CHAPTER 3

HISTOLOGICAL EVALUATION OF EARLY GRAFT COMPATIBILITY OF SCION/STOCK COMBINATIONS IN *UAPACA KIRKIANA* Müell Arg. TREE PROVENANCES

3.1 Abstract

Graft compatibility is important for a stable orchard, and hence a trial was carried out with the objectives of determining graft compatibility and possible causes of scion/stock incompatibility in *U. kirkiana* fruit trees. Field observations of old grafted trees (at least five-years old) and a histological study using one-year old grafts (young grafts) were carried out. Stem diameters at different positions were measured and thin layers of graft union sections were examined under a light microscope. Results indicated considerable growth irregularities, which included overgrowth of stocks, constricted unions and cracks in the bark across the union. Anatomical studies showed phenol compound accumulation and lack of vascular tissue continuity at the scion side of the union. Continuity in wood and bark tissues was evident below the union of partially compatible partners while isolated parenchymatous tissues at the union might be present at the incompatible partners. Callus tissues could be breaking up phenols from the lower side of the union. Necrotic tissues and lacuna areas were present above the union. Accumulation of phenols, poor amounts of callus formation at the graft union and possibly other factors contributed to graft incompatibility in grafted *U. kirkiana* trees.

3.2 Introduction

U. kirkiana trees show wide genetic diversity and variations in geographical adaptation (Akinnifesi *et al*., 2004). Consequently, a participatory clonal selection of *U. kirkiana* from the wild has been initiated in the region to identify superior cultivars for multiplication and wider cultivation (Akinnifesi *et al*., 2006). According to Maghembe *et al*. (1998) and Akinnifesi *et al*. (2004), tree dwarfing and precocious fruiting are desirable characters that potential growers of *U. kirkiana* trees would like to see being addressed in domestication and improvement initiatives spearheaded by the World Agroforestry Centre in southern Africa.

Fruit tree grafting is important to achieve horticultural benefits which include early fruit bearing (precocity), tree dwarfness and improved fruit traits. The early bearing character is particularly important for *U. kirkiana* which is known to have a long juvenile phase when propagated sexually. It is estimated that *Uapaca* require 10-12 years before first fruiting in the wild when grown from seeds (Akinnifesi *et al*., 2004). Different vegetative propagation methods such as air-layering, budding, rooting stem cuttings and grafting have been evaluated at SADC-ICRAF Makoka Station. Budding and rooting stem cuttings have yielded poor results, while air-layering showed some promise, but root development after tree establishment was problematic (Mhango, Akinnifesi & Chilanga, 2000). Grafting using the splice method has been the most promising propagation method for *U. kirkiana* trees (Akinnifesi *et al*., 2004, 2006). It is a fact that rootstocks often impart desirable traits on the scions of different fruit crops, such as dwarfing due to reduced growth rate, improved

fruiting (e.g. fruit sweetness, size, load and colour) and fruiting precocity when they are available (Ferree & Carlson, 1987; Webster, 2001).

Improved graft take (80%) has been achieved in *U. kirkiana* trees by using skilled grafters (Akinnifesi, *et al*., 2004). However, growth irregularities, possibly due to graft incompatibility, have been observed in some grafted trees in the nursery and the field, sometimes with suspected early or late rejection. Graft incompatibility occurring some years after grafting in normal growing trees (Errea, 1998) is a major concern in many grafted trees. Therefore, early evaluation of scion and stock combinations is important for successful orchard establishment. However, there is no known scientific research devoted to graft incompatibility in *U. kirkiana* trees to date. Therefore, evaluating compatibility of scion and stock combinations at an early stage would ensure stable grafted *U. kirkiana* tree production in clonal fruit orchards. Early diagnosis of phenols accumulating at the graft union is important since they adversely affect cell division and new cambium differentiation (Errea, 1998). The main objectives of this study were to determine the graft compatibility of different scion and stock combinations and the possible causes of scion and stock incompatibility in *U. kirkiana* trees.

3.3 Materials and methods

3.3.1 Field study

The field study was carried out at SADC-ICRAF Makoka Research Station in Malawi (refer to section 2.3.2 for site description). Two *U. kirkiana* orchards, one three years or more after grafting and another one-year old orchard (one year after grafting) were used for

the study. With the three-year old orchard (hereafter referred to as 'old graft'), visual observations were carried out and photographs were taken. The stem diameters of scions, stocks and graft unions were measured from the younger trees (one-year old after grafting, hereafter referred to as 'young graft') using a pair of callipers (model: Mitutoyo, OE7343). Bark thickness for both scions and stocks was also measured.

