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In-vitro effects of sucrose and ascorbic acid on induction and shelf-life of micro-tuber of cassava (*Manihot esculenta* Crantz) genotypes

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Abstract

Background: Cassava production faces threat from postharvest physiological deterioration (PPD). The PPD is usually observed within the first 72 hours of harvest. Therefore, extending the shelf-life of cassava root-tubers by few days could reduce appreciable financial losses.

Objective: This study is aimed to investigate effects of sucrose and ascorbic acid on induction and shelf-life of micro-tubers of cassava cultivars using an *In-vitro* model.

Method: Effect of sucrose (30, 45, 60, 75 and 90 g/L) on the micro-tuber induction was determined after 45 days in these cultivars (TME 1333 and TME 2060). The optimal level was later combined with ascorbic acid (0, 25, 50, 75 and 100 mg/L) to investigate its effect on micro-tubers shelf-life during a 10-day storage. Each study was laid out in factorial under CRD with 5 replications.

Results: The 30 g/L sucrose (control) produced the utmost number of micro-tubers (4.33), highest length (6.68 cm), and fresh weight (0.1495 g). The cultivar 'TME 2060' had longer (5.26 cm) and higher fresh weight (0.1079 g) than 'TME 1333' with 5.04 cm and 0.0977 g respectively. Ascorbic acid (100 mg/L) significantly delayed the discolouration of micro-tubers over a 10-day storage. Also, the 100 mg/L ascorbic acid produced the least percentage of deteriorated micro-tubers.

Conclusion: Results demonstrate the roles of sucrose and ascorbic acid in micro-tuber formation and antioxidant activity in delaying PPD. Therefore, we propose for *In-situ* production (via breeding strategy) of vitamin C-fortified cassava varieties in order to control the incidence of PPD and in turn improve farmer`s economy.

Keywords: Cassava; Micro-tuberization; Discolouration; Deterioration; Sucrose; Ascorbic acid

1. Introduction

Cassava (*Manihot esculenta Crantz*), a known root and tuber crop of the Euphorbiaceae family [1]. According to Lebot [2], cassava serves the sixth most useful crop after wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), potato (*Solanum tuberosum* L.) and barley (*Hordeum vulgare* L.). All parts of the cassava plant most especially the

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tuber have a poisonous compound called hydrocyanic glucoside [3]. Cassava tuber is a major source of calories for poor families due to its high carbohydrate content and adaptability [4]. The tubers are mainly rich in starch (complex carbohydrate) with appreciable amounts ranging from 81.0–87.1 g/100g. The leaves are a good source of protein (21 %), rich in lysine, arginine, leucine, valine but deficient in methionine and tryptophan. Also, nutrient content ranges from 167-399 g/kg crude protein, 48-290 g/kg crude fibre, 36-105 g/kg ether extract, 57-125 g/kg ash, and 6.7-10.2 MJ/kg energy [5]. Jyothi et al. [6] and Daramola and Folade [7], independently reported the commercial use of cassava starch in the production of monosodium glutamate, papers, textiles, flavouring agent in Asian recipe and as partial substitution for wheat flour in Africa. Cassava is also a potential source of biofuel and rich source of raw material for industrial starch production [8].

Cassava may be planted as a sole crop and/or intercropped with rubber, maize, vegetables, legumes, oil palm and other important economic plants [9]. And can be propagated through seeds and vegetative parts. The seeds are used for breeding purposes in order to generate improved varieties. The seedlings are raised mostly for the purpose of selecting seedlings with fewer and smaller roots. Biotechnology is another method of vegetative propagation suitable for cassava plants and involves tissue culturing (direct and indirect organogenesis) of plant parts. Tissue culture involves the use of artificial media or non-soil materials under sterile and controlled environmental conditions. Cassava and many tropical plants can be propagated *In-vitro* by different explants such as auxiliary buds [10], nodal segments [11], and shoot apical meristems [12, 13, 14]. Cassava *In-vitro* culture was first reported by Kartha et al. [15]. The authors regenerated shoots from meristems of five cassava genotypes planted on Murashige and Skoog, MS [16] medium supplemented with 0.1 mg/L 6-benzylaminopurine (BAP), 0.04 mg/L gibberellic acid (GA_3) and 0.2 mg/L naphthalene acetic acid (NAA).

