Non-destructive sampling procedure for biochemical or gene expression studies on post-harvest physiological deterioration of cassava roots

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Abstract

Cassava (*Manihot esculenta* Crantz) roots spoil 2-3 days after harvest due to post-harvest physiological deterioration (PPD), a problem for which no solution was in sight until recent reports of genetic variation for tolerance. PPD is a genetically active, oxidative process triggered when the harvested roots are separated from their mother plant. The short shelf life of harvested roots results in large losses and high transport and marketing costs. Recent reports on positive genetic variation for tolerance to PPD will facilitate breeding to extend the shelf life of the roots and also improve our understanding of the biochemical and genetic events leading to PPD. However, PPD scoring is difficult and prone to large experimental errors. Roots from the same plant can have PPD scores of 0 and 100% due to injuries during harvest, variation in dry matter content and, most likely, other variables yet to be identified. Therefore, sampling a root for biochemical or genetic studies and measuring PPD in a different root is not a reliable approach. A device has been developed and tested with which it is possible to extract a core of root parenchyma, fill the space with melted paraffin (to reduce oxygen availability), and then visually quantify PPD in the same root one or two weeks later. Sampling the roots did not have any significant effect on PPD, suggesting that the protocol can be used for biochemical composition and gene expression studies related to the causes of PPD and to the possibility of developing tolerance.

Key words: gene expression; genetic tolerance; post-harvest losses; shelf life.

1. Introduction

Cassava is among the most important staple crops in tropical and subtropical regions of the world. It shows remarkable and widely recognized adaptation to marginal growing conditions due to its perennial growth habit. Under biotic and/or abiotic stresses, the plant can enter a dormant state until favorable growing conditions return. This characteristic gives the plant considerably flexibility in adapting to environmental changes (Ceballos et al., 2011). Compared with other staple foods, cassava is also more flexible with respecting to its harvest date, allowing farmers to keep the roots in the ground until needed (Iglesias et al., 1997). Although the starchy root of cassava is its primary product, fresh leaves are also used for animal and/or human consumption in Africa and Asia (Benesi et al. 2010; Howeler, 2012).

In addition to cassava's important contribution to basic food security, its roots are in growing demand for the production of starch, processed foods, animal feed and ethanol (Balagopalan, 2002; Buitrago 2011a; 2011b; 2011c; Chauynarong et al. 2009; Moorthy, 2004; Sriroth et al., 2010) as well as for making bread (Pasqualone et al. 2010) and snacks (Vitrac et al. 2002). Cassava is the second most important source of starch after maize, and no other starch source is traded more in international markets (Stapleton, 2012). The identification of new root quality traits that offer particular advantages for some of these industries is likely to strengthen and widen the industrial applications of cassava in the near future (Rolland-Sabaté

et al., 2012; Sánchez et al., 2010). Genetic transformation is also an important tool for developing cassava cultivars with new root quality traits (Liu et al., 2011; Koehorst-van Putten et al., 2012).

However, several factors affect the ability of cassava to satisfy new demands. The crop is generally grown in marginal environments, which typically have poor roads and are far from processing centers. Cassava roots are bulky, containing approximately 65% water. In addition, they have a very short shelf life because of a process known as post-harvest physiological deterioration (PPD), which rapidly renders the roots unpalatable and unmarketable (Han et al., 2001; Reilly et al., 2003; 2007).

Consequently, cassava roots must be consumed soon after harvest (van Oirschot et al., 2000). Their short shelf life severely limits marketing options by increasing the likelihood of losses and overall marketing costs (Salcedo et al., 2010). Extending the shelf life of cassava roots to 45 days would result in annual benefits having an estimated value of US$35 million in Thailand alone (Vlaar et al., 2007). The economic impact would be considerably higher in other countries where road and transport infrastructure is not as developed as in Thailand.

The processes involved in PPD, which begin as soon as the root is detached from the mother plant, resemble changes typically associated with the plant's response

to wounding. PPD triggers a cascade of biochemical reactions, in which reactive oxygen species (ROS) are central. Specific genes involved in PPD have been identified and characterized, and their expressions evaluated (Reilly et al., 2001). Several secondary metabolites, particularly hydroxycoumarins, accumulate in the process (Bayoumi et al., 2008a; 2008b; 2010). PPD begins 24-48 hours after harvest (at 20-30 ℃ and 65-80% relative humidity); root handling and storage conditions greatly affect the speed and magnitude of the process. Keeping roots at 10 ℃ and 80% relative humidity delays the onset of PPD by two weeks. Unfortunately, PPD has also been linked with high dry matter content in the roots (van Oirschot et al., 2000; Sánches et al., 2006), which is a common objective of cassava breeding.

