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Lignin biosynthesis rate is responsible for varietal difference in fruit rind and seed coat hardness in the bottle gourd *Lagenaria siceraria* (Molina) Standley

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ABSTRACT

Previous investigations on bottle gourd [*Lagenaria siceraria* (Mol.) Standl.], were mainly focused on genetic resources characterisation and crop husbandry. Accessing the anatomical and chemical determinants of fruit and seed traits that directly influence the yield of *L. siceraria* is a prerequisite for full exploitation of its genetic resources for breeding purposes. This study aimed at gathering the anatomical and chemical bases of fruit rind and seed coat hardness in calabash and egusi varieties of bottle gourd. Variation between the two bottle gourd varieties was observed in fruit rind sclerified outer mesocarp and seed coat thickness. Lignified tissues were thicker in calabash than those of egusi in both fruit rind and seed coat. In addition, the extent of the sclerified outer mesocarp was greater in calabash (up to 503 μm thick) compared to egusi (up to 417 μm thick). In egusi seed coat, only the inner epidermis was lignified while in calabash, both mesophyll and inner epidermis were lignified. Klason lignin procedure confirmed histological analyses, showing a higher amount of lignin in calabash tissues compared to those of egusi, whatever fruit age considered. Fruit rind and seed coat were harder in calabash than in egusi due to the greater degree of lignification in calabash tissues compared to those of egusi.

1. Introduction

Food security, particularly in less developed countries, is one of the challenges faced by scientists and decision makers worldwide. This challenge is increasingly difficult in light of the effects of climate change, whose repercussions are increasingly felt in the agricultural sector (Howden et al., 2007; Gornall et al., 2010). In areas for which predictive models based on the management of cropping systems in relation to the climate change do not exist, dealing with plants that thrive in different agro-ecosystems is a good alternative. Bottle gourd, *Lagenaria siceraria* (Molina) Standley (Cucurbitaceae), which is grown in different agro-ecosystems, is a potentially useful plant model. It is a vigorous, annual, monoecious trailer or climber that is well adapted to various cropping systems. Bottle gourd *L. siceraria* has a wide range of uses and is largely cultivated in the tropics and subtropics (Harika et al., 2012), and landraces of the species various exhibit huge diversity, especially in fruit shape and size. Edibility in *L. siceraria* tends to be associated with variation in size and shape of fruits (Morimoto et al., 2005). Edible forms produce round or egg-shaped fruit, moderate in size, with a thin rind, and seeds rich in lipids and proteins (Loukou et al., 2011, 2013). Non-edible forms are characterised by dipper, club or elongated cylindrical fruit, sometimes bearing handles (Morimoto et al., 2005). The hard rind of the mature fruits allows them to be used to make various types of musical instruments, jugs, domestic utensils for storage of liquid and food materials, and floats for fishing nets (Harika et al., 2012). From a practical point of view, two varieties of *L. siceraria* are distinguished on the basis of their use. One, the so-called calabash, is mainly used as a tool or utensil due to the hardness of its fruit rind. This variety produces mostly inedible seed. The second variety, the so-called egusi in several Western and Central African countries, produces oleaginous seeds that are used in various forms as part of human diet (Zoro et al., 2003, 2006; Achigan-Dako et al., 2008a).

The adaptability of *L. siceraria* to various ecological zones (both temperate and tropical), its wide range of utilisations, and its high phenotypic plasticity make it a likely species to sustain a minimal yield in conditions of variable climate (Cordell et al., 2007; Chimonyo and Modi, 2013; Sithole and Modi, 2015). Due to these attributes, *L. siceraria* has received growing interest from governments, plant genetic resources institutions and researchers over the past decade (Decker-Walters et al., 2001; Dubey and Ram, 2007; Achigan-Dako et al., 2008*b*; Loukou et al., 2011; Xu et al., 2011; Shaikh et al., 2012;

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Chimonyo and Modi, 2013; Yao et al., 2015; Mashilo et al., 2017). The first investigations carried out to promote production of *L. siceraria* were mainly limited to genetic resource characterisation and crop husbandry (Morimoto et al., 2005; Singh et al., 2006; Shaikh et al., 2011; Bhawna et al., 2014; Xu et al., 2014). Now, it is urgent to create high yielding varieties of the bottle gourd to reinforce the nutritional status and economic prosperity of the millions of people growing this plant throughout temperate and tropical rural zones. Accessing the anatomical and chemical determinants of fruit and seed traits that directly influence the yield of *L. siceraria* (Yao et al., 2015) is a prerequisite for full exploitation of the bottle gourd genetic resources for breeding purposes. Data from such a study will be also helpful for ongoing investigations aimed at refining the taxonomic and evolutionary status of the bottle gourd (Achigan-Dako et al., 2008b; Ali and Al-Hemaid, 2011; N'dri et al., 2016). We present results of investigations of the anatomical and biochemical bases of fruit rind and seed coat hardness in bottle gourd, with special reference to fruit maturity.

2. Materials and methods

2.1. Plant material

Two varieties selected from the *L. siceraria* germoplasm collection of Nangui Abrogoua University (Abidjan, Côte d'Ivoire) were used for this study: the oilseed, or so-called egusi (with soft fruit rind), and the calabash (non-edible with hard fruit rind mainly used as instrument). These plant materials were grown at the experimental station of University of Nangui Abrogoua from March to July 2014. In this zone, rainfall is abundant (annual mean > 2000 mm) and the mean temperature is 28 °C, with annual amplitude of 5–10 °C. Flowers were hand pollinated at anthesis. Fruits of different ages (one, two, three, four, five and six weeks after fruit set: WAFS) were harvested from both varieties for histological observations and Klason lignin analysis. Four fruits of each age were used for analyses. All samples used for this study were kept at 4 °C until analyses.