Tissue culture is the most thriving and commercial part of biotechnology with regards to rapid clonal multiplication of selected plant species [17]. The *In-vitro* growth of plants for production of micro-tubers allows the control of the different factors that might influence the tuberization process [18]. They produced micro-tubers of cassava using 0.4 µM BAP, 1.6 µM NAA, and 80 g/L of sucrose. Fotso et al. [19] studied the production of micro-tubers in cassava using 0.1 to 0.6 mg/L NAA, 0.1 to 0.6 mg/L KIN, 0.1 to 0.6 mg/L NAA/KIN ratio and 10 to 60 g/L sucrose, and found that 0.4 mg/L NAA and 0.4 mg/L KIN used separately with 30 g/L sucrose was most effective for micro-tubers production. Also, Elian et al. [20] obtained the highest micro-tubers number (4 to 5) and fresh weight (209.4 to 432.0 mg) when 30 or 40 g/L sucrose were used in combination with 0.5 mg/L BAP and 0.05 mg/L NAA for micro-tuberization of cassava.

Cassava root-tubers undergo rapid postharvest physiological deterioration (PPD) soon after harvest [21]. The exact causes of PPD are uncertain, however, reports suggest that it may be caused by the mechanical damages that occur at harvest and is manifested during storage. The action of PPD progresses from the proximal site of production to the distal end within 3 days, making the tuberous roots unpalatable and unfit for market [21, 22]. A major visual symptom of PPD is the vascular streaking associated with discolouration due to occlusions in the vascular parenchyma, caused by oxidized phenolics [21]. Reports by Sánchez et al. [23] and Uarrota et al. [24] on storage conditions and practices are also recognized to affect responses to PPD. Saravanan et al. [25] observed a decline in phospholipid content during PPD which the authors attributed to membrane degradation and a symptom of oxidative damage. Several studies have been carried out to determine the mode of action and biochemistry of PPD. These studies suggest that reactive oxygen species (ROS) production is a major factor affecting the deterioration process [21, 26, 27, 28, 22]. Generally, ROS are produced as by-products of aerobic respiration [29]. It is also produced in cell systems under stress [30]. The ROS in cell systems include: hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), and free radicals such as the hydroxyl radical ($OH^{\cdot}$) and the superoxide anion (O^{2-}) which also possess specific cellular function known as redox signaling [31]. The hydroxyl radical is the most dangerous because it is an unstable free radical that readily reacts with biological molecules. In a metal-catalyzed redox reaction (Fenton reaction), the hydrogen peroxide serves as the platform for the production of hydroxyl radicals [32, 33].

$$\text{Fenton reaction: } Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Hydroxyl radical is capable of causing lipid peroxidation, oxidizing DNA, and damaging proteins [34, 35, 36]. Under situations of stress, however, the equilibrium between the production and scavenging of ROS is disturbed, manifesting in a rapid accumulation of ROS known as “an oxidative burst” [37].

An antioxidant could be defined as a molecule that inhibits the oxidation of other molecules. Oxidation occurs when electrons or hydrogen is donated to a substance. Oxidation reactions can generate free radicals. These radicals can start chain reactions and when the chain reaction occurs within a cell, it can cause damage to vital components of the cell and/or cell death. Antioxidants can terminate these chain reactions by removing free radical intermediates or inhibit further oxidation reactions. In the process, they may get oxidized. Antioxidants include reducing agents such as thiols,

ascorbic acid, or polyphenols [38]. Antioxidant systems ensure defense against the free radicals before they damage vital component of the cell such as the lipid membrane [39]. Mostly, antioxidant systems are useful in either removing or maintaining the free radicals at optimum physiological level in cells [39]. There are two categories of antioxidants, non-enzymatic and enzymatic antioxidants. The non-enzymatic antioxidant includes vitamin C (ascorbic acid) which possess antioxidant properties and have been found to reduce the presence of free radicals such as hydrogen peroxide (H_2O_2) in the cell [40]. Vitamin C (ascorbate) belongs to the group monosaccharide oxidation-reduction (redox) catalyst, and is observed in both plants and animals. Ascorbate is oxidized by oxygen, hydrogen peroxide and superoxide into monodehydro-ascorbate (MDHA) radicals. Ascorbate oxidase (AOX) is somewhat associated with cell wall growth and expansion. The MDHA radicals, product of AOX reactions, depolarizes the plasma membrane thereby resulting in an ion uptake and cell wall loosening [41]. Vitamin C performs similar function as the hydrogen peroxide in reducing metal ions that generate free radicals through the Fenton reaction [42, 43, 44]. Ascorbic acid prevents oxidative browning of tissue, however, in tobacco cells it was shown to function as a stimulant of mitotic cell division [45, 46, 47]. In addition, it plays a significant role in protecting and/or regenerating oxidized carotenoids or tocopherols, non-enzymatic antioxidants [48].

