Histochemical Evidence of Dramatic Tissue Hardening in Post-harvest Trifoliate (D. dumetorum vars.) Yam Tubers

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Abstract: Post-harvest hardening of Dioscorea dumetorum yam tubers are characterized by lignify cells. In this study, changes in phenolics in freshly harvested and appropriately stored (28±2°C; 72–85% RH and 5±1°C; 85-100% RH) trifoliate yam tubers using histochemical analysis were studied and related to tissue cohesion and hardening in white and yellow cultivars. Results showed an intense lignified cells (deep safranin red and toluidine blue stained cell wall areas) in most parenchyma tissues of stored white and yellow trifoliate yam tubers (28±2°C, 72-85%RH and 5±1°C, 85-100% RH). In addition, histochemical changes in parenchyma tissues were more moderately in phase with an intense tissue cohesion and hardening in white and yellow trifoliate yam tubers at 28±2°C; 72–85% RH and in yellow trifoliate yam tuber at 5±1°C; 85-100% RH. This finding has given insight into the role of lignified tissue in the hardening of post-harvest D. dumetorum yam tubers.

Keywords: post-harvest, histochemical, lignify cell, phenolics, tissue hardening, parenchyma, cohesion

1. Introduction

Yam tubers from Ghana have contributed to the food economies of the world. These yam tubers have netted Ghana an incredible foreign exchanges. Among the yam tubers produced in the tropical yam zones, Dioscorea dumetorum yam tubers are the most nutritious [2,6,16]. Moreover, D. dumetorum yam tubers have numerous therapeutic properties such as antimicrobial [16,17], antioxidant [16,17], anti-inflammatory [16] and antidiabetic [15]. Despite these benefits, one of the conditions that limit trifoliate yam tuber is the sharp changes in tissues hardness [6,10]. Tubers of trifoliate yam are characterized by increase in fiber at storage [3,12]. Synthesized lignin of the cell wall has been reported to cause tissue hardening in trifoliate yam tubers [3,7]. These lignin are crucial for structural integrity of the cell wall by stiffening and strengthening tissues [4]. Characterizing these lignify tissues using histochemical technique in the yam tissue of D. dumetorum serves as a technical basis in solving the problem of post-harvest hardening. Previous report has described cell microstructure of hardened and unhardened trifoliate yam tubers using starch microscopy technique [1]. However, the starch microscopy technique as reported was based on a histochemical procedure [1]. This research aimed to study lignified cells and subsequently relate it to tissue cohesion and hardening in trifoliate yam tubers at storage.

2. Materials and Methods

2.1. Plant Materials

Wholesome, mature D. dumetorum yam tubers (characterized by dry vines and withered leaves), (white and yellow cultivars) were harvested in early January (2013), after 11 months of planting from an Experimental farm (Plant Genetic Resource Research Institute, Bonsu) in Ghana. Care was taken during harvesting, cleaning and transportation not to injure the tubers. Harvesting of the tubers was done between the hours of 8.00am- 9:30 am. During harvesting, soil temperatures of the yam mounds were estimated to be 22 ± 2 °C and with a corresponding dry season RH (40%). Samples were randomly selected and cleaned, kept cold in an ice chest containing ice gels. They were transported (within 3hrs) to the laboratories. The samples were divided into the following treatments: (i) stored at room temperature (28 ± 2°C, 72–85% RH) and (ii) under refrigeration conditions (5 ± 1°C, 85–100% RH) for a period of 0, 1, 2 and 3 days. Day of harvest and arrival at the laboratory was taken as day zero.

2.1.1. Sample Pretreatment for Histochemical Studies

The procedure for histochemical analysis of lignified cells was followed as described [13] with modifications. Tissues of dimensions 4 x 2 x 2 mm were sectioned horizontally from the middle sections of the tubers using a...
dissecting blade. Tissues were first fixed in 10% formalin (v/v) prepared in 50mM acetate buffer (pH 7.01, 0.2M) at 4 °C for 48 hours. Labeled fixed samples in an appropriate basket were washed under running water for 40 minutes and dehydrated using alcohol in an ascending order of concentration starting with 75 %, 85 %, 95 % for 10, 10, 20 minutes and lastly, in three changes of absolute alcohol for 20 minutes per change respectively. Samples were later cleared in three changes of xylene for 10 minutes per change respectively. Lastly, samples were impregnated using three changes of molten paraffin wax at ≤ 60 °C for 20 minutes per change in an oven (Sakura Finetech Co. Ltd, Tokyo-Japan). After processing, the samples were embedded in paraffin wax, blocked and sectioned using a microtome (Bright Instrument company Ltd-England; Model # 5040). Sections of 5-7 μm were cut and de-waxed using two changes of xylene for 1-2 min per change. They were then dehydrated using alcohol, starting with two changes of alcohol of 95 % and 80 % for 3-5 min per change.