Although genotypic variation for PPD has been reported (Booth, 1976; Ekanayake and Lyass, 2003), in no case had the degree of tolerance matched that reported recently by Morante et al., in 2010. Research on PPD and the physiological, chemical, genetic and/or environmental factors affecting it is difficult because of the large experimental errors typically associated with the available scoring protocols. Root handling during harvest and transport, and root dry matter content are among the factors known to influence PPD. Roots from the same plant may often have scores ranging from 0 to 100% PPD. A major problem, therefore, is the inefficiency of the current approach of sampling one root for biochemical or gene expression

111 studies (usually soon after harvest) and measuring PPD in a different root from the
112 same genotype (typically 7-10 d after harvest).
113
114 Given the availability of tolerance and recent advances in our understanding of the
115 process and factors affecting it (Bayoumi et al., 2008a; 2008b; 2010; Ndidi and
116 Akeem, 2011; Reilly et al., 2007), it is important to have a protocol that: **a)** allows
117 non-destructive sampling of root tissue; **b)** can be performed days before PPD is
118 quantified; and **c)** will not induce (or prevent) the normal onset and development of
119 PPD. This article describes an innovative, non-destructive approach for sampling
120 cassava roots soon after harvest (to examine biochemical composition profiles
121 and/or gene expression) without noticeable changes in PPD development during
122 storage periods ranging from one to two weeks. This method will facilitate research
123 aimed at improving our understanding of the factors leading to PPD and the
124 biochemical/genetic factors determining tolerance to this problem.
125

2. MATERIALS AND METHODS

2.1 Germplasm

The genotypes evaluated in this study were selected because of their contrasting reaction to PPD: clones CM 523-7 and HMC1 are susceptible, while AM 206-5 and MPER 183 are tolerant (Morante et al., 2010). AM 206-5 is the genotype for which the amylose-free starch mutation (*waxy starch*) was first reported (Ceballos et al., 2006).

2.2 Root harvest, handling and PPD evaluation

Scoring the reaction to PPD is a destructive process, which was initially developed by Booth et al. (1976) and involves the storage of intact roots (also Booth 1976; 1977). A newer method for quantifying PPD was described by Marriott et al. in 1978 and 1979 and later modified by Weathley in 1985. With this method, the proximal and distal ends of the root are removed to accelerate PPD and avoid microbial contamination, which occurs during long storage periods. The distal open section of the root is covered with cling film to prevent further flow of oxygen. Roots are then stored for 3-7 days.

Several plants from the genotypes described above were harvested from May 30 to July 1, 2012, by which time 60 commercial-size roots in good condition had been selected for each genotype. Roots were harvested manually (according to

typical practice), and care was taken not to cause any injury to the roots, since rough handling creates localized damage to the root tissue that accelerates PPD. Each root was weighted individually and then randomly assigned to one of the four treatments described below (15 roots per treatment), based on combinations of two main factors:

a. Duration and storage conditions

a1. Roots were processed following Wheatley's methodology (described above) and stored for 7 d. The major advantage of this methodology is that it accelerates PPD and prevents the microbial rotting that occasionally occurs when roots are left for long periods.

a2. Roots were stored under normal conditions for 14 d. This treatment simulates the real conditions in which roots are stored before processing in different industries.

b. Sampling of roots soon after harvest

b1. Roots were not sampled at the start of the storage period.

b2. A cylindrical sample of the root was extracted from its midsection, and melted paraffin was then added to fill the space in the root sampled (see description of the procedure below).

