

Optimizing the initial steps of immature endosperm culture of seeded banana (*Musa sapientum* L.) cultivar 'Bhutia' of Bangladesh

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Abstract

The local Bangladeshi banana varieties, possessing similar brix percentage to that of commercial varieties, grow well under adverse conditions with minimum care but are less popular due to the presence of seed. Endosperm culture of seeded banana can produce triploid seedless varieties which can be cultivated commercially in unsuited environments with less agricultural inputs. The current study was conducted to optimize the initial steps of endosperm culture using the immature endosperm of seeded banana cultivar 'Bhutia'. Young fruits at various stages were collected from the local banana gardens to find out suitable developmental stage of endosperm for culture. Endosperms of juvenile fruits at 25 days age, exhibited 'jelly state', was selected for culture because endosperm explants at that age survived the most in MS medium. It was observed that non-treated explants produced larger calli comparatively quickly than that of cold-treated explants. Largest calli (0.41 cm) within shorter time period (27 days after inoculation) was produced in MS medium additionally supplemented with 0.5 ppm 1-Naphthaleneacetic acid (NAA) besides 0.5 ppm 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 ppm Kinetin (Kn). The produced calli gradually became blackish in appearance and higher ascorbic acid content (480 mg/100g) was observed in blackish calli. Avoiding the blackening of calli derived from endosperm of seeded banana would be a challenge to establish a successful triploid production protocol in future.

Key words: *Musa sapientum* L., seeded banana, endosperm culture, seedlessness, triploid, callus blackening, cold treatment

Introduction

Banana (*Musa sapientum* L.) is the number one economically important horticultural fruit in Bangladesh considering its unit price, production, availability and popularity. It is grown both in homestead and commercial farms of Bangladesh that accounts for 41% of the total fruit production from 21% of total acreage (Islam and Hoque, 2003). The average yield of banana is around 14 ton ha⁻¹ (Islam and Hoque, 2003). Bangladesh grows over a million metric tons of banana annually from 58 thousand ha of land (BBS, 2009). The lion share of banana produced in Bangladesh is consumed locally but it also earns some foreign currency from the export to the Middle-East countries (Sattar and Hoque, 2005). Improving local banana varieties genetically, for example induction of seedless version of the local varieties and increasing overall production can, not only improve national nutritional health, but also increase the flow of foreign currency from the banana export. Bangladesh grows a good number of popular banana varieties, namely: Sabri, Amritsagar, Mehersagar, Dudsagar, Kabri, Champa, Agniswar, Genasundari, BARI Kola-1 etc. Besides these commercially grown popular varieties, different types of heavily seeded banana cultivars are also grown in the rural homesteads, roadsides and forests, namely: Baghernokh kola, Bhutia kola, Atia kola etc. Most of these indigenous landraces are taller in plant habit, hardy and drought tolerant. Since, most of the locally grown, seeded indigenous varieties are grown under poor management their productivity is very low. Fruits of these local varieties are often losing popularity due to the presence of many sterile seeds.

Endosperms of the crop plants are unique by its origin, nature of growth and ploidy level. A triploid endosperm cell is generally formed by the fusion of three haploid nuclei, one from the male parent and two from the female parent (Thomas *et al.*, 2000). Triploidy which has immense agricultural use is often considered the best compared to other polyploids as this genomic condition favours for vigour and vegetative productivity (Habashy *et al.*, 2004). The seed sterile triploid plants are undesirable where seeds are of commercial value. But in cases of many different horticultural fruits such as in banana, citrus, apple, papaya, grapes, etc., where the seedlessness is desirable, induction of triploid plants has been practiced (Hoshino *et al.*, 2011). It is well known that triploid endosperms of a diploid variety often produce seedless fruit (Gmitter *et al.*, 1999; Thomas and Chaturvedi, 2008). Endosperm culture provides an easy one-step protocol for triploid plant production (Thomas and Chaturvedi, 2008). To regenerate seedless varieties, the applications of immature endosperm culture have been extensively practiced over last few decades in many different horticultural species, for example in *Cucumis sativus* (Nakajima, 1962), *Malus pumila* (Mu *et al.*, 1977), Citrus (Wang and Chang, 1978), *Morus alba* (Thomas *et al.*, 2000) and *Carica papaya* (Sun *et al.*, 2011).

