The relationship between graft incompatibility and phenols in *Uapaca kirkiana* Müll Arg.

Simon A. Mng’omba a,b,*, Elsa S. du Toit a, Festus K. Akinnifesi b

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**Abstract**

The objective of the study was to determine the relationship between phenols and graft incompatibility in *Uapaca kirkiana*. Phenol quantification and identification were carried out using Folin-Ciocalteau reagent procedure, fluorescence microscopy and reverse phase high performance liquid chromatograph (RP-HPLC) above, below and at the graft union. Results showed no vascular cambium continuity above the scion/stock unions. Significant differences in total soluble phenols and cell wall bound phenols were obtained. Fluorescence microscope indicated the presence of flavonoids and other polymers above the union. The RP-HPLC identified ferulic acid as a major phenol component found in *Uapaca kirkiana* plant cells and responsible for wood discoulouration. High phenol concentrations were obtained in less compatible combinations than in compatible combinations. High peaks of $\beta$-coumaric acid were obtained above the union. It is concluded that phenols, especially $\beta$-coumaric acids and flavonoids caused poor callus formation at the union, and hence implicated in graft incompatibility.

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**1. Introduction**

*Uapaca kirkiana* Müll Arg. (wild loquat), locally known in Malawi as ‘masuku’, is a highly valued indigenous fruit tree of the Miombo woodlands. The fruit is the most preferred by communities in southern Africa (Maghembe et al., 1998) and can be eaten fresh or processed into juice, jam and a variety of wines. However, all the harvest comes from wild populations and there is little effort to renew these vital forest resources.

The propagation of *U. kirkiana* has relied on conventional techniques which include the use of seeds, grafting and air-layering (Akinnifesi et al., 2004). Grafted *U. kirkiana* trees have fruited after 2–3 years compared to those that are raised from juvenile materials (seedlings) which have fruited after 10–12 years (Akinnifesi et al., 2006). Furthermore, grafted trees are dwarf, and hence easier and cheaper to manage than large trees (Usenik and Štampar, 2001; Webster, 2001). About 80% graft-take has been achieved with skilled grafters at Makoka Agricultural Research Station in Malawi (Akinnifesi et al., 2004). However, growth irregularities for the grafted *U. kirkiana* trees have been observed in the field and these growth irregularities could be signs of graft incompatibility (Mng’omba et al., 2007). The recent assessment of grafted *U. kirkiana* trees has shown declining survival trends from 98% at 12 months after field establishment to 67% at 33 months after field establishment (Akinnifesi et al., 2008). This low field survival could be attributed to graft incompatibility which is characterised by visibly poor callus formation, phenol accumulation and necrotic layer at the union (Mng’omba et al., 2007). These signs are prevalent above the union, especially for less compatible scion/stock combinations.

*U. kirkiana* plants release a lot of exudates (phenols) in response to wounding. According to Hamisy (2004), DNA extraction from *U. kirkiana* leaves was adversely affected due to high production of phenols. Also, fruit darkening after harvest *U. kirkiana* fruits has been associated with high production of phenols. Histological study on grafted *U. kirkiana* trees revealed accumulation of deposits (phenols) at the union leading to cell or tissue death and lacuna layers above the union (Mng’omba et al., 2007). Phenols have been implicated in graft incompatibility (Pina and Errea, 2005) and differences in quantity or specific phenols above and below the union area play a role in reducing graft compatibility (Facteau et al., 1996; Usenik et al., 2006).

Graft incompatibility is complex and involves many physiological and biochemical processes (Usenik and Štampar, 2000), but the main cause largely depends on the plant species (Andrews and Marquez, 1993). Although *U. kirkiana* plants produce a lot of...
phenols in response to wounding, there has been no study to establish the relationship between the visible graft incompatibility and high phenol production. We hypothesise that plants that accumulate a lot of phenols in response to wounding have graft incompatibility problem and that specific phenols could cause graft incompatibility. The objective of this type of work was to determine the relationship between phenol production and graft incompatibility in *U. kirkiana*.