169 Roots were stored on shelves in a structure with a roof but without walls, allowing
170 air to circulate freely. Before storage, each root was weighed individually.
171 Sampled roots (treatment b1 above) were weighed after the core sample was
172 taken. Roots were evaluated 7 d after harvest (using Wheatley's protocol), or 14 d
173 after harvest (leaving the tips of the roots untouched). For each genotype and
174 treatment, 15 roots were included at the beginning of the experiment. Before
175 measuring PPD reaction, roots were weighed again to quantify weight loss during
176 storage.
177
178 Scoring PPD reaction is an inherently destructive process. Seven transversal
179 slices were cut along the root, starting at the proximal end. Each slice was
180 assigned a score of 1 to 10, corresponding to the percentage of the cut surface
181 showing discoloration (1=10%, 2=20%, etc). The mean PPD score for each root
182 was calculated by averaging the scores of the seven transversal sections
183 (Wheatley et al., 1985). This method, is time-consuming and laborious, however,
184 and also prone to large experimental errors (i.e., roots from the same plant may
185 have PPD scores ranging from 0 to 100%) - the problem that prompted the
186 research reported here.
187
188 Roots showing symptoms of microbial rotting (very different from those related to
189 PPD) or affected by insects were not used for quantification of PPD. Only the

190 visual signs of deterioration (bluish grey vascular streaking) were assessed in this
191 study.
192
193 *2.3 Dry matter content*
194 A sample was taken from the roots for quantification of dry matter content after
195 measurement of PPD. For this purpose, 20-30 g of chopped and grated fresh roots
196 were dried in an oven at 60℃ for 24 h. Dry matter was expressed as the
197 percentage of dry weight relative to fresh weight.
198
199 *2.4 Statistical analysis*
200 The PPD values were expressed as percentages following the scoring procedure
201 described by Wheatley et al. in 1985. The data were arcsine-square root
202 transformed prior to analysis (Steel and Torrie, 1960). Analysis of variance was
203 conducted using the PROC GLM from SAS (SAS, 2008). The experimental unit
204 was each individual root from different cassava cultivars subjected to one of four
205 treatments (a1-b1, a1-b2, a2-b1 or a2-b2).
206
207 *2.5 Extraction of a cylindrical sample of root parenchyma.*
208 PPD is an oxidative process, which is actively controlled by the expression of
209 genes in the root. It is triggered by the separation of the root from the mother plant
210 and is clearly linked to the presence of reactive oxygen species (ROS) (Reilly et
211 al., 2003). Studies designed to monitor chemical composition or gene expression

212 in cassava roots require that these be sampled soon after harvest (for evaluation
213 of a phenomenon that becomes evident several days later. However, this sampling
214 requires that ROS be prevented from promoting or accelerating PPD around the
215 area injured when the sample is extracted. A device was therefore developed for
216 extracting a core of root tissue and then immediately pouring melted paraffin into
217 the space left by extraction of the root sample (Figure 1). Key features of this
218 device are: **a)** a stainless steel, heated container on top to hold the melted
219 paraffin; **b)** a heated valve for manual release of the melted paraffin immediately
220 after the root sample is taken; **c)** a stainless steel cylinder with an internal
221 diameter of 8mm, whose penetrating edge is slanted and sharpened; **d)** a lever
222 connected to the sampling cylinder; and **e)** a pusher rod inside the cylinder that is
223 used to push the core root sample out of the cylinder.
224
225 **[Place Figure 1]**

3. RESULTS

Table 1 presents a summary of key environmental parameters that have been linked to the development of PPD. Temperature ranged from 19° to 30°C both in the first and second week of the experiment. Data for the second week are relevant only for the stored roots whose extremes had not been cut. Air moisture was above 90% at 7:00 AM; between 50 and 60% (first week) and in the 60s (second week) at 1:00 PM; and in the 70s at 7:00 PM.

At the beginning of the study, 15 roots were selected for each combination of treatments. One common problem with PPD assessment in cassava roots is the rotting caused by microbial infections. The symptoms of rotted roots are readily distinguished from those of PPD. Yet, PPD cannot be assessed in rotten roots, so in few cases, less than 15 roots could properly be scored. Averages for the roots representing each combination of treatments along with those for the three main effects are presented in Table 2. This table also presents information on the averages for dry matter content (DMC) and number of rotten roots, both of which were quantified at the end of the experiment.