Culturing endosperm tissues with proper nutritional and hormone concentrations and combinations can potentially produce triploid seedlings (Hoshino *et al.*, 2011). With a vision to produce triploid seedless banana varieties in future, the present study was planned to optimize the initial steps of culturing endosperm of a local-seeded banana variety of Bangladesh.

Materials and methods

The present study was carried out during the period from April, 2013 to October, 2014 at the tissue culture laboratory of the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh, Bangladesh. To compare fruit size, brix percent, percent pulp occupied by seeds in seeded banana variety 'Bhutia' with the two most popular varieties: 'Sagor' and 'Sabri', five ripe and randomly chosen fruits were collected from the local market. Fruits of the varieties are shown in Fig. 1. Individual fruit peel, pulp and seed were collected and their dry weights were recorded. The brix percentage was also measured using a refractometer. This comparative study of the fruit size, seed content and brix percentage was conducted to show that the seeded banana has immense potential in terms of its quality as compared to the existing popular seedless varieties of Bangladesh.

Plant material: Seeded green bananas (local name: Bhutia kola, kola is the Bengali name of Banana) at 15, 20, 25 and 30 days of age (days after flowering) were collected to isolate immature endosperm (Fig. 1A). The seeded banana was used to optimize initial steps of endosperm culture. Developing endosperm at 15, 20 and 25 days of fruit age was cultured in MS medium to observe their survivability. The fruits of 15, 20, 25 and 30 days of age were cross-sectioned and the developmental stages of endosperm were visually observed.

Cold treatment of banana: A set of 10 banana fruits were kept at 4 °C for 72 hours while another set of 10 fruits were non-treated.

Media preparation: Two different hormone compositions such as: i) MS medium (Murashige and Skoog, 1962) containing 0.5 ppm 2,4-D + 0.5 ppm Kn (Treatment 1) and ii) MS medium containing 0.5 ppm 2,4-D + 0.5 ppm NAA + 0.5 ppm Kn (Treatment 2) were used for culturing endosperm of seeded banana.

Sterilization of explants: Surface sterilization of seeded banana fruits was carried out under a Laminar Air Flow Cabinet. Fruits were rinsed in 70% ethanol for 2 minutes. Ethanol treated banana was immersed into 0.1% HgCl₂ solution for 3 min followed by 3 rinses in sterile distilled water to remove traces of HgCl₂ which would be toxic to the explants if kept for longer duration.

Inoculation and culture of endosperm: The selected seeded bananas were cut into slices to expose the developing seeds. Endosperm at jelly-state was aseptically removed from banana

seeds using a scalpel and a fine forceps and inoculated into vials each containing 25 mL MS medium with desired hormone concentrations (Fig. 2). The inoculated culture vials were kept at 25 °C temperature, 1000 lux light intensity with a 16:8 hours day:night cycle. A convenient relative humidity and restricted access were also maintained in the culture room.

The endosperms under incubations were monitored regularly and any contaminations were removed immediately. The experiment was conducted following a completely randomized design with 10 replicates for two hormone compositions for both cold-treated and non-treated explants. Days to callus formation, size of callus, colour of callus and percent survival of callus were recorded.

Estimation of ascorbic acid: Ascorbic acid content of five replicates of each of the the blackish callus produced from the endosperm culture of seeded banana and the white calli produced from the garlic was measured (Plummer, 1971). Whitish calli was chosen as 'control' considering that those were not suffering from any oxidation whereas it was assumed that calli blackening was because of oxidative stress produced during culture.

Statistical analysis: Data were analyzed using MINITAB 15 statistical software package (Minitab Inc., State College, Pennsylvania, USA). To find out variations among varieties, treatments imposed on explants, hormonal compositions and ascorbic acid content in calli separate one-way analysis of variance (ANOVA) was conducted. Tukey's pairwise comparisons were conducted when mean difference was significant.

Results

Morpho-physiological comparison of banana fruits: The objective of this study was to optimize the initial stages of endosperm culture of a local, seeded banana variety. Prior to that, three banana varieties namely, 'Sagor', 'Sabri' and seeded-banana were assessed based on their morphological and physiological traits. Analysis of variance showed significant varietal difference for all of the following traits: brix percentage, fresh weight, dry pulp weight, dry peel weight (data not shown). The total fresh weight, dry pulp weight and dry peel weight were highest in seeded banana followed by variety 'Sagor', and 'Sabri' (Fig. 3). Brix percentage of 'seeded banana' was statistically similar to that of 'Sabri' and was higher than that of variety 'Sagor'. The seeds of the seeded banana variety were found to occupy around 31% of the total pulp mass (Fig. 3).