3.3.2 Histological and anatomical studies

Ten young grafts of *U. kirkiana* trees, grafted by the splice method, were randomly selected at Makoka nursery and the identity, sources (locations) and codes are shown in Table 3.1. Samples (at least three trees per graft combination) were collected by cutting approximately 4-5 cm below and above the scion/stock graft union. These stem sections were immediately immersed in formalin acetic acid (FAA; 5% formalin, 5% acetic acid and 90% ethanol) and later rinsed in sterile water to remove the acid. The samples were then mounted on a slide microtome stage (model: E-Leitz Wetzlar, 17815) using high pressure freezing carbon dioxide gas. Several thin layer transverse sections were cut at a right angle to the graft union and the thin layer tissues (approximately 10µm) were then mounted on microscope slides. Specimens were viewed under a light microscope (Olympus microscope Model: ach 1x, SZX7) connected to a digital camera and microphotographs of union interfaces were taken using low power magnification.

Visual scoring for graft compatibility included a visible union line in the bark and wood (scale of 1 to 4: 1 = visible, 2 = faint, 3 = very faint, 4 = absent); browning intensity of deposits at the union interfaces (scale of 1 to 4: 1 = visibly high, 2 = medium, 3 = low, 4 = visibly absent) and amount of callus proliferation (scale of 1 to 4: 1 = high, 2 = medium, 3

= low, 4 = absent). Visual scores were converted to percentages (0 - 25% = absent, 26 - 50% = very faint/low, 51 - 75% = medium/faint, 76 -100% = high). There were three samples scored per graft combination.

3.3.3 Statistical analyses

Data on diameters of scions, stocks and graft unions, and bark thickness of scions and stocks were arranged in a completely randomised design before subjecting the data to analysis of variance (ANOVA). Data on visual scores for the graft unions were analysed using correspondence analysis (GenStat 4.24DE, Rothamsted Experimental Station). Variables (union line, callus proliferation and deposit intensity) were used to discriminate the compatible from incompatible combinations and to show the distribution of these grafted trees (Lebart, Morineau & Warwick, 1984).

3.4. Results and discussion

3.4.1 Field study

There was a wide range of growth disorders at the graft unions in the old grafts of *U. kirkiana* trees (Figure 3.1A-B). Stem diameters of the graft unions were visibly greater than either the scions or stocks. This could be attributed to accumulation of necrotic tissues or deposits or metabolites (presumably phenols and carbohydrates) as there might be partial cambial continuity at the union. In some trees, there were grooves at the union area. Morphologically, a graft combination of MW26/22 (Figure 3.1A) showed good rate of callusing and healing at the graft union, and hence this combination shows good compatibility at the union. MW1/61 (Figure 3.1B) graft combination showed growth

irregularities (swellings and cracking of the bark) at the unions. Morphologically, these irregularities are indicative of incompatibility. Generally, old *U. kirkiana* trees have cracks in bark running almost vertical to the tree axis and this is attributed to genotypic traits. However, horizontal cracks across the union were visible and could be implicated in graft incompatibility.

Figure 3.2 shows contrasting growth irregularities at the unions of the old grafted *U. kirkiana* trees and these could be indicators of graft incompatibility. Their growth differences (Figure 3.2A-C) suggest variations in specific reactivity or growth rates of the grafted partners. Matching scions and stocks in *U. kirkiana* trees is a problem due to scions, which are always thicker than stocks (Akinnifesi, *et al*., 2004) but an overgrowth of stocks and constricted graft unions are least expected.

The graft union swelling could be due to a number of factors including the accumulation of phenols, but this can be temporary as changes may occur as trees grow old, as evidenced by Figure 3.2B-C. Further, the lacuna might get filled up with parenchymatous (callus) tissues and hence this would increase the area of vascular continuity at the union. Differences in diameter between scions and stocks might also be due to differential growth rates, especially after grafting, as evidenced by an overgrowth of the rootstock in Figure 3.2B. Constriction of the union in older grafted partners might be due to degeneration of vascular cambium.

For the young grafted trees, there were significant differences ($P \leq 0.05$) with respect to stem diameter and bark thickness (Table 3.2). There was a significant increase in diameter at the

unions (1.50 cm) compared to the scions (1.10 cm) and stocks (1.21 cm). Significant differences in diameter between the scions and stocks were also obtained. Tshokoeva and Tsonev (1995) reported marginal differences between scion and stock diameters in grafted apricot trees, but a significant increase in diameter at the union. An increase in stem diameter at the union could be attributed to metabolite accumulation (presumably phenols and carbohydrates) as a result of partial cambium continuity at the union. Errea (1998) reported that translocation constraints caused accumulation of some compounds. Moreover, a high amount of callus forming into the undifferentiated parenchymatous cells could also cause the union to swell.