2. Materials and methods

2.1. Plant material

Three-year-old grafted *U. kirkiana* trees were collected at ICRAF nursery located at Makoka Agricultural Research Station in Malawi in February 2006. This site lies at 1029 m above sea level, 15°30’S and 35°15’E. It has an annual rainfall of 560–1600 mm (Akinnifesi et al., 2004). All the rootstocks were from seedlings and they were at least 1-year old before splice grafting. Both homografts (scion and stock combinations from the same mother trees within a population) and heterografts (graft partners from different mother trees and from different tree populations) were collected at ICRAF nursery and transported within 2 days to the University of Pretoria in South Africa for phenol quantification and identification.

2.2. Phenol extraction

*U. kirkiana* homografts namely, MW26/26, MW12/12, MW13/13, MW76/76 and MW32/32, and also heterografts namely, MW84/57, MW56/49, MW32/28, MW80/82 and MW7/10 were collected. At least three samples per scion/stock combination were collected, freeze-dried (Edwards, Modulyo, Pirani 10) and dissected into three segments (above, below and the union). The bark samples comprising the phloem and the vascular cambium were ground using a mortar and pestle (Willey mill, 20 mesh). The fine powder (0.05 g) was placed in Eppendorf tubes for phenol extraction. Samples were extracted with 1 ml of methanol–acetone–water solution (7:7:1 v/v/v) three times and then evaporated. The mixture was made up to 1 ml with deionised water and mixed in ultrasonic cleaner VWR (USC900TH, VWR International bvba/spri, B-3001 Leuven) (4 min). Samples were stored at 4°C and then centrifuged for 4 min using a bench centrifuge (Combi-spin, type: FVL-2400N, Rochelle Chemicals & lab equipment, Germany). The supernatants were mixed and 1 ml was collected for total soluble phenol quantification.

2.3. Quantification of total soluble phenols

Folin-Ciocalteau reagent (Sigma) based on reduction of phosphomolybdene/phospho-tungstate (De Ascensao and Dubery, 2003) was used for quantification. Deionised water (175 μl) was dispensed in ELISA plate wells. The sample extract (5 μl), Folin-Ciocalteau reagent (25 μl) and then sodium carbonate (50 μl of 20%, w/v) were added. A blank in which water replaced the sample was used as a control. Four wells were used per sample and incubated at 40°C for 30 min. Absorbance was read using Multiskan Ascent spectroscopy (V1.24 345-00007T) set at 690 nm. Gallic acid was used as a phenol standard to construct a standard curve passing through the origin \(y = 1.3527x – 0.0109\). Total soluble phenol concentration was expressed as mg gallic acid equivalent per g dry weight of sample. The experiment was laid out as a randomised block design and the four wells formed the replicates.

2.4. Fluorescence microscopy

Three *U. kirkiana* scion/stock combinations namely, MW12/12 (incompatible), MW26/26 (partially compatible) and MW7/10 (intermediate compatibility) were selected based on their contrasting relative compatibility as illustrated in Fig. 1. Incompatible partners had no visible continuity in the bark and wood above and below the union (Fig. 1A), while partially compatible partners showed at least continuity in the bark below the union (Fig. 1B). At least three samples per graft combination were collected and immersed in formalin acetic acid (5% formalin, 5% acetic acid and 90% ethanol). Thin (ca. 10 μm) layer sections were dissected at a right angle to the union using a sliding microtome (Leitz Wetzlar, 17815). Sections were viewed under a Zeiss Axiosvert 200 (Zeiss, Göttingen, Germany) microscope fitted with a sensys camera. Images of emission 397 nm, 515 nm and 565 nm were captured with Nikon ACT-1 version 2. White or blue light was used before and after staining the samples with Vanillin-HCl (1% of concentrated HCl) purchased from Sigma Aldrich Chemie (Steinheim, Germany).

2.5. RP-HPLC phenol identification

Cell wall bound phenols were extracted from MW12/12, MW26/26 and MW7/10 *U. kirkiana* graft combinations according to De Ascensao and Dubery (2003) methodology. Samples were obtained after extraction of total soluble phenols. Precipitates (0.01 g) obtained were dried and then 0.5 M NaOH was added. The

Fig. 1. Longitudinal sections and external view of the graft unions of *Uapaca kirkiana* showing (A) incompatible combination with poor callus formation at the union and (B) partially compatible combination (arrows show necrotic tissues and differences in callus proliferation for the external view).
mixture was suspended in a water bath (Julabo V, Labotec Pty Ltd., model 101) for 1 h at 96 °C. The supernatant was acidified with HCl to pH 2.6, centrifuged for 10 min and extracted with 1 ml anhydrous diethyl ether. The mixture was dried and the precipitates were suspended in 200 µl 80% aqueous MeOH. Folin-Ciocalteau reagent was used to determine the total cell wall-esterified phenolic acids. The remaining solution was separated using a reverse phase high performance liquid chromatograph (RP-HPLC).