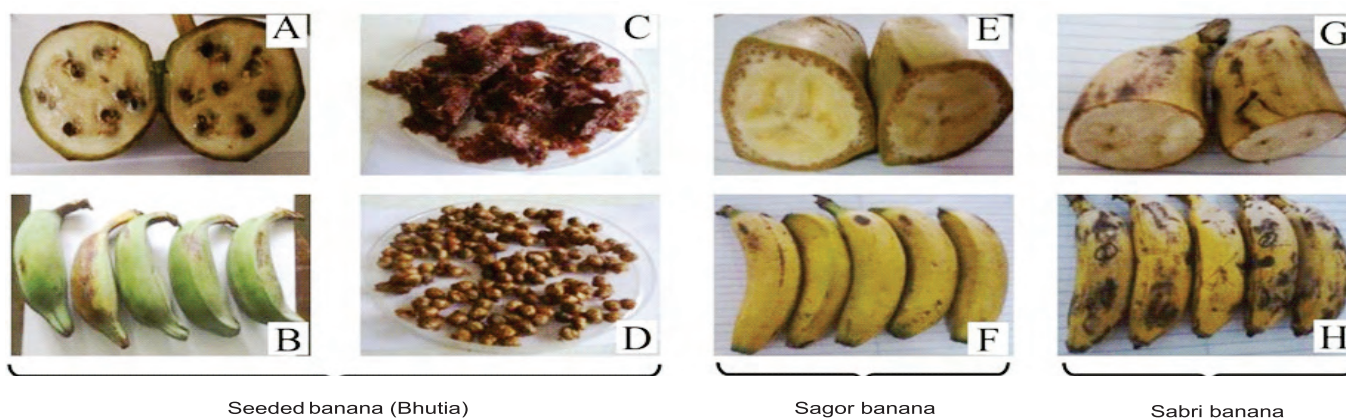


Fig. 1. A comparative study between seeded banana and two popular varieties, Sagor and Sabri. Cross section of the fruit and the entire fruit of seeded banana (A&B), 'Sagor' banana (E&F) and 'Sabri' banana (G&H) and the dry flesh (C) and the seeds (D) of seeded banana. 'A' in comparison with 'E' and 'G' shows presence and absence of seeds inside the edible flesh. 'B', 'F' and 'H' compare the individual fruits.

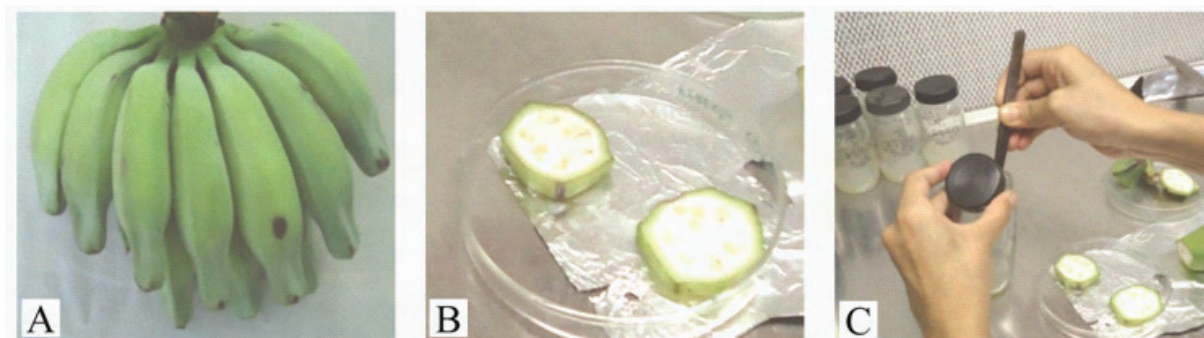


Fig. 2. A) 25 days old seeded banana, B) Cross-sections of 25 days aged seeded-banana and C) Inoculation of endosperm into tissue culture media.

Endosperm development: Endosperm like structures started to develop on 15th day. The developing seeds of 25 days old seeded banana fruit were found to be filled with material of soft and gelatinous consistency inside (Fig. 2B) and that of the 30 days old seeded banana fruits were found to become solid. Percent survived endosperm-explants in culture was estimated and it was found that more than 85% explants survived when endosperms were collected from 25 days old fruits (Fig. 4).