The stocks had significantly thicker barks (0.25 cm) than scions (0.18 cm) and this could be attributed to differences in growth and amount of callus formation of the scion/stock partners after grafting. According to Akinnifesi *et al*. (2004), matching the cambial cells between scion and stock has been a challenge in grafting *U. kirkiana* trees since scions are usually thicker than the stocks. Therefore, correct matching depends on selecting scions and stocks with almost similar stem diameter and bark thickness. This is to improve proximity of vascular tissues of the scions and stocks. Bark thickness at the union was not measured, but this could be a factor contributing to an increase in union diameter since the presence of non-functional tissues can increase the union diameter. Simons and Chu (1981) reported an overgrowth of the union due to radial growth of vascular tissues.

3.4.2 Histological and anatomical studies

Figure 3.3 illustrates an external view and longitudinal section of the graft unions of *U. kirkiana* trees. There are variations in the amounts of callus proliferation and union line

visibility although these trees were alive. There were no perfect unions for all of the ten *U. kirkiana* tree provenances sampled except some formed a good union below the graft (Figure 3.3B) and this was termed a 'partial' graft union (Ünal, 1995). Graft partners with partial union showed a good amount of callusing at the union (both external view and longitudinal section of the union). Therefore, a poor union might be associated with poor amounts of callus formation at the union. Figure 3.4 illustrates incompatible (A) and partially compatible (B) grafted *U. kirkiana* partners.

MW84 scion on MW57 (MW84/57) stock shows incompatibility, possibly due to wide unfilled areas (poor amounts of callusing) at the union (Figure 3.4A). This poor union is illustrated by a visible line between partners showing no continuous bark and wood tissues. Survival of such partners could be attributed to the presence of some portions of undifferentiated tissues (parenchymatous tissues) into cambium and vascular tissues. Errea, Felipe & Herrero (1994a) reported presence of some parenchymatous tissues in incompatible combinations that made the unions in *Prunus* species weak. In the present study, the bark tissues at the upper part of the union were dead and this was also observed in partially compatible trees. MW26/22 stock (Figure 3.4B) showed partial continuity in bark tissues and a small area of necrotic tissues at the pith and the upper part of the union. This combination might form a good graft union with time.

Data in Table 3.3 shows mean separation of different graft combinations with respect to the four attributes (absence of visible line in bark and wood, callus proliferation and phenol accumulation). Mean separation was done using Student-Newman-Keuls test (SAS, 1999). Graft combinations are in three main groups (Table 3.4) with respect to the absence of a

visible line in the bark, namely (i) MW26/26, (ii) MW7/10, MW84/57 and MW12/57, and (iii) MW 1/61, MW2/U, MW28/32, MW57/49 and MW71/U. For callus proliferation, the graft combinations are in two groups, namely (i) MW26/22 and (ii) MW12/57. Graft combinations, MW2/U and MW7/10, are also in two groups with respect to phenol accumulation. With respect to absence of visible line in the bark, there are three main groups, namely (i) MW26/26, (ii) MW26/22 and (iii) MW1/61, MW7/10, MW2/U, MW84/57, MW12/57 and MW57/49. From this statistical analysis, it is difficult to group graft combinations into a compatible or incompatible category when all the four attributes are simultaneously considered. Moreover, it is difficult to interpret histological sections because of variability induced during grafting and variations in incompatibility symptoms (Ermel *et al*., 1995).

Figure 3.5 shows a simultaneous representation and descriptive summary of data from the correspondence analysis output. MW26/22, MW26/26 and MW7/10 have been grouped together indicating compatibility. Correspondence analysis is based on transformation of Chi-square values and produces dimensions which represent the Chi-square distances (Lebart, Morineau & Warwick, 1984). In this trial, the principal inertias were 0.06 (79.5%) and 0.14 (18.0%) at one- and two-dimensions, respectively. A two-dimensional correspondence analysis was appropriate since it represents 97.5% of the profiles (i.e. 97.5% simultaneous representation and descriptive summary of the data).