The HPLC (Hewlett Packard Agilent 1100 Series) was equipped with 20 µl loop injection value, DAD detection (diode array detector, 280, 325, 340 nm) and Luna 3u C-18 (Phenomenex®) reverse phase column (150 mm by 2 mm, Ref. 550). It was also fitted with solvent degasser (G1322A). A gradient elution was performed with HPLC water acidified with orthophosphoric acid (pH 2.6) and acetonitrile (ACN) as follows: 0 min, 7% ACN; 0–20 min, 20% ACN; 20–28 min, 23% ACN; 28–40 min, 27% ACN; 40–45 min, 29% ACN; 45–47 min, 33% ACN. The flow rate was 0.6 ml min⁻¹. The column was stabilized and 40 µl of each sample was injected through a 20 µl loop. The UV detector was set at 280 nm and phenol identification was carried out by comparing retention time and UV apex spectrum to those of authentic standards purchased from Sigma Chemical Company, USA.

2.6. Statistical analysis

Data collected were subjected to analysis of variance (ANOVA) using GenStat release 4.2DE, 2005 (Rothamsted Experimental Station) and LSD (P ≤ 0.05) test was used for mean separation.

3. Results

3.1. Total soluble phenols

Significant differences (P < 0.05) were obtained in total soluble phenol concentration above, below and at the union (Table 1). For the homografts, 80% of the combinations showed no significant differences in total soluble phenol above and below the union. Sixty percent of the heterografts also showed no significant differences in total soluble phenol concentration above and below the union. MW26/26 had significantly higher total soluble phenol concentration below than above the union. MW12/12 showed no differences in phenol concentration above and below the union (Table 1). For heterografts, MW7/10 showed no significant differences in total soluble phenol concentration below, above and at the union.

Table 1

<table>
<thead>
<tr>
<th>Graft combination</th>
<th>Gallic acid equivalent of dry weight (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Above the union</td>
</tr>
<tr>
<td>Homografts</td>
<td></td>
</tr>
<tr>
<td>MW26/26</td>
<td>97.0b</td>
</tr>
<tr>
<td>MW12/12</td>
<td>79.7b</td>
</tr>
<tr>
<td>MW13/13</td>
<td>71.4a</td>
</tr>
<tr>
<td>MW76/76</td>
<td>110.0a</td>
</tr>
<tr>
<td>MW32/32</td>
<td>79.2a</td>
</tr>
<tr>
<td>Heterografts</td>
<td></td>
</tr>
<tr>
<td>MW84/57</td>
<td>70.8ab</td>
</tr>
<tr>
<td>MW56/49</td>
<td>72.5b</td>
</tr>
<tr>
<td>MW32/28</td>
<td>62.7b</td>
</tr>
<tr>
<td>MW80/82</td>
<td>59.9b</td>
</tr>
<tr>
<td>MW7/10</td>
<td>94.0a</td>
</tr>
</tbody>
</table>

Means with the same letters within a row are not significantly different at P ≤ 0.05.

3.2. Cell wall bound phenols

No significant differences (P < 0.05) were obtained in cell wall bound phenol concentration above and below the union for MW26/26 (Fig. 2). A similar trend was also obtained for MW7/10 combination. MW12/12 showed significantly higher cell wall bound phenol concentration below than above the union (Fig. 2). MW12/12 had higher cell wall bound phenol concentration at the union interface than MW26/26 and MW7/10 combinations. Furthermore, MW26/26 and MW12/12 combinations accumulated significant amounts of cell wall bound phenols at the union compared to above the union.

3.3. Fluorescence microscopy

The darkened and blackened parts under UV light showed cell death due to crushed cells during grafting (Fig. 3). Cell death was pronounced above the union in all the scion/stock combinations (Figs. 3 and 4). Generally, there were no differences in colour staining above the union for MW26/26 and MW12/12 (Fig. 3). Brown staining was obtained under UV light, while green staining was obtained under white light (WL) and these indicate that light was passing through the holes (lacuna) without any refraction (Fig. 3). MW12/12 shows a wider area of necrotic layer indicated as green staining under WL above the union. Differences in colour staining were obtained below the union (Fig. 4). There was purple staining below the union for MW12/12 under WL (Fig. 4). There were also small necrotic (lacuna) layers below the union.