Assessment of endosperm-derived calli: Days to callus induction varied significantly between cold-treated and non-treated explants (Table 1). It was observed that non-treated explants produced larger calli (Fig. 5A) comparatively quickly than that of cold-treated explants (Table 1). Treatment 2 of the non-treated explants induced the largest calli within the shortest time period, after 27 days, compared to treatment 1 (Fig. 5B).

Table 1. Days to callus initiation from the endosperm of seeded banana variety, 'Bhutia' in MS media under two hormonal treatments Data presented as mean \pm SE (n=10)

Explant treatment	Days to callus induction		P value
	Treatment 1	Treatment 2	
Non-treated explants	29 \pm 0.3	27 \pm 0.4	0.045
Cold treated explants	33 \pm 0.5	32 \pm 0.6	0.32

Both hormone compositions in MS tissue culture medium produced calli which were blackish in colour. Ascorbic acid content of blackish calli (480 \pm 12.4 mg/100g) was significantly higher than that of whitish calli of garlic (400 \pm 7.8 mg/100g, $P < 0.01$).

Discussion

Fruit age factor: As for the percent survival, endosperm collected from 25 days old fruits of seeded banana performed better than

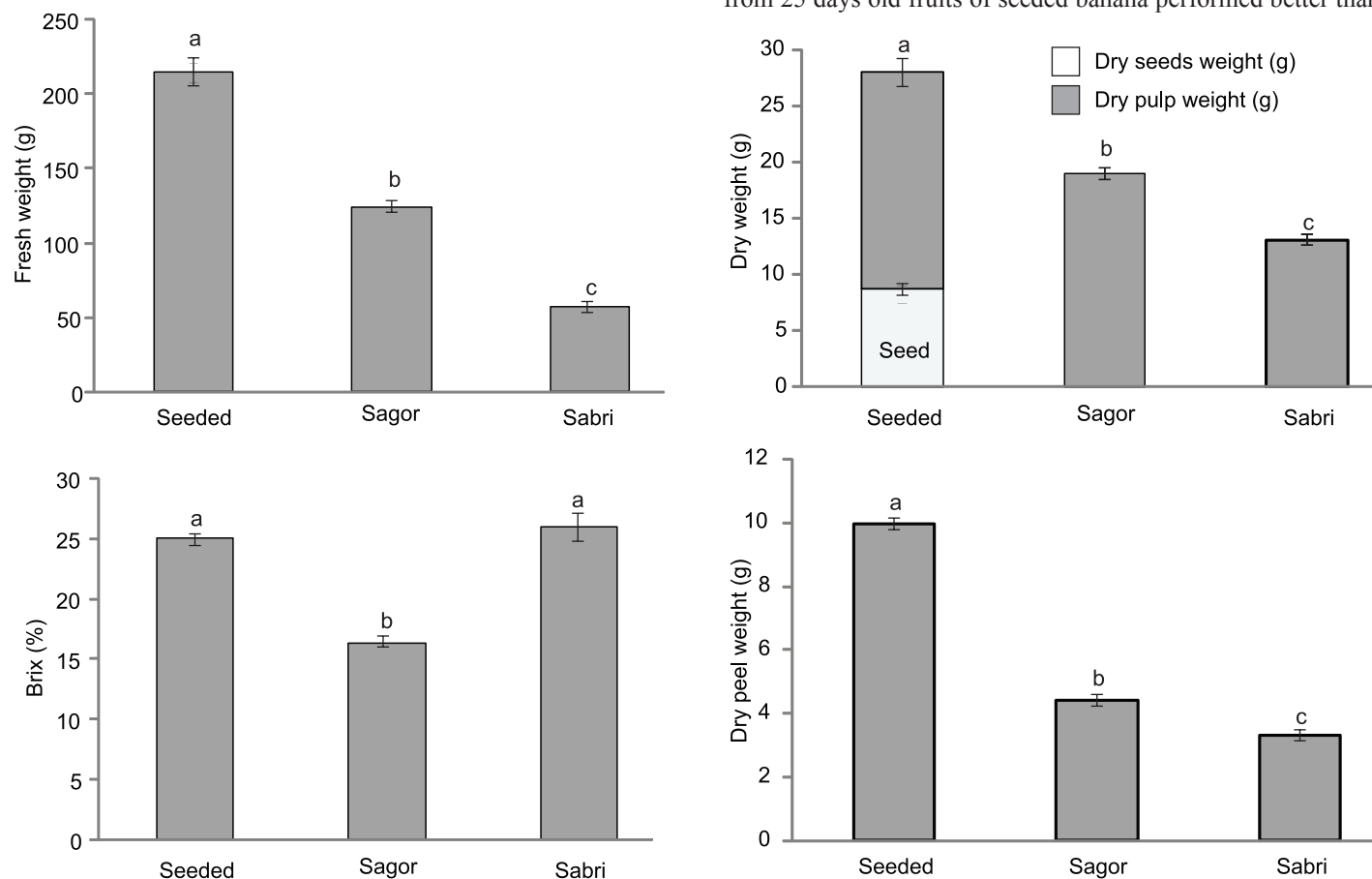


Fig. 3. Fresh weight, dry pulp weight, dry peel weight of individual fruits and brix percent of seeded, 'Sagor' and 'Sabri' banana varieties. Data presented as mean \pm SE (n=5). Different letters indicate statistically significant difference after Tukey's pairwise comparisons.

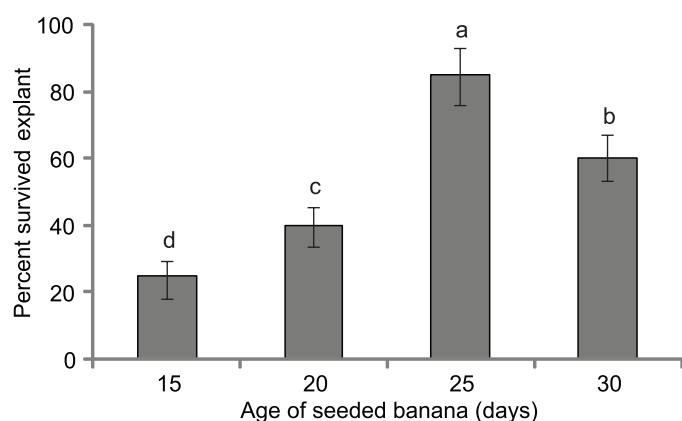


Fig. 4. The bar chart showing the percent survival of 15, 20, 25 & 30 days old endosperm of seeded banana. Data presented as mean \pm SE (n=5). Different letters indicate statistically significant difference after Tukey's pairwise comparisons.