According to Lebart, Morineau & Warwick (1984) and Greenacre (1984), correspondence analysis gives a simultaneous perceptual map showing relationships between the objects (rows) and variables (columns) of a data matrix. It provides an informative and descriptive

summary of a data set containing many interrelationships, which are difficult to interpret with other statistical methods. It is used in several fields including compatibility of tissues (Greenacre, 1984). It is clear that correspondence analysis (Figure 3.5) improves interpretation and provides a descriptive summary of the graft combination data. In this trial, different graft combinations were categorised into a compatible or incompatible group with respect to all the attributes. According to Ermel *et al*. (1997), correspondence analysis offers a better procedure to discriminate compatible from incompatible graft combinations.

Time of grafting of *U. kirkiana* trees could have played a role with respect to accumulation of phenols since the trees were grafted in different months of the year. However, incompatibility might be compounded by variability in imperfect grafting although skilled grafters were used to graft these trees. Using a visual classification described by Ünal (1995), there was no perfect union observed in this study. It is only suspected that partially compatible partners might form a perfect union with time.

Figure 3.6 shows a common trend for callus cell proliferation at the graft unions observed under a light microscope. In all the combinations, callus cells were prolific below the graft union where a good union had been formed. However, tissues above the union were necrotic and highly stained, and hence there was no continuity in the bark and wood. Observations showed that *U. kirkiana* plants exude a lot of metabolites (phenols) in response to wounding. Hamisy (2004) reported high amounts of phenols in *U. kirkiana* leaves during DNA extraction. Therefore, it is suspected that phenols could play a role in graft incompatibility of *U. kirkiana* trees since grafting involves wounding of plant tissues. Figure 3.6A shows more lacuna or unfilled areas than the other scion/stock combinations

(Figures 3.6B-C). According to Errea (1998), phenols have been implicated in union formation processes, which include insufficient callus proliferation, cell necrosis and metabolic interactions. These are known to bring about disorders and damage at the graft unions. Therefore, phenol accumulation observed in this trial might play a role in graft incompatibility.

Poor callus formation was observed in some partners (e.g. MW84/57) and possibly, phenols were oxidised to other forms (such as quinones) which were toxic and eventually disrupted chemical reactions (Errea, 1998). According to Errea *et al*. (1994b), a significant amount of flavanol (phenol) in phloem was found in apricot as a response to graft incompatibility. Phenols prevent cambial connection continuity formation and a high accumulation occurs at the union of incompatible or less compatible combinations (Errea *et al*., 1994b). They induce cell damage and alter phloem cambium around the graft union. Our study showed a high phenol accumulation above the union where the bark and phloem tissues were both dead. This agrees with the findings by Ermel *et al*. (1997) where cell necrosis and discontinuity of vascular connections at the union were the main indicators of incompatibility. Figure 3.7A shows accumulation of phenols at the union, and hence prevents continuity in the tissue connection. Gebhardt & Feucht (1982) reported that a high concentration of phenols above the union is the cause of graft incompatibility. Furthermore, some incompatible combinations may grow without any external indication of incompatibility, but the presence of phenols accumulating at the union serves as an indicator of problems in graft combinations (Considine, 1983; Errea, 1998).

Phenol accumulation above (high) and below (low) the graft unions are shown in Figure 3.7A-B. There were unfilled areas and necrotic tissues at the unions (Figure 3.7A) and callus tissues broke up the phenols from the lower side of the union (Figure 3.7C). The quantity of phenols is high above the union and coincidentally there was no continuity of cambial connections. A good connection was found below the union in partial compatible partners. An accumulation of phenols, especially above the union has been implicated in reduced graft compatibility in many heterogenetic grafts (Usenik & Štampar, 2001).

Hartmann, Kester & Davies (1990) reported that maintaining a film of water at the union during grafting is necessary for callus formation. This water could possibly dilute some phenols as they accumulate below the union, especially water-soluble phenols. This could aid in breaking up of phenols by prolific callus tissues and consequently, grafted partners are able to establish cambial continuity. High accumulation of deposits (phenols) at the union of some *U. kirkiana* trees indicates that such partners might take time for phenols to be broken up completely and form complete vascular tissue continuity. This could be the reason that the union diameter was larger than either the scions or stock in young grafted trees. Figure 3.8A shows a section of a partial MW26/22 compatible combination (Figure 3.8B). Union line visibility increases above the graft union. Figure 3.8A shows small pockets of deposits and invisible union line. Figure 3.8C shows a line of deposits and necrotic layers along the union line.

Graft set in November - December period was found to be the best for *U. kirkiana* trees (Akinnifesi *et al*., 2004). This could suggest seasonality in phenol accumulation and the

quality of phenols at a particular time of the year. Trees used in this trial were grafted during the months of June, August and early October.