3.4. RP-HPLC phenol analysis

RP-HPLC indicated that ferulic and p-coumaric acids were prominent in the samples (Figs. 5–7). Ferulic acid had high peaks (directly related to high concentrations) in all the chromatograms. There were high p-coumaric acid peaks for MW12/12 (Fig. 5A), but low peaks for MW26/26 (Fig. 5B). A similar trend is observed below the union for MW12/12 and MW26/26 (Fig. 6). The unknown phenol with 44 min retention time (phenol 44) was obtained from MW12/12 and MW26/26 (Figs. 5 and 6), but phenol 44 was not present in MW7/10. Another unknown phenol eluted at 47 min (phenol 47). A general trend shows that the unknown phenols had higher concentrations below the union than above the union. Both ferulic and p-coumaric acids show higher peaks in MW12/12 than in MW26/26 combinations. Apart from these two phenolic compounds, there was also 3,4-dihydroxybenzoic acid present.
below the union for MW12/12 (Fig. 6A) and above the union for MW7/10 (Fig. 7B).

4. Discussion

The results from this study show that the presence of phenols could disrupt chemical reactions, callus formation and differentiation of vascular tissues (Usenik and Štampar, 2000). Phenols can adversely affect movement of cytokinins and auxins which are important in cell differentiation and growth. Cytokinins and indole-3-acetic acid (IAA) are known to induce differentiation of sieve tubes and vessels (Usenik and Štampar, 2000). Furthermore, phenolic acids inhibit plant growth and increase oxidative decarboxylation of IAA.

In this study, the homografts showed no significant differences in total soluble phenol concentration between above and below the union except for MW26/26. Gebhardt and Feucht (1982), and Usenik and Štampar (2001) reported similar content of phenols between above and below the union for homografts. Morphological observations showed that MW12/12 with low total soluble phenol content was incompatible, while MW26/26 with high total soluble phenol content was partially compatible.

Fig. 3. U. kirkiana sections above the graft union of MW12/12 (incompatible) and MW26/26 (partially compatible) combinations viewed under fluorescence microscope showing similar phenol staining.

Fig. 4. U. kirkiana sections below the graft union of MW12/12 (incompatible) and MW26/26 (partially compatible) combinations viewed under a fluorescence microscope showing differences in phenol colour staining.
Continuity in the bark and wood below the union for MW26/26 enables cell metabolism and cell sap movement to take place. This could result in some phenols being deposited around the union interface as a result of cell metabolism, and this could also be due to cell reaction to an infection. However, this is unlikely for MW12/12 because many cells above and below the union were already dead.

Concentration of total soluble phenols above and below the union could be influenced by other factors. For example, some phenols could be polymerised and deposited into cell walls, and hence there could be a change in concentration and form. The cell wall bound phenols might play an important role in graft incompatibility. In this study, there were more cell wall bound phenols in less compatible combination (MW12/12) than in partially compatible combination (MW26/26). Furthermore, *U. kirkiana* samples were 3 years old after grafting, and hence wounding was over. Therefore, further release of phenols onto the union interface could be related to graft incompatibility or other factors rather than wounding response.

Plants are able to modify phenols deposited at the wounds by converting them into different forms. This has been demonstrated through shikimate pathway (Harborne, 1989). In contrast to the total soluble phenol, MW12/12 had higher cell wall bound phenol concentration than MW26/26 combination. De Ascensao and Dubery (2003) reported an increase in cell wall bound phenols at the wounds. Since phenols are known to adversely affect cell differentiation and proliferation at the graft union, they could be implicated in graft incompatibility.

Isolated cambial continuity in the bark could support some tree growth in the incompatible pairings up to a certain stage (Mng’omba et al., 2007). However, poor continuity in wood could adversely affect tree survival as the trees mature and begin to fruit. In this study, incompatible homografts could be attributed to differences in rootstocks. *U. kirkiana* is a dioecious tree and there is a possibility of different pollinators from different male trees onto flowers of a single female tree. This means that not all the rootstocks grown from seeds collected from a single clonal mother tree will be the same. Therefore, rootstocks could influence great differences in phenol content between scion/stock combinations. Usenik and Štampar (2000) also reported that rootstocks influence different concentration of $\rho$-coumaric acid both below and above the graft union.