Fenton reaction: Fe^{3+} + Ascorbate → Fe^{2+} + Dehydro-ascorbate

During the harvest of cassava storage roots, oxidative burst occurred at about 15 minutes after harvest [28]. Zidenga et al. [49] reported that cassava cyanogen played an important role in oxidative burst leading to the onset of PPD. The cyanide released during mechanical damage of cassava root-tubers can cause a build-up of ROS. A reduction in ROS accumulation was found to delay the onset of PPD by 14 to 21 days after harvest of greenhouse-grown plants [49]. Also, the delay in PPD of transgenic plant tubers under field conditions were due to expression of alternative oxidase gene (*AOX*), which is cyanide insensitive, however, the root-tuber`s size was reduced [49]. Increase in enzyme activities such as those of catalase, peroxidase and superoxide dismutase modulate ROS levels [21, 27, 22]. In addition, high content of β-carotene (non-enzymatic antioxidant) reported in cassava cultivars, were shown to be less susceptible to PPD [50]. Therefore, it may be possible to promote safe or longer shelf-life in farmer-preferred varieties using antioxidant by way of helping to reduce losses due to PPD [21, 22]. Ascorbic acid is a very imperative antioxidant because it reacts not only with hydrogen peroxide, but also with oxygen free radicals, or hydroxide radicals to detoxify all [41]. It is water-soluble vitamin and can raise the antioxidant profile of plant tissues thereby improving food quality during storage and conservation [51].

On the other hand, micro-tubers are important materials for maintaining gene-bank accessions in a small space and it can be used for producing virus-free plants among asexually propagated species [52, 53]. Another benefit of *In-vitro* propagation technique is that it is not influenced by season [54, 55]. Protocol for cassava micro-tuber production was reported with wide variation in the response on the induction medium, for instance in cassava cv. TMS 96/0023 [19] and cv. TMS 95/0211 [20]. Standardizing the micro-tuber induction medium would improve the availability of micro-tubers for germplasm exchange and crop improvement.

In this present study, we investigate the effect of sucrose on cassava micro-tuber induction. Thereafter, due to the major challenge sighted on PPD in cassava root-tubers. Using an *In-vitro* model, we examine the effect of ascorbic acid on cassava micro-tubers shelf-life.

2. Material and methods

This research was conducted at the Tissue Culture Laboratory of the Department of Crop and Horticultural Sciences, University of Ibadan and the Biotechnology Laboratory of the Department of Crop Science and Horticulture, Faculty of Agriculture, Nnamdi Azikiwe University, Awka, Anambra State of Nigeria. The Nnamdi Azikiwe University is located at latitude 6.24^0 North and longitude 7.12^0 East, altitude of 183m above sea level. Its annual mean rainfall is about 1,798.52 mm, with average mean relative humidity of 82% and an average temperature of about 26 °C.

2.1. Source of plant materials

Cassava cultivars (TME 1333 and TME 2060) were obtained from the *In-vitro* collections of the Genetic Resource Center, International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State of Nigeria.

2.2. Basic equipment and chemicals

The basic equipment used are as follows: aluminum foil, autoclavable plastic/glass containers, autoclave, electronic weighing balance, dissecting kit, forceps (long and short), refrigerator, laminar airflow cabinet, magnetic stirrer, measuring cylinder, media dispenser, pH meter, spoon, spatula and weighing boat, scalpel and surgical blades (number

10 and 11), pair of scissors, spray bottle, test tube (16 x 125 mm) and cap, water distiller, deionizer and water tank. The chemicals used include: phytagel, growth regulators such as naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP), MS basal medium, Myo-inositol, sugar, ascorbic acid, potassium hydroxide, hydrogen chloride, potassium dichromate, sulphuric acid, and distilled/deionized water.

2.3. General sterilization procedure

New glasswares were properly washed using detergent and rinsed with clean running tap water followed by a dip in a mixture of chromic acid for 8-10 hours. Chromic acid was prepared by mixture of potassium dichromate (20 g) and concentrated sulphuric acid (300 ml) in a 2:1 (v/v) ratio respectively. After this treatment, the glasswares were washed thoroughly with water for the removal of all traces of chromic acid and then rinsed with distilled water thrice. Routinely used laboratory glasswares were also washed with detergent and rinsed thrice with distilled water before any experiment. The cleaned glasswares were transferred and stored in a dust-free cupboard. For the sterilization of working area and surgical tools. The laminar airflow cabinet which is the main working area for aseptic manipulation (culturing) was scrubbed thoroughly with 70% ethanol and switched on for about 30 minutes before each manipulation. The surgical tools (scalpel, forceps, spatula, needles, etc.) and paper towel were sterilized by wrapping them with aluminum foil and autoclaved at 121 °C for 20 minutes. The hot forceps and other tools were allowed to cool at room temperature in laminar airflow cabinet for few minutes and then used for culturing. The working tools are flamed at transfer intervals to maintain sterility (free from contamination).