A key finding, as shown in Table 2, is that in no case did sampling of the roots change the PPD score (increasing or decreasing it). Average PPD scores from sampled roots were similar to those for un-sampled ones when roots from individual clones were analyzed after 7 or 14 d of storage. Therefore, average PPD

248 scores did not show statistically significant differences between clones or during
249 the storage period. In other words, sampling the roots caused no noticeable
250 change in the evolution of PPD. Although the analysis of variance for PPD was
251 made on the arcsine-square root transformed data, Table 2 presents the original
252 values for PPD to facilitate understanding of the results. The statistical significance
253 of those means, however, relates to the transformed data.
254
255 As reported in the literature, PPD seems to be correlated with DMC (van Oirschot
256 et al. 2000; Sánchez et al. 2006). It is important, therefore, to report DMC values
257 as a reference point for analysis of PPD in cassava roots. For AM 206-5 average
258 DMC after 7 days of storage differed significantly between roots that were sampled
259 (43.2%) and those that were not (33.4%). DMC of sampled roots from this clone
260 after 14 d of storage was also higher when sampled (35.0%) than when not
261 sampled (31.5%). Similar results were observed for roots from HMC1 stored for 7 d
262 (41.6 vs. 34.4%). It is unlikely that these differences resulted just from random
263 sampling variation. Therefore, as expected, the overall average for sampled roots
264 also showed significantly higher levels of DMC than for roots that had not been
265 sampled (41.2 vs. 38.6%). It is not clear why these differences occurred and only
266 in roots from AM 206-5 and HMC-1. One explanation is that in some cases
267 sampling the root allowed water loss through the injured tissue. This would explain
268 the increases observed in DMC. In no case, however, those changes affected PPD
269 scores.

A summary of the analyses of variance is presented in Table 3. Four variables were analyzed: weight loss expressed in grams or as a percentage of the initial weight, PPD (expressed as a percentage and transformed) and DMC. The clone source of variation showed highly significant effects ($P>0.01$) for the four variables. The length of storage period also had highly significant effects on both ways of measuring weight loss but not for PPD or DMC. Sampling a cylinder of root parenchyma had a significant effect ($P>0.05$) only for weight loss expressed as a percentage of the initial weight and for DMC. No interaction showed statistical significance for any variable, except for changes in DMC. It is important that extracting a cylinder of root parenchyma did not have any noticeable effect on PPD development, which, as expected, was highly affected by genetic differences.

As indicated by the analysis of variance, sampling the roots and filling the space with paraffin did not result in significant changes in PPD (11.9 versus 12.5%, Table 2). When individual treatments (clones and duration of the storage period) are considered, then larger variation between averages of sampled and un-sampled roots can be observed. For example, in the case of AM 206-5, roots stored for 14 d showed 9.1% PPD when they had been sampled, though the average PPD of un-sampled roots was only 3.9%. However, these differences were not statistically significant. The standard deviations presented in Table 2 provide further evidence

292 of the experimental errors associated with PPD scoring. However, in some cases
293 sampling resulted in higher PPD values while in others lower levels. The variation
294 observed (also note the standard deviations within a given treatment) illustrates the
295 error that is typically associated with PPD and that prompted this study.
296
297 HMC-1 and CM523-7 proved very susceptible to PPD (18.8 and 21.0%,
298 respectively). These results agree with those reported by Morante et al. in 2010,
299 providing further evidence that there is genetic variation for PPD reaction in
300 cassava. Figure 2 illustrates the differences between roots from a tolerant (AM206-
301 5) and susceptible (CM523-7) genotype (whether sampled or not). Figure 2 also
302 shows what appears to be healing tissue around the paraffin cylinder, which is
303 frequently observed on roots from susceptible genotypes. This tissue acquired a
304 "*chalky*" appearance and consistency.
305
306 **[Place Figure 2]**
307
308 **4. Discussion**
309 Root sampling increased the frequency of rotten roots (16 versus 8 in Table 2).
310 This makes sense, since injury can serve as an entry point for microbes and fungi.
311 MPER183, as observed in the past, showed good tolerance to PPD, though its
312 roots tend to rot considerably more than those of other genotypes (particularly
313 HMC1). MPER183 also lost a lot of weight. Both MPER183 and AM206-5 where

314 clearly tolerant to PPD (4.6 and 4.2%, respectively), though AM 206-5 lost
315 considerably less weight (both in grams and in percentage terms). It can be
316 concluded, therefore, that weight loss is probably unrelated to PPD tolerance.
317
318 As is also evident from the analysis of variance, the storage period had a clear
319 effect on weight losses (whether expressed in grams or as a percentage). Roots
320 stored for 14 d showed higher weight loss (43.81 grams or 9.75%) than for 7 d
321 (33.02 grams or 8.64%). However, the difference in PPD levels between storage
322 periods (7 or 14 d) was not statistically different (11.2 versus 13.1%). This last
323 finding reinforces the longstanding perception that cutting the tips of the roots and
324 covering the distal cut with cling film accelerates PPD. Since no genotype-by-
325 treatment interaction was significant, it can be concluded that the two storage
326 methods provide similar information on PPD.
327
328 PPD is an enzymatically mediated oxidative process, which parallels plant wound,
329 senescence and defense responses. It is a very active and complex process, in
330 which as many as 72 non-redundant expressed sequence tags were either
331 induced or down regulated (Reilly et al. 2007). Salcedo et al., (2010) published
332 data indicating a negligible correlation between the accumulation of
333 hydroxycoumarins (assessed through fluorescence) and the visual symptoms of
334 PPD. These authors concluded that accumulation of hydroxycoumarins is not a
335 reliable marker for evaluation of PPD.