3.5 Conclusion

Indicators of graft incompatibility in *U. kirkiana* trees include growth irregularities at the union, poor callus formation, presence of necrotic tissues and accumulation of phenols. Such findings confirm existence of graft union problems despite the fact that some trees were surviving in the nursery and the field. For graft incompatible partners, portions of parenchymatous tissues supported the graft unions. MW26/26, MW26/22, MW7/10 and MW28/32 were partially compatible. However, phenolic compounds were major factors influencing graft incompatibility.

Tables

Table 3.1 Tree identification (ID) of *Uapaca kirkiana* stocks and scions from different districts and locations (natural forest or cultivated field) in Malawi

Tree ID	Accession name	District	Area	Fruit trait
MW1	ICR02NkhumbaMW1	Zomba	Forest	sweetness
MW2	ICR02KanyotaMW2	Zomba	Forest	sweetness
MW7	ICR02MalemiaMW7	Zomba	Forest	sweetness
MW10	ICR02MalemiaMW10	Zomba	Forest	sweetness
MW12	ICR02SitolaMW12	Zomba	Forest	sweetness
MW22	ICR02ElsoniMW22	Dedza	Forest	sweetness
MW26	ICR02HardwickMW26	Dedza	Field	sweetness, load, size
MW28	ICR02HamiyoniMW28	Dedza	Field	sweetness
MW32	ICR02YesayaMW32	Dedza	Forest	sweetness, size
MW49	ICR02NkhumbaMW49	Phalombe	Forest	sweetness, fruit load
MW57	ICR02NkhumbaMW57	Phalombe	Forest	sweetness, fruit early
MW61	ICR02MigowiMW61	Phalombe	Forest	sweetness, fruit early
MW71	ICR02NkhumbaMW71	Phalombe	Forest	sweetness, fruit load
MW84	ICR02NazombeMW84	Phalombe	Forest	sweetness

Table 3.2 Average scion, stock, and graft union diameters and bark thicknesses of young *Uapaca kirkiana* fruit trees (one-year old after grafting). Measurements taken approximately 5 mm below and above the graft union and means are calculated with standard errors (N = 40)

Plant parts	Stem diameter (cm)	Bark thickness (cm)
Scion	1.10 ± 0.04^{c}	0.18 ± 0.01^{b}
Stock	1.21 ± 0.06^{b}	0.25 ± 0.02^{a}
Union	1.50 ± 0.07^{a}	-
CV (%)	13.3	20.5
$LSD_{0.05}$	0.08	0.04

Numbers with different letters within a column are significantly different ($P \leq 0.05$)

- Not measured since one side of the graft union is a scion and on the other is a stock

Table 3.3 Mean scores of *Uapaca kirkiana* graft combinations with respect to absence or presence of visible line in the bark and wood, callus proliferation and phenol accumulation

Graft combination	Bark	Wood	Callus	Phenol
MW1/61	45.0^{c}	5.0^{c}	60.0^{ab}	70.0^{ab}
MW26/22	80.0^{ab}	30.0^{b}	80.0^{a}	65.0^{ab}
MW2/U	48.3^{c}	5.0^{c}	60.0^{ab}	80.0^{a}
MW26/26	90.0^{a}	50.0^{a}	65.0^{ab}	60.0^{ab}
MW71/U	48.3^{c}	5.0^{c}	65.0^{ab}	65.0^{ab}
MW7/10	70.0^{b}	30.0^{b}	60.0^{ab}	50.0^{b}
MW84/57	65.0^{b}	5.0^{c}	50.0^{ab}	60.0^{ab}
MW12/57	70.0^{b}	5.0^{c}	45.0^{b}	65.0^{ab}
MW28/32	45.0^{c}	40.0^{ab}	75.0^{ab}	65.0^{ab}
MW57/49	48.3^{c}	5.0^{c}	50.0^{ab}	60.0^{ab}
Probability	0.0001	0.0001	0.0245	0.0001
CV (%)	11.88	34.02	18.51	13.98

Numbers with the same letters within a column are not significantly different ($P \leq 0.05$)

Figures

Figure 3.1 Morphology of *Uapaca kirkiana* graft unions (A) HardwickMW26 (scion) on ElsoniMW22 and (B) NkhumbaMW1 (scion) on MigowiMW61 (stock)