2.4. Plant culture media preparation

Cassava micropropagation medium consists of Murashige and Skoog [16] basal medium (4.43 g/L), Myo-inositol (100 mg/L), naphthalene acetic acid (0.01 mg/L), 6-benzylaminopurine (0.05 mg/L), sucrose (30 g/L), and phytagel (7 g/L) at pH 5.70 ± 0.1 (communication from IITA, Nigeria). For micro-tuber induction, the method of Fotso et al. [19] was followed with modifications in growth regulators, sucrose and agar concentrations. The medium consists of MS basal medium (4.43 g/L), Myo-inositol (100 mg/L), naphthalene acetic acid (0.01 mg/L), 6-benzylaminopurine (0.04 mg/L), sucrose (30, 45, 60, 75 and 90 g/L) and phytagel (1.00 g/L) at pH 5.70 ± 0.01. The optimal sucrose level determined in the micro-tuber induction experiment, was combined with ascorbic acid concentrations (0, 25, 50, 75 and 100 mg/L) to determine the effect on micro-tubers shelf-life. The pH of each medium was adjusted to 5.70 ± 0.01 with 1N KOH or 1N HCl. Ascorbic acid was added during media preparation, before adjusting the pH as it greatly alters the pH of the medium. The medium was dispensed in pre-sterilized culture vessels (~20 ml for regular sub-culture and 80 ml for micro-tuberization). The medium in the culture vessels, covered with fitted polypropylene caps were sterilized by autoclaving at 121 °C for 20 minutes. The sterilized medium was allowed to cool at room temperature in laminar airflow cabinet before culture operations.

2.5. Explants and culture conditions

Polypropylene caps were removed from each culture vessel containing the plantlets for sub-culture and with the help of sterile forceps, the explants were brought out and nodal segments (about 10 cm each) were dissected and transferred unto each solidified medium. Three weeks after culture, the plantlets were transferred to a sterilized semi-solid medium for micro-tuber induction. Each culture vessel was carefully covered after slightly heating the vessel`s tip to prevent contamination. All cultures were exposed to a 16-hour light (illuminated with cool white fluorescent bulbs at 54 µmol m^{-2} s^{-1}) and 8-hour dark photoperiod. All cultures were maintained at 22 ± 2 °C in the growth chamber for 45 days.

2.6. Experimental design and treatments application

To determine the effects of sucrose on cassava micro-tuber induction. A 2 x 5 factorial in a completely randomized design (CRD) was followed and replicated five (5) times. Factors are two (2) cassava cultivars (TME 1333 and TME 2060) and five (5) levels of sucrose (30, 45, 60, 75 and 90 g/L). The 30 g/L sucrose was set as the control.

For the effect of ascorbic acid on cassava micro-tubers shelf-life. The 30 g/L sucrose was combined with ascorbic acid (0-100 mg/L) to raise new micro-tubers for shelf-life study. A 2 x 5 factorial in a completely randomized design (CRD) was also followed and with five (5) replicates. Factors are two (2) cassava cultivars (TME 1333 and TME 2060) and five (5) concentrations of ascorbic acid (0, 25, 50, 75 and 100 mg/L). The 0 mg/L ascorbic acid was set as the control. Treatment duration to raise new micro-tubers was 45 days after explant culture while the shelf-life study lasted for 10 days following the micro-tuber induction phase. Ascorbic acid was added to the culture medium before adjusting the pH to 5.70 ± 0.01. Media were autoclaved at 121 °C for 20 minutes. Freshly harvested micro-tubers were used for the shelf-life study. A set of 3-5 uniform-sized (cm) micro-tubers from each treatment was used. The micro-tubers were stored in sterile dry glassware vessels and kept under room temperature in the laboratory.

2.7. Data collection and analysis

Data on the number of micro-tubers produced per treatment, length (cm) of micro-tubers and fresh weight (g) of micro-tubers were taken. The data for micro-tubers shelf-life were collected at a 2-day interval for 10 days following storage at room temperature (25 °C). Days to onset of deterioration among each stored set was determined and percentage (%) of micro-tubers deteriorated. All data were subjected to analysis of variance (ANOVA) using GenStat Statistical Package 4th Edition [56]. Means were separated using Fisher`s Protected Least Significant Difference at 5% level of probability.