2.1.1. Staining of Lignified Cells
The sections were stained with 1 % Safranin in 50 % ethanol for 30 minutes, rinse in running tap water, then counter stained with 1% aniline blue in 90% ethanol for 10 minutes and then dehydrated using ethanol in an ascending order of concentration, starting with 95 % (2 minutes) and then two changes of absolute alcohol (a total of 5 minutes) and then three changes of xylene (a total of 3 minutes) and then mounted using DXP. After mounting, the slides were examined using a Fluorescence microscope (Olympus BX41, Japan; Model U-LH100HG) in objective 40 and micrographs taken. Using Adobe Photoshop CS4 Extended Version 11.0 software, tris colour (red intensity in arbitrary units) of the micrograph was measured (four times) as lignified cell index.

2.1.1.2. Staining of Phenolics
The sections before staining was hydrated with distilled water and were then stained with 0.5% toluidine blue stain in acetate buffer (pH 4.4) for 20 minutes [10]. Stains were rinsed with running tap water for 3 minutes and then sections were mounted in DXP and micrographs were taken with a Fluorescence microscope (Olympus BX41, Japan; Model U-LH100HG) in objective 40. Using the Adobe Photoshop CS4 Extended Version 11.0 software, tris colour (blue intensity in arbitrary units) of the micrographs was measured (four times) as phenolic index.

2.2. Sample Preparation for Textural Studies
White and yellow cultivars of D. dumetorum tubers were sectioned into sizes of 2cm × 2cm × 4cm (breadth× width× length, volume 16 cm³) and were then cooked for 30 minutes with the aid of an electric stove (Ariston Thermo, Italy) [18]. After which samples were subsequently cooled to ambient temperature (25-30°C) and were then cut into cubes of 1.5 cm for texture analysis [18].

2.2.1. Texture Analyzer
Tissue cohesion and hardness of white and yellow cultivars of D. dumetorum were determined as described by [18] using a TX.XT2 Texture analyzer (Stable Micro Systems, England) equipped with the following parameters: Pre-test speed (mm/s) -2, Test speed (mm/s) -1, Post-test speed (mm/s) -2, Rupture test distance (mm) - 40, Force (g) -100, Time (sec) -3, Distance (mm) - 20, Load cell (kg) -25, Temperature (°C) -25. A 5-kg load cell was used to calibrate the probe (35 mm diameter compression base). Measurements (10) were made and tissue hardness were recorded as force in Newton (N).

2.3. Statistical Analysis
Analysis of variance were carried out on the data. Where necessary, post-hoc analysis LSD multiple range test was employed to determine significant differences (p≤0.05). The tissue hardness was model on tissue cohesiveness (cohesion) using fitted line plot. All data analyses were done using Minitab (version 15) and Microsoft office Excel (2010).