Continuity in the bark was observed below the union for partially compatible (MW26/26) combination. This is despite a high total soluble phenol content below the union compared to MW12/12. Specific phenols could be responsible for poor callus formation, proliferation and cell differentiation to ensure vascular continuity. The presence of high $\rho$-coumaric acid peaks or concentration for MW12/12 could be responsible for the absence of vascular continuity even below the union.

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**Fig. 5.** Chromatograms of cell wall bound phenols extracted from above the graft union of *U. kirkiana* (A) MW12/12 and (B) MW26/26.

**Fig. 6.** Chromatograms of cell wall bound phenols from below the graft union of *U. kirkiana* (A) MW12/12 and (B) MW26/26.
compatibility is not clear. The predominance of ferulic acids in all combinations. However, the influence of this acid on graft possibly inhibited.


Absence of differences in colour staining above the unions for MW26/26 and MW12/12 could be attributed to the lack of continuity in the bark and wood above the union. Continuity in wood and bark was only observed below the union for MW26/26. Flavonoid shown as purple staining (Dixon and Paiva, 1995) or anthocyanin (Kangatharalingam et al., 2002) was prominent. Flavonoids have been implicated in graft incompatibility in apricot (Errea et al., 1994a,b; Andrews and Marquez, 1993). From the present results, flavonoids could also be implicated in U. kirkiana graft incompatibility. According to Errea et al. (1994b), high flavonoid content was found in the phloem of apricot as a response to graft incompatibility. Furthermore, phenols might be oxidized to quinones which are also toxic to plant cells due to chemical reaction disruption (Errea, 1998).

In this study, unknown phenol with retention time 44 min (phenol 44) and another with retention time 47 min (phenol 47) appeared only in half of the compatible graft combinations, and hence they are likely to have less significant influence on graft incompatibility. The chromatograms show that p-coumaric acid had higher peaks for the incompatible combination (MW12/12). DeCooman et al. (1996) reported p-coumaric acid accumulation in less compatible Eucalyptus gunnii. Usenik et al. (2006) reported that p-coumaric acid concentration was high above the union of heterograft in apricot and sweet cherry. Our results agree with their findings. According to Méndez et al. (1986), p-coumaric acids strongly hinder cell elongation. Ramina and Masia (1982) reported that p-coumaric acids, isolated from peach fruits, were inhibitory to cell elongation and cress seed germination. Furthermore, cell wall bound p-coumaric acids were related to pit hardening in peach fruits. In this study, the presence of p-coumaric acids at the union interface could have adversely affected the graft compatibility of U. kirkiana since cell elongation and plasticity were possibly inhibited.

There was 3,4-dihydroxybenzoic acid in MW12/12 and MW7/10 combinations. However, the influence of this acid on graft compatibility is not clear. The predominance of ferulic acids in all the samples provides evidence that ferulic acids were bound to the cell walls. Ferulic and p-coumaric acids have been cited as IAA antagonists (Usenik and Štampar, 2000). Furthermore, they reported that other compounds such as prunin and genisten could be synthesised from p-coumaric acids under stress conditions. This indicates that the activities of phenols and their different forms could be influenced by other factors. This makes graft incompatibility complex, and hence the use of different methods to capture different forms of phenols is warranted in relating graft compatibility to phenol amount. The present study has shown that high phenol production in trees has a negative effect on graft compatibility.

5. Conclusion

Graft incompatibility in U. kirkiana is attributed to high ferulic and p-coumaric acid concentrations, flavonoids, anthocyanins or their derivates at the union interface. Phenols inhibit callus proliferation and disrupt cell chemical reactions or functions. There was no scion/stock combination with complete graft union in both homograft and heterograft combinations of U. kirkiana. It is concluded that phenols such as p-coumaric acid, flavonoid and anthocyanin are implicated in graft incompatibility.

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References


Fig. 7. Chromatograms of cell wall bound phenols extracted from the bark of MW7/10 U. kirkiana combination (A) above the union and (B) below the graft union.