3. Results

3.1. Mean squares from analysis of variance on the effects of sucrose and ascorbic acid on induction and shelf-life of micro-tubers of cassava

The mean squares for the sucrose, genotype and interactions are presented in Table 1. The sucrose and genotype on cassava micro-tuber induction, revealed a highly significant difference (P<0.001) across studied variables. Also, the interactions recorded a significant difference (P<0.05). Exception was on number of micro-tubers, the genotype and interactions were shown to remain same.

On the effect of ascorbic acid on cassava micro-tubers shelf-life (Table 1). The mean squares for ascorbic acid showed a highly significant differences (P<0.001) across the parameters. The mean squares for genotype and interactions remained non-significant across variables.

Table 1 Mean squares from analysis of variance on the effects of sucrose and ascorbic acid on induction and shelf-life of micro-tubers of cassava

Effects of sucrose on micro-tuber induction					**Effects of ascorbic acid on micro-tubers shelf-life**			
Source of variation	**df**	**No. of MT**	**MT length (cm)**	**MT weight (g)**	**Source of variation**	**df**	**No. of DD (days)**	**% MT.D (%)**
Sucrose (30-90 g/L)	4	4.4500***	6.14399***	0.00701970***	Ascorbic acid (0-100 mg/L)	4	40.0333***	376.867***
Genotype	1	0.1333 ns	0.34261***	0.00077013***	Genotype	1	1.2000 ns	5.633 ns
S x G	4	0.0500 ns	0.01684 *	0.00002380 *	A x G	4	0.0333 ns	0.133 ns
Residual	20	0.4667	0.02397	0.00004653	Residual	20	0.7667	1.867

***, * – significant at P (<0.001; <0.05 respectively), ns – non-significant, df – degrees of freedom, MT – micro-tuber, DD – days to deterioration, MT.D –micro-tuber deteriorated.

3.2. Effects of sucrose on cassava micro-tuber induction

The number of micro-tubers produced per treatment significantly varied (Table 2). The 30 g/L sucrose (control) produced a significantly higher (P<0.001) number of micro-tubers (4.33) compared to other sucrose levels. On the other hand, the number of micro-tubers produced by other sucrose levels (45-90 g/L) were the same. For the length (cm) of micro-tubers, there were significant differences (P<0.001) among treatments. The micro-tuber length of 6.68 cm produced on 30 g/L sucrose was significantly (P<0.001) longer than those of other sucrose levels. This was followed by the micro-tuber length of 5.57 cm produced on 45 g/L sucrose and length of 4.94 cm produced on the 60 g/L sucrose. The 75 and 90 g/L sucrose produced similar micro-tuber lengths (4.26 and 4.29 cm respectively). The fresh weight (g) of micro-tubers also had significant differences (P<0.001). The 30 g/L sucrose produced a significantly higher (P<0.001) fresh weight of micro-tubers (0.1495 g) compared to other sucrose levels. The 45 g/L sucrose had 0.1225 g fresh weight of micro-tubers and was followed by the 60 g/L sucrose with 0.1013 g fresh weight of micro-tubers. There was no significant difference between the 75 and 90 g/L sucrose with mean values of 0.0698 and 0.0708 g respectively.

Responses of cassava cultivars on micro-tuber induction (Table 2). The number of micro-tubers produced by both cassava cultivars were the same. The cultivars (cv. TME 1333 and TME 2060) responded differently (P<0.001) with regards to the length (cm) of micro-tubers. The cv. 'TME 2060' produced a significantly longer (5.26 cm) micro-tubers than cv. 'TME 1333' with a length of 5.04 cm. The fresh weight (g) of micro-tubers was significantly varied (P<0.001). The cv. 'TME 2060' weighed (0.1079 g) higher than cv. 'TME 1333' which had a fresh weight of 0.0977 g.

The interactions considered were the within treatment interactions. They responded differently (P<0.05) across the variables (Table 2). The number of micro-tubers across the interactions were the same respectively. The length of micro-tubers significantly (P<0.05) varied. The 30 g/L x TME 2060 interaction had longer (6.82 cm) micro-tubers than the counterpart (30 g/L x TME 1333 interaction) with 6.53 cm. Also, the 60 g/L x TME 2060 interaction recorded higher length (5.12 cm) of micro-tubers than the 60 g/L x TME 1333 interaction (4.77 cm). On the other hand, the fresh weight of micro-tubers showed a significant difference (P<0.05). The 30 g/L x TME 2060 interaction weighed (0.1560 g) higher than the 30 g/L x TME 1333 interaction with 0.1430 g. Likewise, the 90 g/L x TME 2060 interaction had higher fresh weight (0.0780 g) of micro-tubers than the 90 g/L x TME 1333 interaction with 0.0637 g. Interactions not stated were the same across the studied variables.