336

337 The information provided in Table 2 (particularly the standard deviations) illustrates
338 the large experimental error associated with PPD. In early work, PPD was
339 assessed in one group of roots while biochemical or gene expression
340 measurements were made in a different one, particularly if the latter analyses had
341 to be made earlier (at harvest or soon thereafter) than those for PPD (typically at
342 least 7 d after harvest). These studies acknowledged the weakness of the
343 assumption that data taken on one root could be associated with that from a
344 different one provided they were from the same genotype and harvested at the
345 same time. With the kind of experimental errors shown in Table 2, this assumption
346 was clearly questionable, and researchers knew it. The methodology proposed
347 here offers the advantage that the root can be sampled earlier and then PPD
348 assessed in the same root several days later. Since sampling the root has little
349 influence on PPD, the possibility of making both measurements in the same root
350 has a clear appeal.

351

352 Results on DMC (Table 3) were not surprising. During storage roots lose DMC
353 because of respiration and active hydrolysis of starch to produce simple sugars.
354 On the other hand, water loss occurs as well, which would tend to increase DMC. It
355 has been demonstrated that the hydrolysis of starch into simpler sugars may occur
356 at different rates in roots from different genotypes (Sánchez et al., 2013). This
357 could explain the statistical differences observed in DMC.

One pending issue needs to be addressed in future research. Sampling the roots seems to increase weight losses slightly (when expressed as a percentage), though it is not clear why or how. Roots were weighed after the sample was taken and the paraffin treatment completed. So, the higher weight losses are not a result of the paraffin being lighter than the extracted root sample. Since paraffin is very hot when applied to the root, perhaps the high temperature temporarily induced some transpiration/evaporation as it was applied. This is a weak explanation, however, since the temperature would remain high only briefly.

5. Conclusions

The recent report of wide genetic variation for PPD tolerance has generated interest in better understanding the genetic and biochemical factors influencing PPD and in exploiting this variation to extend the shelf life of cassava roots. The sampling protocol described here allows early root sampling (when gene expression and/or biochemical characteristics likely determine later evolution of PPD) with no effect on visual PPD scores taken afterwards for the same root. This procedure overcomes the major problem of conducting genetic and/or biochemical analysis for different roots from those in which PPD score is taken (which is affected by unacceptably high experimental errors). This study also further confirmed the differences in susceptibility to PPD within cassava germplasm.

This research justifies a follow-up study to assess the impact of taking several samples sequentially (two or three root samples during storage) before finally assessing PPD. Such a study would be of particular interest for tracking carotenoids content during storage. High carotenoids content has been linked with PPD tolerance (Morante et al., 2010; Sánchez et al., 2006), and these pigments may be metabolized during storage (CIAT, unpublished data). Sequential samplings of the same roots may also be helpful for a chronological study of gene expression.

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555 **Table 1**. Key environmental factors during the duration of this experiment.

Week	Hour	Average	Minimum	Maximum
Relative air moisture (%)				
First	7:00 AM	95.1	92	97
	1:00 AM	57.7	53	62
	7:00 PM	75.1	72	79
Second	7:00 AM	96.7	95	98
	1:00 AM	62.6	58	71
	7:00 PM	75.1	70	81
Daily temperature (° C)				
First		24.7	19.2	30.8
Second		24.4	19.4	30.0

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Table 2. Least square means and standard deviations (within parenthesis) of the experiment to assess PPD in roots from four cassava genotypes. At the bottom of the table the averages across genotypes, duration of storage period and sampling versus not sampling the roots is provided for comparison[1]. Roots stored for 7 d were subjected to Wheatle's method, whereas roots stored for 14 d were not cut at the extremes.