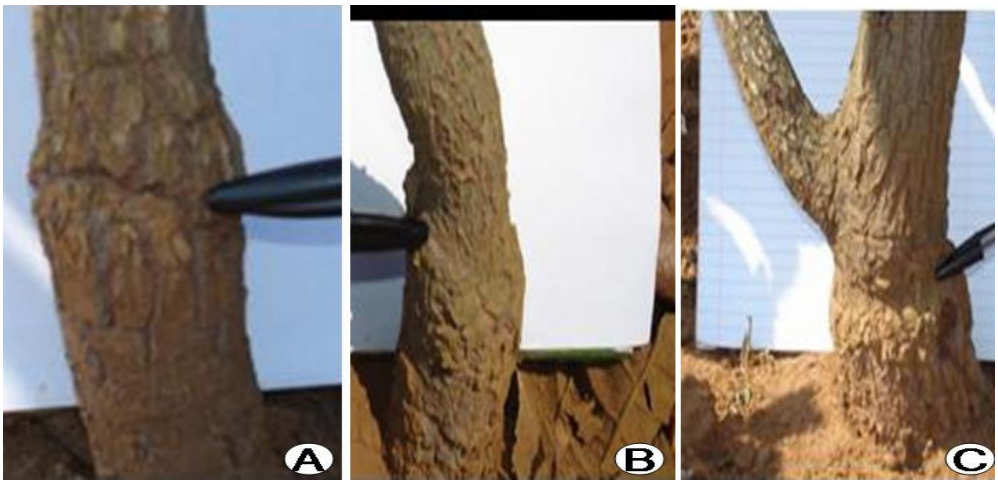

Figure 3.2 Growth irregularities at the graft unions of *Uapaca kirkiana* fruit trees (A) a groove across the union; (B) a small scion on an overgrown stock; (C) a constriction at the union (pen points at the union)

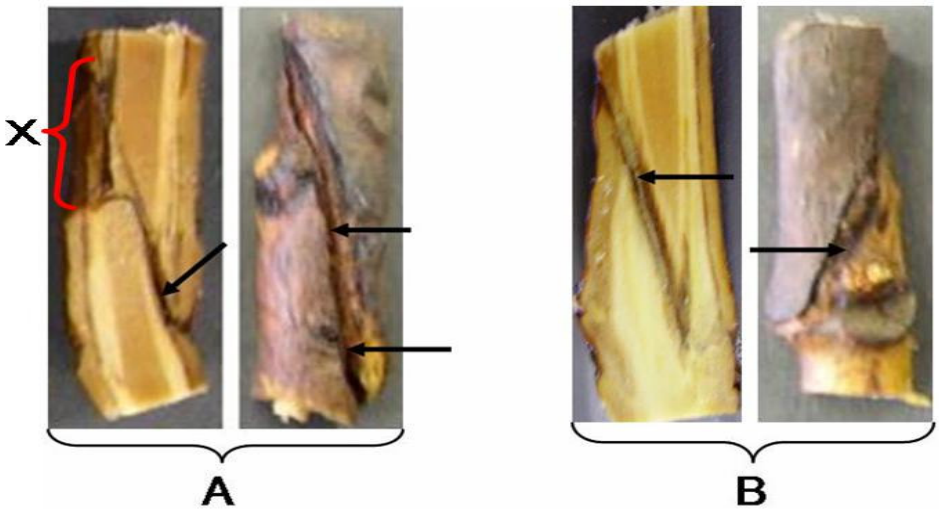

Figure 3.3 Longitudinal sections and external view of the graft unions of *Uapaca kirkiana* trees showing (A) poor rate of callusing at the union interface; (B) good amount of callus formation at the union. (Arrows show necrotic tissues for the internal sections and differences in callus proliferation for the external sections, X = dead bark area of the incompatible graft combination)

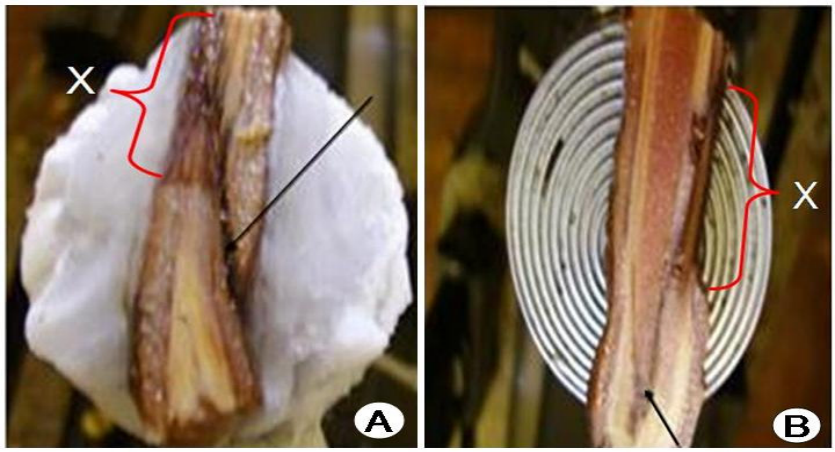

Figure 3.4 *Uapaca kirkiana* scion/stock combinations (A) a visible line between the scion (NazombeMW84) and stock (NkhumbaMW57); (B) a faint line between HardwickMW26 and ElsoniMW22 (X = death of bark and vascular tissues, arrows show visible union lines)