Table 2 Effects of sucrose on cassava micro-tuber induction

Treatment	No. of MT	MT length (cm)	MT weight (g)
Sucrose (g/L)			
30 g/L (Control)	4.33	6.68	0.1495
45 g/L	2.83	5.57	0.1225
60 g/L	2.67	4.94	0.1013
75 g/L	2.33	4.26	0.0698
90 g/L	2.17	4.29	0.0708
SE	0.279	0.0632	0.00278
$LSD_{(0.05)}$	0.823 ***	0.1865 ***	0.00822 ***
Genotype			
TME 1333	2.93	5.04	0.0977
TME 2060	2.80	5.26	0.1079
SE	0.176	0.0400	0.00176
$LSD_{(0.05)}$	0.520 ns	0.1179 ***	0.00520 ***
Interactions (S x G)			
30 g/L x TME 1333	4.33	6.53	0.1430
30 g/L x TME 2060	4.33	6.82	0.1560
45 g/L x TME 1333	3.00	5.52	0.1200
45 g/L x TME 2060	2.67	5.62	0.1250
60 g/L x TME 1333	2.67	4.77	0.0957
60 g/L x TME 2060	2.67	5.12	0.1070
75 g/L x TME 1333	2.33	4.19	0.0663
75 g/L x TME 2060	2.33	4.32	0.0733
90 g/L x TME 1333	2.33	4.19	0.0637
90 g/L x TME 2060	2.00	4.39	0.0780
SE	0.394	0.0894	0.00394
$LSD_{(0.05)}$	1.163 ns	0.2637 *	0.01162 *

***, * – significant at P (<0.001; <0.05 respectively), ns – non-significant, MT – micro-tuber, TME – Tropical *Manihot esculenta*, SE – Standard error, $LSD_{(0.05)}$ – Fisher`s least significant difference (P<0.05), g/L – gram/litre.

a. (i) A 5-week old cassava (cv. TME 1333) *In-vitro* culture undergoing micro-tuber induction. a. (ii & iii) Cassava micro-tubers (cv. TME 1333) induced after 45 days of initial culture with 30 g/L sucrose. b. (i) A 5-week old cassava (cv. TME 2060) *In-vitro* culture undergoing micro-tuber induction. b. (ii & iii) Cassava micro-tubers (cv. TME 2060) induced after 45 days of initial culture with 30 g/L sucrose.

Figure 1 Cassava (cv. TME 1333 and TME 2060) micro-tuber induction after 45 days of initial culture