Clon	Sample taken	Storage (days)	Rotten roots	**Weight loss** [2]		**PPD** [2]	**DMC** [2]
				(g)	**(%)**	**(%)**	**(%)**
AM206-5 (Tolerant)	+	7	0	24.3(5.4)	7.3(1.6)	3.0(4.6)	43.2(1.9)
	-	7	1	21.8(5.3)	6.2(1.1) *	0.6(1.9)	33.4(3.9) **
	+	14	3	41.9(15.2)	8.9(1.9)	9.1(9.3)	35.0(2.3)
	-	14	1	28.6(9.8) *	7.3(1.7)	3.9(8.4)	31.5(4.3) *
CM523-7 (Suscept.)	+	7	0	35.0(12.4)	9.0(1.6)	24.8(11.2)	43.9(1.7)
	-	7	1	25.3(11.2) *	8.5(1.4)	20.3(10.9)	44.3(1.6)
	+	14	4	35.3(19.4)	9.7(2.1)	18.2(18.3)	43.0(1.7)
	-	14	1	36.4(20.5)	10.1(2.4)	20.6(16.1)	43.5(2.0)
HMC1 (Suscept.)	+	7	1	33.0(8.8)	8.6(1.3)	13.6(10.9)	41.6(3.0)
	-	7	0	36.7(14.4)	8.4(1.4)	17.4(12.5)	34.4(5.1) **
	+	14	0	58.3(25.1)	10.0(2.6)	17.2(11.3)	41.7(2.9)
	-	14	0	50.8(28.1)	9.5(2.3)	27.1(19.0)	41.7(1.9)
MPER183 (Tolerant)	+	7	3	41.0(17.1)	10.1(2.0)	4.3(4.6)	39.5(2.0)
	-	7	1	45.3(14.7)	10.8(1.7)	5.8(10.6)	39.0(4.5)
	+	14	5	54.5(25.9)	12.5(2.5)	4.7(6.8)	41.8(2.8)
	-	14	3	47.7(32.0)	9.7(2.4) *	3.8(7.7)	41.4(1.9)
AM206-5			5	29.2[b]	7.4[c]	4.2[b]	35.8[c]
CM523-7			6	33.0[b]	9.3[b]	21.0[a]	43.7[a]
HMC1			1	44.7[a]	9.1[b]	18.8[a]	39.8[b]
MPER183			12	47.1[a]	10.8[a]	4.6[a]	40.5[b]
Roots stored for 7 d			7	32.8[b]	8.6[b]	11.2[a]	39.9[a]
Roots stored for 14 d			17	44.2[a]	9.7[a]	13.1[a]	40.0[a]
Roots sampled			16	40.4[a]	9.5[a]	11.9[a]	41.2[a]
Roots not sampled			8	36.6[a]	8.8[b]	12.5[a]	38.6[b]

[1] Treatments followed by the same letter are not statistically different.

[2] Difference between means of sampled versus not sampled roots (for individual clones and specific duration of storage period) significant at $P < 0.05$ (*) or $P < 0.01$ (**)

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570 **Table 3**. Mean squares from the analyses of variance for weight losses (Expressed
571 in grams or in % related to initial weight) and post-harvest physiological
572 deterioration (PPD) and dry matter content (DMC) expressed in %.

Source of variation	df	Weight loss		Arcsin (√PPD)	DMC
		gr	%	%	%
Clone (C)	3	3886**	90.14**	34.83**	516.5**
Root/Clone	56	214	2.90	0.73	10.1
Length of storage (S)	1	6668**	64.44**	0.62	0.9
Root sample taken (R)	1	739	24.84*	0.41	295.6**
C * S	3	522	1.04	2.60	198.6**
C * R	3	119	4.73	2.03	141.0**
C * S * R	4	386	9.31	0.52	312.3**
Error	144	365	3.94	0.04	8.3

