Agenda with its 17 Sustainable Development Goals (SDGs) was adopted [9]. Goal 2 of the SDGs is aimed at promoting sustainable agriculture, ending hunger, ensuring food security as well as improved nutrition by the year 2030 [10]. Considering its premium nutritional value, indigenous Nigerian pumpkin has the potential to tackle the problem of hunger and malnutrition if the right biotechnology approaches are applied to enhance its production and conservation.

Regeneration of whole plants via shoot organogenesis is a more suitable and rapid biotechnological approach [11]. Small segments of plant tissues (explants) possess the capacity to regenerate shoots and roots, and therefore whole plants under defined *in vitro* cultural conditions. This process whereby whole plants are regenerated via culturing small pieces of tissues such as meristems, root and shoot tips, anthers, nodes, embryos and leaf tissues on nutrient medium is known as micropropagation [12]. Some of the advantages of micropropagation over the traditional means include: conservation of endangered species and plant genetic resources, [12, 13, 14] production of plants in the absence of seeds, [12] and rapid multiplication of plants in a relatively short time and on an all year round basis, regardless of season and weather [13]. Compared to other fruits and vegetables, indigenous Nigerian pumpkin is not readily available owing to a decline in cultivation and utilization. Therefore, establishment of an efficient regeneration protocol for its mass production, improvement and conservation is crucial [15, 16].

We have hitherto reported multiple shoots formation from cotyledonary node explants of indigenous Nigerian pumpkin cultivar maintained on PGR-free medium [16]. In that study, explants cultured on medium fortified with BAP in combination with 2,4-D showed no shoot response. Thus, we reasoned that the non-responsiveness might have been due to the high levels of BAP employed, and envisaged that better outcomes could be obtained if cotyledonary node explants are cultured on medium augmented with BAP alone at lower concentrations. The objective of this study therefore, was to investigate the influence of low BAP levels on *in vitro* multiple shoots induction from cotyledonary node explant of indigenous Nigerian pumpkin.

2. Materials and Methods

2.1 Source of primary biological material

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

2.2 Media preparation

The study was performed in the Plant Tissue Culture Laboratory of Covenant University, Ota, Ogun State. MS basal medium [17] with vitamins and sucrose was used. The medium was dissolved in 850 mL of deionized water. pH of solution was adjusted to 6.0 before adding 500 mg of agar. The beaker containing the medium was heated over a magnetic stirrer hot plate to dissolve the agar. Subsequently, the medium was dispensed into autoclavable glass culture vessels and autoclaved at 121℃ and 15 psi for 15 min. Medium was left to cool after sterilization, after which BAP was added.

2.3 Seed collection and preparation

Seeds were separated from the fruits and cleansed with distilled water for 10 min prior to sterilization. The seeds were dipped in 70% ethanol for 2 min and thereafter surface sterilized with 15% sodium hypochlorite for 20 min. Following sterilization, seeds were given six rinses of distilled water. Subsequently, the seeds were inoculated onto MS medium in culture vessels. After inoculation, the vessels were closed, and their rims sealed with parafilm to prevent contamination. The seeds were left to germinate into seedlings at room temperature for 3 weeks.

2.4 Explant Source

Three week-old *in vitro* grown seedling was used as source of explant. Cotyledonary nodes excised from the seedlings were used as explants and inoculated onto the surface of MS medium in the culture vessels.

2.5 Effect of BAP on direct multiple shoots induction

Explants were cultivated in culture vessels containing MS medium amended with 0.35, 0.45 and 0.55 mg/L BAP. MS medium without BAP (0.00 mg/L) served as control. For each BAP treatment, three replicates were set up with three explants inoculated per replicate. A total of twelve culture vessels were set-up for the experiment. The vessels were kept in an incubator (ThermoSCIENTIFIC, USA), and cultures were maintained at 25±2℃ for 8 h and 16 h photoperiod supplied by cold fluorescent lamps of 120 μmol/m^2/s intensity for 8 weeks.

2.6 Data collection

After 8 weeks of incubation, data on number of shoots per explant for each treatment replicate were collected for statistical analysis.

2.7 Experimental Design and statistical analysis

Completely randomized block design was employed. For the different BAP concentrations, variation in mean number shoots per explant was analyzed using analysis of variance (ANOVA) at a confidence level of 95%. Duncan's multiple range test (DMRT) was used to compare the mean number of shoots per explant. Values are expressed as means±standard error of means.

3. Results and Discussion

From the result obtained, all BAP concentrations employed enhanced shoot organogenesis (Table 1) (Figure 1). Cytokinins perform a prime role in the development of plants. Such roles include modulation of shoot formation and proliferation, as well as stimulation of cell division and expansion [18]. BAP is the most extensively used synthetic cytokinin in micropropagation. The rate of proliferation of adventitious buds and shoots under BAP influence is a fundamental factor that determines the efficiency of any micropropagation procedure [19].

Table 1: Effect of BAP on direct multiple shoots induction

BAP concentration (mg/L)	Mean number of shoots/cotyledon explant
0.00	**1.25^{a}±0.313**
0.35	$2.83^{a,b}$±0.307
0.45	**3.25^{b}±0.921**
0.55	$2.50^{a,b}$±0.548

Values = Mean values ± Standard error of means for each of the three replicates. Means with the same superscript are not statistically significant (P≤0.05) using DMTR.