3.3. Mean performance on effects of ascorbic acid on cassava micro-tubers shelf-life

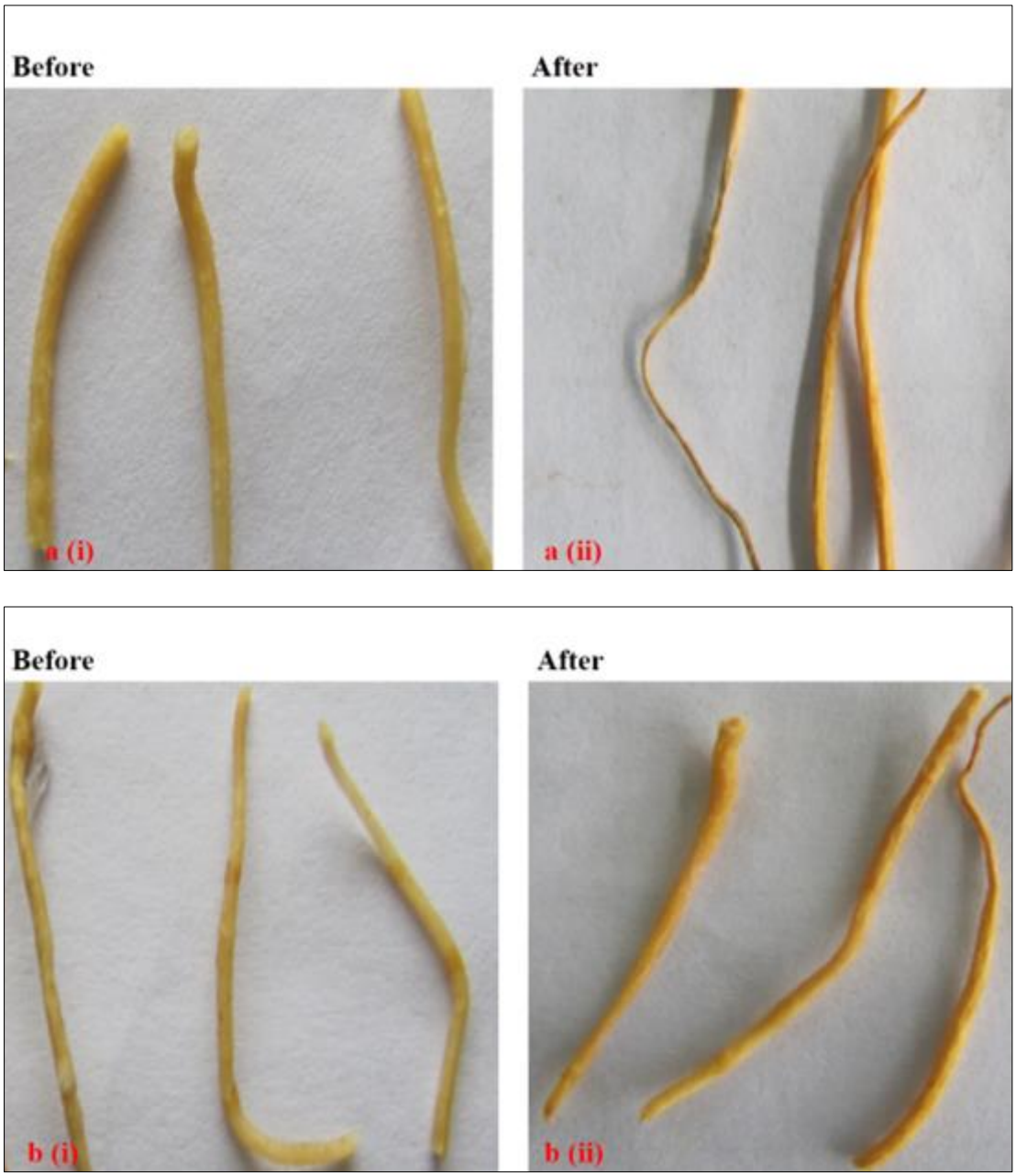
There were significant differences (P<0.001) among treatments on the number of days to deterioration (Table 3). The 100 mg/L ascorbic acid significantly delayed discolouration (onset of PPD) of micro-tubers (at 10 days) compared to other treatments (control, 25, 50, and 75 mg/L). There was no significant difference between 50 mg/L and 75 mg/L ascorbic acid. However, 50-100 mg/L ascorbic acid significantly delayed discolouration/deterioration longer (7-10 days) than the control and 25 mg/L ascorbic acid (3-4 days). The percentage of micro-tubers deterioration on day 10 (Table 3) showed a highly significant differences (P<0.001) among treatments. A significantly (P<0.001) lower number

of micro-tubers (72%) appeared deteriorated after 10 days storage period with 100 mg/L ascorbic acid compared to other treatments including control (80-92%).

Table 3 Mean performance on effects of ascorbic acid on cassava micro-tubers shelf-life

Treatment	No. of DD (days)	% MT.D (%)
Ascorbic acid (mg/L)		
0 mg/L (Control)	3.83	92.50
25 mg/L	4.50	90.17
50 mg/L	7.50	82.50
75 mg/L	8.17	80.17
100 mg/L	10.00	72.83
SE	0.357	0.558
$LSD_{(0.05)}$	1.055 ***	1.645 ***
Genotype		
TME 1333	6.60	84.07
TME 2060	7.00	83.20
SE	0.226	0.353
$LSD_{(0.05)}$	0.667 ns	1.041 ns
Interactions (A x G)		
0 mg/L x TME 1333	3.67	93.00
0 mg/L x TME 2060	4.00	92.00
25 mg/L x TME 1333	4.33	90.67
25 mg/L x TME 2060	4.67	89.67
50 mg/L x TME 1333	7.33	83.00
50 mg/L x TME 2060	7.67	82.00
75 mg/L x TME 1333	8.00	80.67
75 mg/L x TME 2060	8.33	79.67
100 mg/L x TME 1333	9.67	73.00
100 mg/L x TME 2060	10.33	72.67
SE	0.506	0.789
$LSD_{(0.05)}$	1.491 ns	2.327 ns

*** – significant at P<0.001, ns – non-significant, DD – days to deterioration, MT.D – micro-tuber deteriorated, TME – Tropical *Manihot esculenta*, SE – Standard error, $LSD_{(0.05)}$ – Fisher`s least significant difference (P<0.05), mg/L – milligram/litre.



a. Effect of 0 mg/L ascorbic acid (control) on cassava (cv. TME 2060) micro-tubers shelf-life. (i) Freshly harvested micro-tubers after initial culture. (ii) 10 days after storage at 25 **°C**.

b. Effect of 100 mg/L ascorbic acid on cassava (cv. TME 2060) micro-tubers shelf-life. (i) Freshly harvested micro-tubers after initial culture. (ii) 10 days after storage at 25 **°C**.