573 * Significant at the P>0.05; ** Significant at the P>0.01

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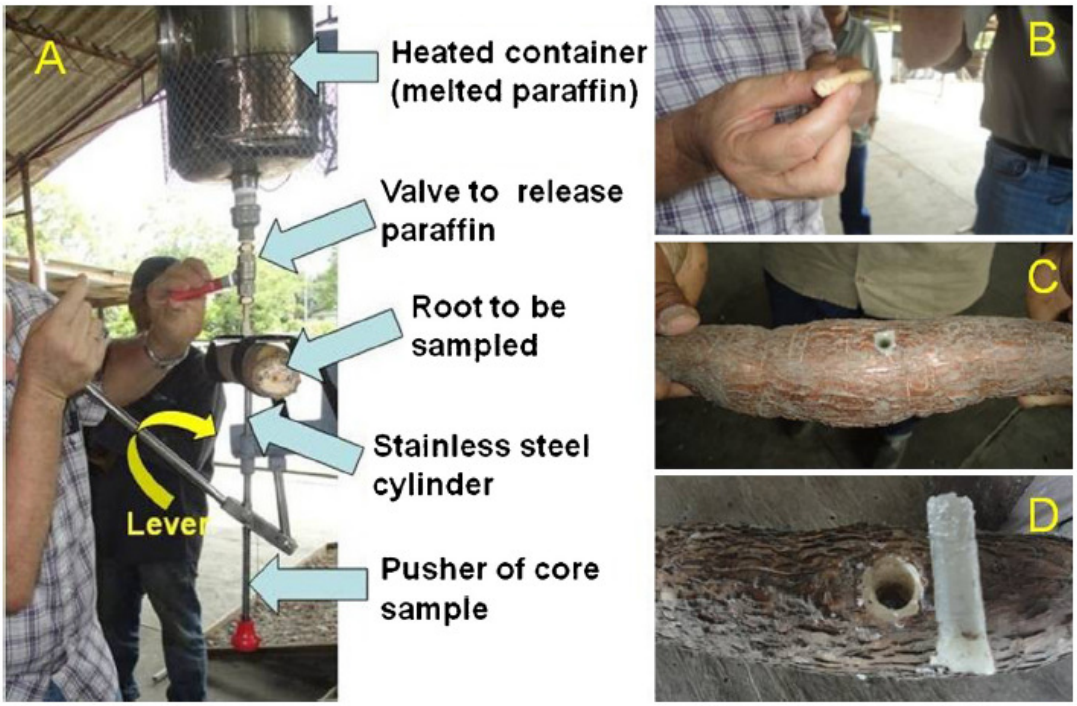
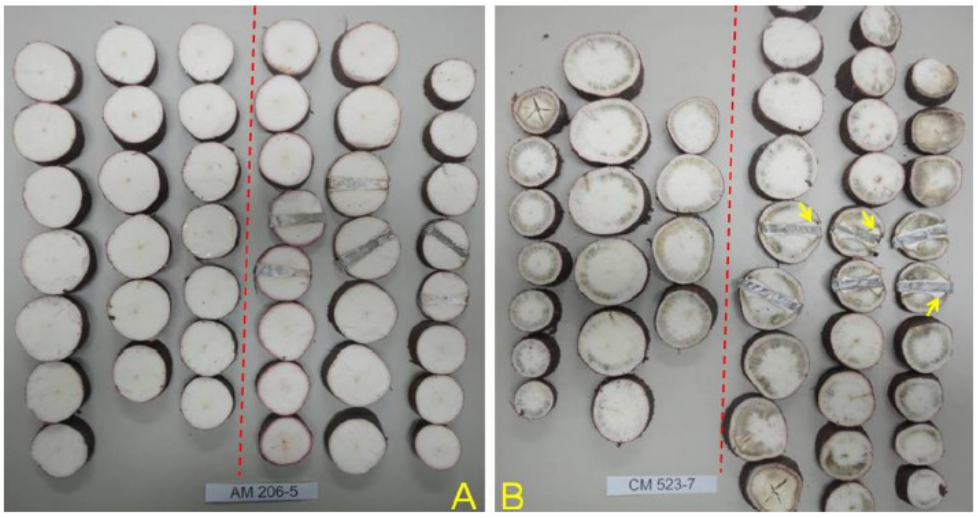


Figure 1. (A) Illustration of the system to extract core samples of root parenchyma and ifll the space with melted paraffin; (B) Example of core of the root parenchyma extracted; (C) Appearance of the root after paraffin had been applied and solidified; (D) Cooled down and solidified cylinder of paraffin extracted from the root for illustration.



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584 Figure 2. Photographs of roots from (A) tolerant genotype AM 206-5 and (B) susceptible
585 genotype CM523-6. For each genotype roots on the left had not been sampled and those
586 on the right had been sampled and the paraffin cylinder is clearly visible. In some cases, a
587 "chalky" healing tissue could be observed around the paraffin cylinder (arrows on right).