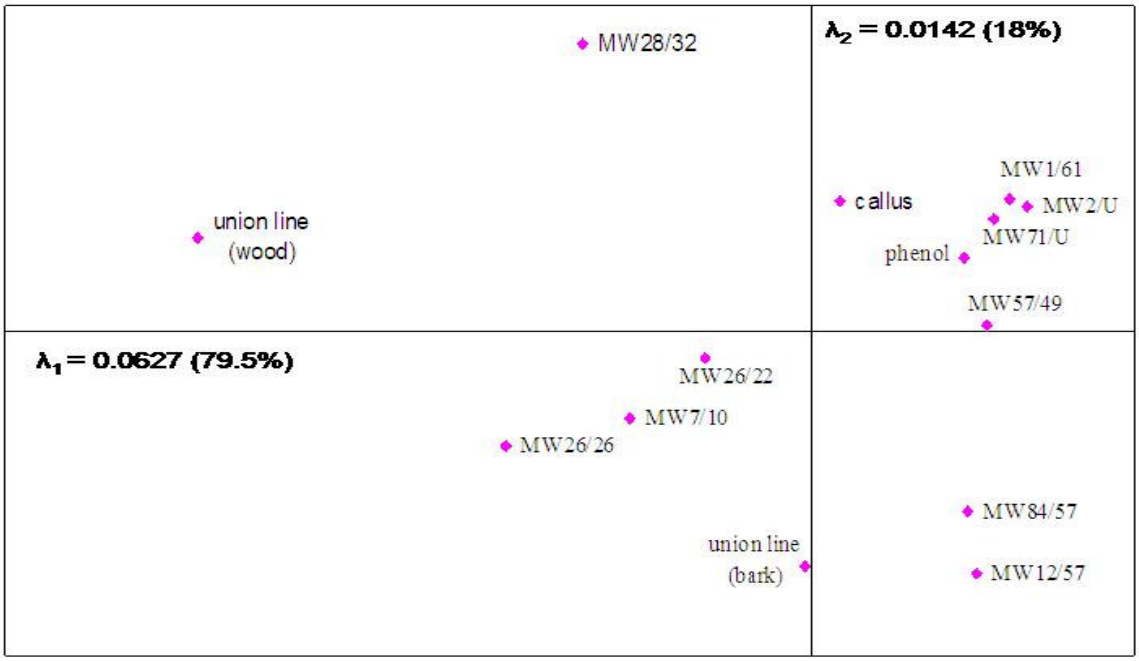

Figure 3.5 Two-dimensional correspondence analysis showing distribution and association of different *Uapaca kirkiana* graft combinations with respect to union line in the bark and wood, presence of phenols and callus proliferation at the union (U = unknown stock, λ = inertias)