3. Results and Discussion
3.1. Micro-morphology and Histochemical Changes in Trifoliate Yam Tissues
The degree of lignified cells in white trifoliate yam at 28±2°C and 72–85% RH was more intense than in white trifoliate yam at 5±1°C and 85-100% RH (Figure 1). This implies that lignin in tissues of trifoliate yam was elicited when stressed at post-harvest. The post-harvest stress could be due to cold storage, ambient temperature storage, relative humidity, mechanical damage, gases (CO2) and artificial light intensity. Again, white and yellow cultivars of the tuber just after harvest were not significantly different (p≥0.05) in degree of lignified cells (Figure 1). This confirms that lignified cells in white and yellow trifoliate yam tubers were initiated when stressed. Dimensions of lignified parenchyma cell in respect to length of white trifoliate yams at 28±2°C and 72–85% RH were not different from those refrigerated (3 days). The lignified parenchyma cell length of white trifoliate yam at 28±2°C and 72–85% RH; and at 5±1°C and 85-100% RH ranged from 10-20µm and 5-15µm respectively (Figure 2 and Figure 4). On the contrary, lignified parenchyma cell length of yellow trifoliate yam stored (3 days) at 28±2°C and 72–85% RH; and at 5±1°C and 85-100% RH ranged from 5-7.5µm and 10-22µm respectively (Figure 2 and Figure 4). The height of the parenchyma cells (lignified and un lignified) as observed ranged from 5-7.5 µm (Figure 2 and Figure 4). However, white and yellow trifoliate tubers just after harvest had lignified parenchyma cell length ranging from 5-10 µm and 5-20 µm respectively (Figure 2 and Figure 4). The shapes of lignified parenchyma cells from trifoliate yam tissue at 28±2°C and 72–85% RH were thinly irregular oval shapes. Also, shapes of un lignified parenchyma cells from trifoliate yam tissue at 5±1°C and 85-100% RH were thinly regular oval shapes. Besides, some of the lignified parenchyma cells in both stored yam tissues were unevenly elongated (Figure 2 and Figure 4). Furthermore, lignify parenchyma tissues of the trifoliate yam tissues were observed in certain localized areas (tissues with deep red stains) in appropriately stored yams (Figure 2). This proves that those parenchyma tissues with deep red stains (tubers stored for 3 days) were highly lignified, suggesting that phenolics in such tissues are of lignin nature. This
observation confirms the finding that post-harvest hardening in trifoliate yam occurs within 2-3 days after harvest [6]. Also, the difference in colour reactions appeared to be associated with the ratio of monomeric units of lignin in the cell walls [5,9]. In addition, lignify cells of yam tissue were not only restricted to the secondary cell walls but included the middle lamella and primary cells (Figure 2). Despite the occurrence of lignified cells in trifoliate yam tubers under storage conditions, some of the parenchyma tissues were unlignified. Other studies have indicated in various morphological regions of *broussonetia papyrifera* that lignin content follows a decreasing order: CC (cell corner) > CML (compound middle lamella) > S2 (secondary wall) [14]. Once again, no significant difference (p≥0.05) in phenolic index was observed in white trifoliate yam tuber at 5±1˚C and 85-100% RH and those tubers immediately harvested (Figure 3). Phenolic index in white trifoliate yam tuber at 5±1˚C and 85-100% RH was less than in white trifoliate yam tuber at 28±2˚C and 72–85% RH (Figure 3). Also, phenolic index in white trifoliate yam tuber stored at 28±2˚C and 72–85% RH; and 5±1˚C and 85-100% RH for three (3) days was less than white trifoliate yam tuber stored at the same conditions (Figure 3). Yellow trifoliate tubers had more phenolic index than white trifoliate yam tubers just after harvest (Figure 3). This could also mean that not all phenolics in the yellow trifoliate yam tubers were of lignin nature. It was also evident that freshly harvested yellow trifoliate yam tuber has the same phenolic indices as white trifoliate yam tuber stored at room temperature for three (3) days (Figure 3). The phenolic indices in tissues of white and yellow trifoliate yam tubers showed that phenolics are not only intense in the cell walls alone but also the middle lamella (Figure 4). Also, lignified cell wall regions (deep blue stains) in trifoliate yams were more pronounced in freshly harvested yellow trifoliate yam tuber, white and yellow trifoliate yam tuber stored at 28±2˚C and 72–85% RH for three (3) days and trifoliate yam tubers (yellow) stored at 5±1˚C and 85-100% RH for three (3) days (Figure 4) respectively. This implies that phenolic compounds in tissues of trifoliate yam tubers appropriately stored for three (3) days are of lignin nature (lignin precursors). Interestingly, differences in reaction colour of the phenolic compounds and toluidine blue stain in micrograph of trifoliate yam tissues might indicate differences in phenolic compositions [11]. Moreover, the walls of the primary cell were mostly thin and smooth but some were irregularly thickened (Figure 4). In addition, the secondary cell wall in lignified trifoliate yam tubers were also irregularly thickened (Figure 4). With this information, future research should aim at using other advance techniques (Scanning Electron Microscopy, Confocal Raman Microscopy, Transmission Electron Microscopy and NMR Spectroscopy) to study greater particulars of lignify cells in trifoliate yam tubers under various stressors.

3.2. Histochemical Changes as Associated to Tissue Cohesion and Hardening in Trifoliate Yam Tuber at Storage

Once more, the study revealed that lignify cell of tissue of the yam tissues at day 3 of storage (room temperature) were found to be highly linked to tissue cohesion of a day 3 stored tuber. The relation was high in yellow trifoliate yam tissues at room temperature storage (Gradient of slope (11.941)). Again, the slope as observed in white trifoliate yam tissues at room temperature and refrigeration conditions had gradients of 8.802 and 4.941 respectively. These lignify cells were reported to stiffen and strengthen tissues [4] thus the basis of tissue cohesiveness (cohesion). This implies that yellow trifoliate yam tissues at post-harvest conditions (room temperature and refrigeration) had a higher tendency to lignify thus leading to higher tissue cohesion than in white trifoliate yam tissues. Similarly, high lignin depositions in juice sacs of citrus