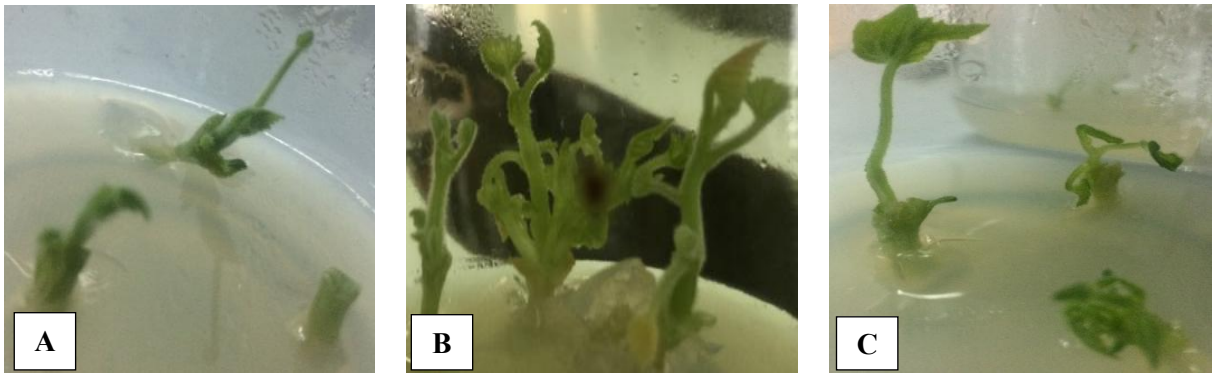

Figure 1(A-C): Shoot induction from cotyledonary node explant of indigenous Nigerian pumpkin cultivated on MS medium incorporated with low BAP concentrations. (A) Shoot formation from cotyledonary node explant on MS + 0.35 mg/L BAP (B) Cotyledonary node explant with multiple shoots on MS + 0.45 mg/L BAP (C) Shoot formation from cotyledonary node explant on MS + 0.55 mg/L

Generally, the effect of BAP treatments on mean number shoots per explant was not statistically significant ($P \leq 0.05$), except for the control (0.00 mg/L) and medium amended with 0.45mg/L (Table 1). According to Lakshimi and Mythili [20], amendment of culture medium with cytokinin such as BAP promotes *in vitro* shoot multiplication in many plant species. Zhang et al. [21] reported similar observation and noted that BAP was vital to stimulate shoot induction from cotyledon explant of *C. moschata*, nevertheless, the concentrations (0.50 – 2.00 mg/L) did not significantly influence the number of shoots per explant or the mean time for shoot formation. The authors attributed these phenomena to the responsiveness of organogenic competent cells within the explant to BAP at a certain concentration. Hence, the cells were readily stimulated to form shoots even though the concentrations did not significantly impact on shoot organogenesis [21].

The mean number of shoots per explant ranged from 1.25±0.313 in 0.00 mg/L BAP to 3.25±0.921 in 0.45 mg/L BAP (Table 1). From the result presented, 0.45 mg/L BAP was optimum for shoot induction and gave a maximum number of shoots per explant of 3.25±0.921 (Figure 1C). This result contradicts the report of Stipp et al. [22] that maximal shoot organogenesis from cotyledon segments (with attached hypocotyl fragment) derived from 4-day-old *C. pepo* cv. Caserta seedling occurred on MS medium incorporated with 1.00 mg/L BAP. This discrepancy may be due to differences in exogenous PGR concentration, explant type as well as genotype [21, 23]. It is now known that explant type is critical for the induction of *in vitro* morphogenic response, as competent cells for adventitious shoot organogenesis in cucurbits appears to be limited to a particular cotyledon locale [24] Variation in explant sensitivity to exogenously applied PGRs is determined by differences in endogenous hormonal levels, which in turn, is responsible for variation in regenerative potentials of cells [21]. More so, efficiency of explant regeneration is genotype dependent for the fact that cultivars of a given genus or species do not all induce similar responses under a defined culture condition [22].

Lastly, increase in mean number of shoots per explant was concentration dependent up until 0.45 mg/L (Table 1), beyond which a decrease was observed in medium amended with 0.55 mg/L. This finding disagrees with Mokkhan, [25] who reported a higher number of shoots when 3-to-5-day old seedling-derived cotyledonary node explants of *C. pepo* (dark green zucchini) were maintained on MS media amended with 0.50 – 2.00 mg/L BAP. The decrease observed in this study may be attributed to the age of explant used (cotyledonary node derived from 3-week-old seedling). Compared to older explants, young explants have been demonstrated to exhibit greater morphogenic potential, as they may possess greater number of metabolically active cells with nutritional and hormonal states that enhance organogenesis [26]. The inhibitory effect of BAP at 0.55 mg/L may have also contributed to a

reduction in the number of shoots per explant, since a specific concentration is required to stimulate optimum shoot response [27].

4. Conclusion

The present study has shown that low levels of BAP were capable of eliciting shoots formation from cotyledonary node explant of indigenous Nigerian pumpkin. However, the numbers of shoots formed were not statistically significant for all the concentrations employed, except for control and 0.45 mg/L BAP. In furtherance, a concentration of 0.45 mg/L was most efficient for inducing multiple shoots formation whereas, 0.55 mg/L BAP had a repressing effect on *in vitro* shoot proliferation. Future prospect would then be to investigate the likelihood of lower BAP concentrations than used in the present study to induce higher shoot responses. Determination of an appropriate BAP concentration for enhanced multiple shoots induction is critical for the development of an efficient regeneration protocol in indigenous Nigerian pumpkin.

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Conflict of Interest

No conflict of interest exists among the Authors.

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