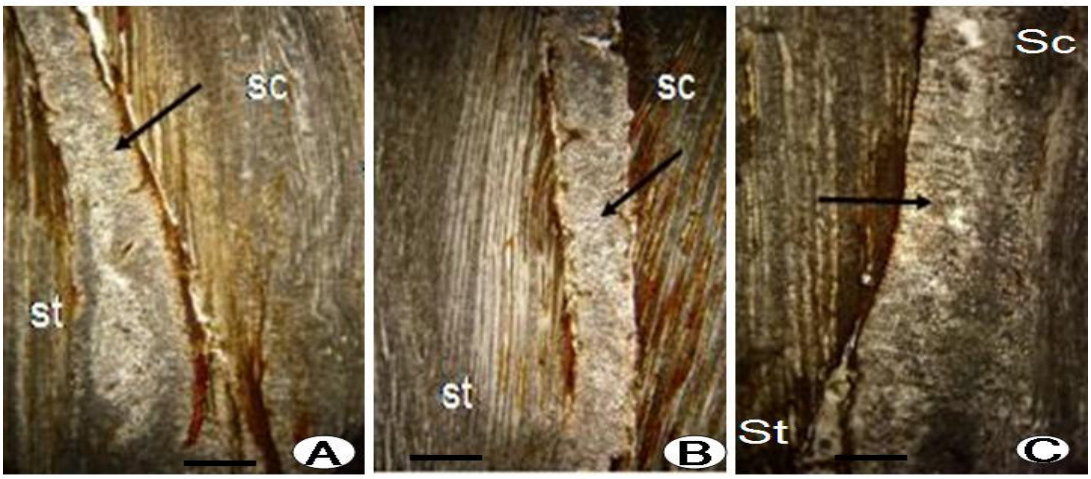

Figure 3.6 Anatomical observations of callus tissues very prolific below the union area (A) numerous unfilled (lacuna) areas; (B) a few unfilled areas; (C) absence of unfilled areas (arrows indicate callus tissues and the stains at the union interface are phenols, St = stock, Sc = scion, bar = 5 µm)

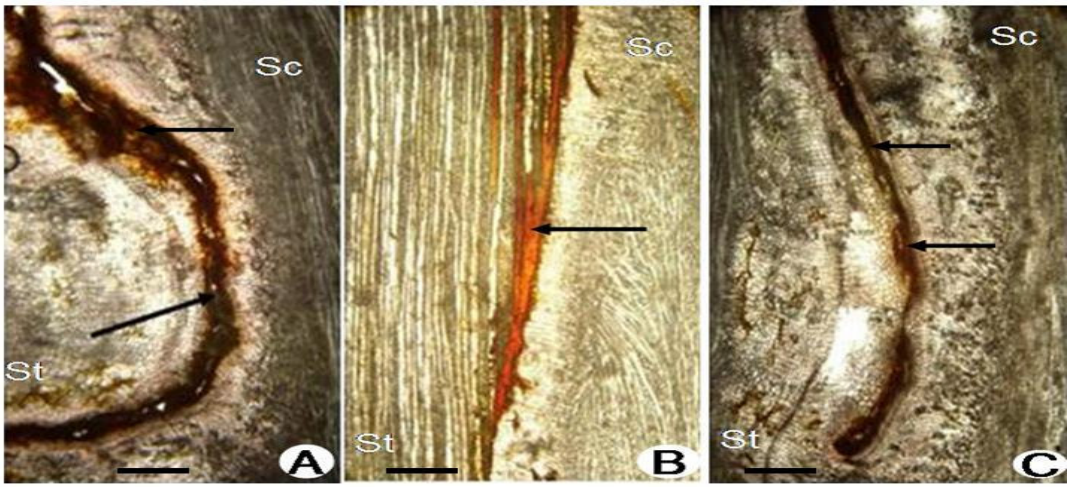

Figure 3.7 Deposits at *Uapaca kirkiana* scion/stock union (A) incompatible partner (NkhumbaMW1/MigowiMW61) with high amounts of deposits; (B) partial compatible union with high amounts of deposits above and at the union; (C) callus cells breaking up deposits from below the union area (arrows indicate deposits, St = stock, Sc = scion, bar = 5 µm)

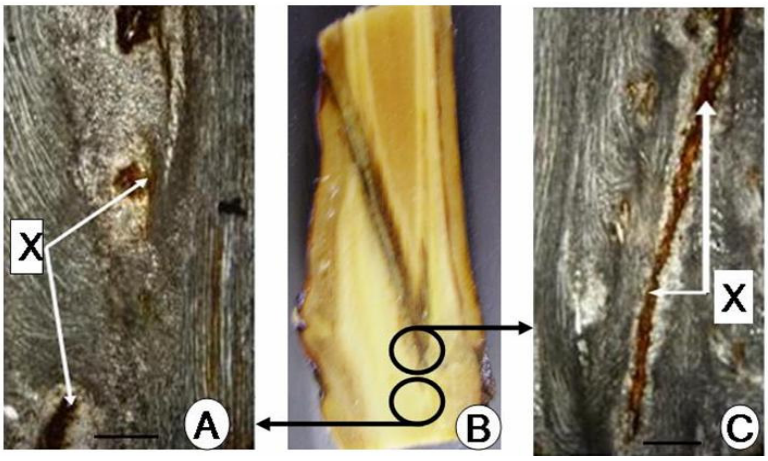

Figure 3.8 Sections below the union of a compatible HardwickMW26/ElsoniMW22 *Uapaca kirkiana* combination (A) invisible union line; (B) HardwickMW26/ElsoniMW22 section; (C) a faint union line (X = pocket/line of deposits, bar = 5 µm)