2.2. Microscopy

The rind and seed coat of selected fruits were cut into small blocks (3 × 3 × 3 mm) and fixed for 72 h in a mixture of 10% (v/v) glutaraldehyde in 1% (w/v) NaH_2PO_4 and 8% (w/v) paraformaldehyde in 2% (w/v) Na_2HPO_4. The pH was adjusted to 6.8. During fixation, the tissue samples were vacuum infiltrated for 2 h. Fixed tissues were dehydrated using a graded ethanol series of 30, 50, 70, 90, 95 and 100% (v/v) and embedded in pure Technovit 7100 resin (HeraeusKulzer GmbH, Wehrheim, Germany). Sections of 3 μm were cut using a rotary microtome (Microm HM 360, Thermo Scientific, Dreieich, Germany), collected on glass slides and incubated on hotplates (40 °C) for 12 h. Sections were stained with 0.05% (w/v) toluidine blue solution (O'brien et al., 1964). With toluidine blue staining, the lignified cells stain blue-green and cellulose is coloured reddish purple (O'brien et al., 1964).

Micrographs were taken with a Leitz DMBR (Leica, Wetzlar, Germany) equipped with an Evolution MP camera (Media Cybernetics, Bethesda, USA) and Q-Capture Pro acquisition software (version 7, Media Cybernetics Inc., Maryland, USA). Image processing was done with Adobe Photoshop (Adobe Systems Inc., USA).

2.3. Lignin analysis

Extractives and lignin content were determined according to the US Department of Energy, National Renewable Energy Laboratory analytical procedure (Sluiter et al., 2008, 2010). Analyses were carried out on duplicate samples of rind and seed coat. Fruit rind (3 mm depth) and seed coat of each stage from egusi and calabash were oven dried and milled to a fine powder. Extractives (non-structural compounds of the plant material) may interfere in the analysis of lignin, and their removal prior to lignin analysis improves precision of those methods. Accordingly, the powder (2 g) of each sample was submitted to successive soxhlet-extraction with water and acetone for 8 h, respectively to remove extractives components and to minimise the formation of pseudo lignin during the analyses. The solvent was removed from extractives using a rotary evaporator under reduced pressure and the extractives content was evaluated gravimetrically in relation to the samples' dry weight. Lignin content was assayed by Klason procedure involving 100 mg of air-dried lignocellulosic materiel treated with 72% (v/v) H_2SO_4 and then vacuum filtrated to collect the insoluble lignin residue in a washed pre-weight sintered crucible. The dry crucibles were weighed to determine Klason (acid-insoluble lignin) lignin gravimetrically. Acid-soluble lignin was measured spectrophotometrically by reading the UV absorbance at 205 nm in the filtrate (Sluiter et al., 2008).

3. Results

3.1. Anatomy

Transverse sections of fruit rind in both calabash and egusi revealed an epicarp covering a sclerified outer mesocarp and a parenchymatous middle (and inner) mesocarp containing vascular bundles. At 1 WAFS there were no visible differences between rind of calabash and egusi (Supplementary data Fig. S1A, D). From 2 WAFS, visible differences in lignification emerge between calabash and egusi. In both varieties the pericarp cells begin lignification, giving rise to a sclerified pericarp (Fig. 1A, D). However, the extent of lignification is greater in calabash (153 μm thick) than in egusi (67 μm thick) (Fig. 1A, D). In addition, variation between the two gourd varieties was seen in the extent of lignification, with lignified cells being more extensive (up to 503 μm 6 WAFS) in calabash compared to egusi (up to 417 μm thick 6 WAFS) (Fig. 1A, F). These observations were consistent at fruit of all ages (Fig. 1A to F and Supplementary data Fig. S1A to F). However, it appeared that the extent of lignification of parenchyma cells of the middle mesocarp declined with the fruit age. Thus for example, in calabash, the lignification increase declined from 173 μm (from the third to the fourth week) to 20 μm (from the fifth to the sixth week). In egusi this extent decreased from 44 μm to 3 μm during the same period (Fig. 1A to F and Supplementary data Fig. S1A to F).

Transverse sections of seed coat in calabash and egusi revealed the same anatomy, the inner and outer integument divisible into five layers as follows: a three-layered outer integument comprising the outer epidermis, mesophyll, and inner epidermis, and a two-layered inner integument with an outer parenchyma layer and an inner collenchyma layer. At 1 WAFS, there was no visible difference between the seed coat of both varieties of bottle gourd, and all cell layers stained reddish purple indicating an absence of lignin (Supplementary data Fig. S2A, D). From 2 WAFS, cells of inner epidermis in both varieties stained blue-green, attesting their lignification. In addition, in calabash but not egusi some cells of the mesophyll began lignification (Fig. 2A, D). From the fourth week, all the cells of mesophyll were lignified in calabash while in egusi, none of them was lignified (Fig. 2B, E). Also in egusi, the inner epidermis was two cell layers thick while in calabash it was three cell layers thick (Fig. 2C, F). Until the sixth week the lignified cell walls thickened progressively. At maturity, in egusi only the cells of the inner epidermis layer were lignified while in calabash, the cells of both the inner epidermis and mesophyll were lignified (Fig. 2B, C, E and F and Supplementary data Fig. S2B, C, E and F).

3.2. Histochemistry

The amount of lignin in the fruit rind increased with age in both egusi and calabash tissues (Fig. 3A). The amount of lignin in fruit rind after one week was 25.89% and 22.08% in calabash and egusi, respectively, and by six weeks this had increased to 78.67% and 75.85% in