comparatively younger endosperms (Fig. 4). Therefore, 25 day old endosperm of seeded banana is recommended to use as explants for any future attempt to regenerate triploid banana. Younger endosperms at this stage is gelatinous in consistency which indicates its immature state in contrast to the endosperms collected from 30 days old fruits which turns to become solid. It is known that the maturity of the embryo is an important factor in regeneration and also a determining factor of success of endosperm culture and the frequency of callus induction is higher in case of immature embryos which decreases with embryo maturity (Escalant and Teisson, 1989; Hoshino *et al.*, 2011; Uma *et al.*, 2011; Dayarani *et al.*, 2014).

Can induction treatment be beneficial: An induction treatment (cold or heat) is generally beneficial for anther culture (Sunderland and Roberts, 1979; Huang and Sunderland, 1982) and the cold treatment was found to control the darkening of callus and increase the survival of the callus in pear (Poudyal *et al.*, 2008). The cold treatment was thus tested to see if there's any positive effect on the success of endosperm culture. But the cold treatment did not account any positive effect on callus proliferation and callus size whereas the non-treated endosperm induced the callus quickly and produced larger calli (Fig. 5).

Use of NAA in endosperm culture: In terms of the hormone composition in MS media, it was observed that the inclusion of 0.5 ppm NAA along with 0.5 ppm 2,4-D and 0.5 ppm Kn can improve the callusing parameters positively (Fig. 5B, Table 1). Inclusion of NAA was also found to be effective in inducing callus during the production of triploid dessert banana (Navarro *et al.*, 1997), triploid papaya (Sun *et al.*, 2011) and in mature endosperm derived callus induction from *Annona squamosa* (Nair *et al.*, 1986).