Figure 2 Effect of ascorbic acid on cassava (cv. TME 2060) micro-tubers shelf-life (10 days of storage at 25 °C)

4. Discussion

This study determined the roles of sucrose and ascorbic acid on cassava storage micro root-tubers during the onset of PPD. Although, studies on *In-vitro* micro-tuberization has been reported for similar root and tuber crops such as potato [57, 58], yam [59, 60] and cocoyam [61]. This study found that the production of micro-tubers of cassava can occur at 30 g/L sucrose which is quite unusual for other crops. Fogaça et al. [18], reported that cassava cultivars (Parazinha and Mantiqueira) produced the most micro-tubers after 45 days of growth when sucrose (80 g/L) in combination with 0.4 µM BAP and 1.6 µM NAA were used in the induction medium. The results obtained in this study is in agreement with the findings of Fotso et al. [19] who used 0.4 mg/L NAA or KIN in combination with 10 to 60 g/L sucrose for the culture of a cassava cv. TMS 96/0023 and obtained highest micro-tubers (4 to 5) and fresh weight (403.3 to 408.1 mg) on 30

g/L sucrose. Elian et al. [20] achieved the highest micro-tubers number (4 to 5) and fresh weight (209.4 to 432.0 mg) when 30 to 40 g/L sucrose were used in combination with 0.5 mg/L BAP and 0.05 mg/L NAA for micro-tuberization of cassava cv. TMS 95/0211.

Cassava production faces serious threat due to PPD which can cause economic losses of up to 25% of the global total production. The PPD is usually observed within the first 72 hours of harvest [21, 22]. Therefore, extending the shelf-life of cassava root-tubers by several days could reduce financial losses which was estimated at $2.9 billion in Nigeria over the last 20 years [62]. One of the earlier strategies employed by local farmers to reduce the incidence of cassava PPD include harvesting root-tubers in small quantities at a time. However, leaving tubers in the soil longer than necessary may adversely affect the quality and flavour as tuberous roots may become woody [63]. A widely accepted and cost-effective control strategy is to use varieties that genetically have long shelf-life [63]. In literature, non-enzymatic antioxidants (salicylic acid, glutathione, flavonoids and carotenoids) have been shown to protect against PPD [64, 65, 66] as they are able to fix problems relating to oxidative stress. Also, Sanchez et al. [50] reported that cassava cultivars with yellow roots attributed to the presence of β-carotene have delayed onset of PPD for 1 to 2 days. Vitamin C is commonly used during *In-vitro* culture to improve growth and reduce oxidative browning [67]. This study showed that ascorbic acid (100 mg/L) significantly reduced discolouration/deterioration of micro-tubers stored at room temperature (25 ^{o}C). In a similar study, ascorbic acid improved the survival of recalcitrant *Nephelium ramboutan-ake* shoot tips following cryopreservation-induced stress [68]. Correspondingly, vitamins C and E reduced lipid peroxidation of blackberry shoot tips following ultra-low temperature-induced oxidative stress [30]. The *In-vitro* derived micro-tubers of many root and tuber crops such as cassava, potato, yam, etc. are often the same biologically as those produced under conventional/field conditions. Thus, an *In-vitro* model of this nature could be directly applicable to field-grown crops and through plant breeding strategies can be improved with vitamin C.

5. Conclusion

An optimized micro-tuberization protocol was developed for cassava cultivars (TME 1333 and TME 2060) using a basic tissue culture medium supplemented with 0.04 mg/L BAP, 0.01 mg/L NAA and 30 g/L sucrose. These cultivars had variable responses on the number of micro-tubers produced, length (cm) of micro-tubers, fresh weight (g) of micro-tubers, number of days to deterioration and percentage (%) of deteriorated micro-tubers among treatments indicating genotype differences. The cassava cv. TME 2060 generally produced longer and higher fresh weight of micro-tubers than cv. TME 1333. When 30 g/L sucrose was combined with ascorbic acid (50-100 mg/L), it extended the shelf-life of micro-tubers up to 10 days. The micro-tubers with least percentage of deterioration occurred on medium with 100 mg/L ascorbic acid, a non-enzymatic antioxidant. These findings suggest that genetic improvement with ascorbic acid on farmer-preferred cassava varieties could prevent or reduce the incidence of PPD.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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