![Figure 1](image-url)
fruits have been reported to reduce its edibility [4]. Furthermore, tissue cohesion in white and yellow trifoliate yam tubers were significantly high in room temperature stored (3 days) compared to freshly harvested tubers (Figure 5A and Figure 5B). Additionally, these dramatic increase in tissue cohesion in the white and yellow trifoliate tubers were found to be associated to sharp tissue hardening (Figure 5A and Figure 5B). The association was greater in yellow trifoliate tissue at room temperature condition than in the white cultivar (Gradients; 0.718, 0.687, R^2 0.375, 0.154) (Figure 7C & Figure 7A, Table 1). This explains role of lignify cells in tissue cohesion and hardening at post-harvest. As seen in Figure 6 A, no significant difference (p≥0.05) were found in tissue cohesion and hardening in refrigerated white trifoliate tuber. However, an inverse relation was found between tissue cohesion and hardening in refrigerated white trifoliate tuber (Gradients; -0.442, R^2 0.192) (Figure 7B, Table 1). This indicate that tissue cohesion in refrigerated white trifoliate tubers were not associated to sharp tissue hardening. Also, no significant differences (p≥0.05) were found in tissue cohesion in freshly harvested yellow trifoliate tuber and those refrigerated (3 days) (Figure 6B). But, a strong relation was found between tissue cohesion and hardening in refrigerated yellow trifoliate tuber (Gradients; 1.115, R^2 0.55) (Figure 7D, Table 1). This meant that tissue cohesion in refrigerated yellow trifoliate tubers were associated to dramatic tissue hardening.

**Figure 2.** Histochemical exhibit of stained tissue in cell wall region (arrow) of (A1-2) white trifoliate yam tuber after harvest (Day 0), (B1-2) white trifoliate yam tuber at refrigeration (Day 3), (C1-2) white trifoliate yam tuber at room temperature (Day 3), (D1-2) yellow trifoliate yam tuber after harvest (Day 0) (E) yellow trifoliate yam tuber at refrigeration (Day 3), (F) yellow trifoliate yam tuber at room temperature C (Day 3). Safranin-aniline blue stain (No evidence (no red stain) of lignified tissues and Evidence (red stain) of lignified tissues) **Scale bar:** 20µm
**Figure 3.** Phenolic index of white and yellow trifoliate cultivars after harvest and those stored for three days at room and refrigeration conditions

Key: **WRT**-White trifoliate cultivar stored at room temperature, **WRF**- White trifoliate cultivar stored at refrigeration temperature, **YRT**- Yellow trifoliate cultivar stored at room temperature, **YRF**- Yellow trifoliate cultivar stored at refrigeration temperature

**Figure 4.** Histochemical exhibit of stained tissue in cell wall region (arrow) of (G) white trifoliate yam tuber after harvest (Day 0), (H) white trifoliate yam tuber at refrigeration (Day 3), (I) white trifoliate yam tuber at room temperature (Day 3), (J) yellow trifoliate yam tuber after harvest (Day 0), (K) yellow trifoliate yam tuber at refrigeration (Day 3), (L) yellow trifoliate yam tuber at room temperature (Day 3)

(Toluidine blue stain showing lignified tissue) **Scale bar: 20µm**
Figure 5. Relating Changes in (A) Hardness of white trifoliate yam cultivars to tissue cohesion at room temperature (B) Hardness of yellow trifoliate yam cultivars to tissue cohesion at room temperature

Table 1. Regression coefficient of fitted line plot between tissue cohesiveness and log10 tissue hardness (N) of trifoliate yam tubers at storage

<table>
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<tr>
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<th>log10 HWRT</th>
<th>log10 HYRT</th>
<th>log10 HWRF</th>
<th>log10 HYRF</th>
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<td>Tissue cohesion</td>
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<td>0.7183</td>
<td>-0.442</td>
<td>1.115</td>
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<tr>
<td>Constant</td>
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<td>1.156</td>
<td>1.468</td>
<td>1.114</td>
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<td>$p$ (value)</td>
<td>0.608</td>
<td>0.387</td>
<td>0.561</td>
<td>0.261</td>
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</table>

HWRT - Tissue hardening of white trifoliate yam tuber at room temperature storage, HYRT - Tissue hardening of yellow trifoliate yam tuber at room temperature storage, HWRF - Tissue hardening of white trifoliate yam tuber at refrigeration condition and HYRF - Tissue hardening of yellow trifoliate yam tuber at refrigeration condition
In summary, lignify cells in most parenchyma tissues of stored white and yellow trifoliate yam tubers (28±2°C, 72-85%RH and 5±1°C, 85-100%RH) were characterized by a deep safranin red and toluidine blue stained cell wall areas. In addition, these lignified cells were evident of histochemical changes in parenchyma tissues as seen to be moderately in phase with the dramatic tissue cohesion and hardening in white and yellow trifoliate yam tubers at 28±2°C; 72–85% RH and in yellow trifoliate yam tuber at 5±1°C; 85-100% RH. This finding has given insight into the role of lignified tissue in the hardening of post-harvest D. dumetorum yam tubers.
Figure 7. Fitted line plot of tissue hardness (N) and tissue cohesion in (A) white trifoliate yam cultivars at room temperature storage (B) white trifoliate yam cultivars at refrigeration condition (C) yellow trifoliate yam cultivars at room temperature storage (D) yellow trifoliate yam cultivars at refrigeration condition.
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Conflict of Interest

The authors declare no conflict of interest.

References