Calli blackening: In all culture conditions of the present study blackening of the derived calli was observed. In literature, a similar phenomenon, blackening or browning of the callus, has been previously reported in many fruit species including banana (Munguatosha *et al.*, 2014; Chikezie, 2012; Khatri *et al.*, 2005; Nisyawati and Kariyana, 2013), pear (Gao *et al.*, 2003; Li and Qiao, 2001; Yan and Li, 1998), avocado (Castro *et al.*, 1995), guava (Meghwal *et al.*, 2000), cashew (Aliyu, 2005) and litchi (Chandra and Padaria, 1999). Further investigations are required to reveal the physiological reasons of blackening or browning. An attempt to know the difference in ascorbic acid content in the

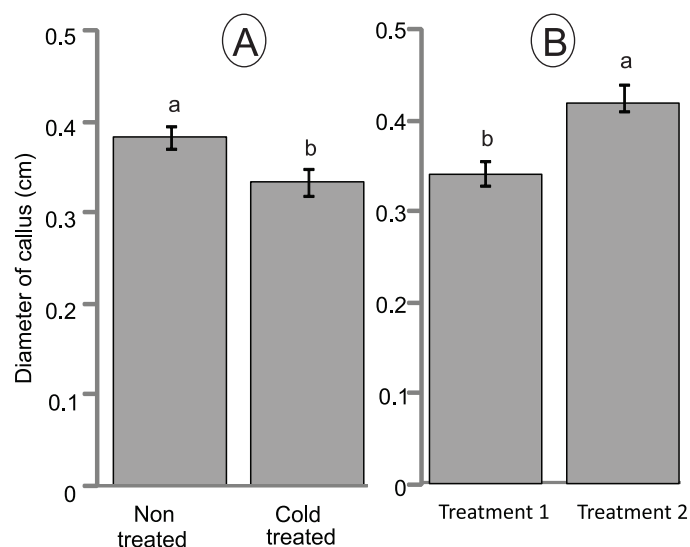


Fig. 5. Diameter of calli produced from the endosperm of seeded banana varieties: A) in non-treated and cold treated explants ($P=0.03$). and B) in MS media under two hormonal treatments: MS media + 0.5ppm 2,4-D + 0.5ppm Kn (Treatment 1) & MS media + 0.5ppm 2,4-D + 0.5ppm Kn + 0.5ppm NAA (Treatment 2) for the non-treated explants ($P=0.01$). Data presented as mean \pm SE (n=10). Different letters indicate statistically significant difference after Tukey's pairwise comparisons.

blackish calli obtained from the endosperms with normal whitish calli (from garlic) revealed higher concentration of ascorbic acid content in blackish calli. The results suggested the endosperm derived calli in MS medium under both hormone compositions may have been suffering from the oxidative stress. Whitish calli of garlic was used as control.

Several remedies have been suggested in literature that can reduce the blackening or browning of calli which includes the addition of cysteine and methionine (Khatri *et al.*, 2005), polyvinyl pyrrolidone (PVP) (Poudyal *et al.*, 2008; Wei *et al.*, 2007; Zhou, 2007; Zhang *et al.*, 2003), vitamin C (Peng *et al.*, 2007), activated charcoal (Aliya, 2005) and antioxidant 8-hydroxyquinolin (8-HQS) (Li and Qiao, 2001) in the culture medium. Pre-treatment of explants, for example, cold treatment (Poudyal *et al.*, 2008; Zhang *et al.*, 2003; Li and Qiao, 2001; Liu and Han, 1986) and manipulation of culture conditions, such as: dark culturing (Nisyawati and Kariyana, 2013; Poudyal *et al.*, 2008; Zhang *et al.*, 2003; Aliyu, 2005) were found beneficial. Besides, addition of ascorbic acid in the culture medium as an antioxidant was found to effectively control the darkening of the callus (Munguatosha *et al.*, 2014; Peng *et al.*, 2007; Aliya, 2005; He *et al.*, 1995); contrarily, Chikezie (2012) reported no beneficial effect of ascorbic acid on the effective minimization of explant blackening.

The study revealed that 'jelly like' endosperm explants from around 25 days old seeded banana fruits survived the best in MS culture medium. NAA supplemented MS medium produced calli of larger diameter comparatively quickly compared to the medium lacking NAA. Calli became blackish in appearance probably due to oxidative stress as indicated by higher ascorbic acid content in blackish calli compared to white calli. Further research is needed to avoid blackening of callus which is a challenge for establishing a complete triploid plantlet regeneration protocol through endosperm culture of seeded banana.

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