CROP POST HARVEST PROGRAMME

Identifying Target Points for the Control of Post-Harvest Physiological Deterioration in Cassava

R6983 (ZB0098)

FINAL TECHNICAL REPORT

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Executive Summary

Post-harvest physiological deterioration (PPD), which can limit the shelf life of roots of cassava (*Manihot esculenta*) to approximately 48 hours, is a major constraint to the development of this important staple food from essentially a village-based crop to one with major urban importance and industrial potential. The purpose of this project was to generate the intellectual and practical context and tools necessary for the rational improvement of cassava germplasm with respect to PPD. Thereby, enabling the identification of key features as potential candidates for modulating PPD via breeding or genetic modification. To this end, cassava varieties with contrasting PPD responses were examined structurally and in terms of their biochemical changes during the time course of deterioration. In addition the genetic contribution to PPD was analysed via the progeny (Family K) of a cross used to generate the molecular genetic map of the cassava genome, and via specific crosses set up to study the inheritance of factors contributing to the PPD response. The results from these experiments highlighted the role that reactive oxygen species (ROS) and enzymes and compounds that modulate them, play in PPD. In particular, antioxidant carotenoids were found, above a certain threshold, to be correlated with reduced PPD. The crosses confirmed that there is a genetic contribution to the PPD response, but also highlighted the strong environmental influence that can complicate analyses.

While crosses and breeding can provide useful information for the understanding of PPD and the testing of genetic components that contribute to this trait, this conventional approach to crop improvement is unlikely to solve the problem due to the high heterozygosity of cassava and due to the close association between high dry matter (a desirable characteristic) and high PPD responses. However, this project and its synergistic interaction with other Bath-CIAT PPD research have highlighted the important roles that oxidative stress, senescence, phytohormones and signalling compounds play in the deterioration response. Thereby identifying these and the genes involved in these processes as being likely target candidates for the modulation of the PPD response via genetic modification experiments; for example via antisense genes driven by PPD-specific promoters. Crosses and breeding should play a supportive, though important, role.

This project has led to several publications and many contributions to conferences and presentations to institutions. These dissemination activities have aroused the interest of other institutions, encouraged related research and the planning of major international collaborative proposals to tackle aspects of PPD.

Background

Cassava roots suffer from a rapid post-harvest physiological deterioration (PPD), which renders them unpalatable and unmarketable within a short time after harvesting, typically 24 – 48 hours. With changes in societies, PPD increasingly has become a major constraint to the development of cassava for farmers, processors and consumers alike, due largely to distances between farm and market. While no formal economic analysis exists of the discounting, waste and added costs caused by PPD, several estimates of the impact of this problem are available. Estimated losses vary from 5 – 25% of harvested roots [1]. Most of the deteriorated roots end up as animal feed with a price reduction greater than 50%. For example in Colombia where 1.3 million tons are consumed fresh, approximately 10% is lost due to PPD with a reduction in price from US$ 80 per ton (fresh market) to US$ 40 (animal feed), generating a loss of around US$ 5 million in just one country [2]. The marketing margin can be as high as 60% of the final retail price [3]. An informal survey of small-scale farmers' cooperatives, producers' associations and processing companies in Colombia in 2001, revealed that PPD had a significant negative impact on producers and processors alike and that losses could be between 30 – 60% depending on climatic conditions and distances to market. In particular processors highlight the problems inherent in the variable input quality of cassava roots, and all would welcome cassava with reduced a PPD response [4]. Similar losses are reported for Africa [1], for example, 7- - 90% discounting on three-day-old cassava in Tanzania [5]. Thus, while farmers, markets and industry have learnt to cope with PPD, it is at the price of substantial wastage and loss.

Therefore, research directed towards introducing resistance to PPD, or delaying the response, is considered a priority by international bodies such as the FAO and the Cassava Biotechnology Network [1, 6]. Increasing the shelf-life of cassava to one or two weeks, either by conventional breeding or via biotechnology, is considered an achievable long-term objective by these bodies. Such genetic improvement of cassava should impact positively on all concerned with its production, processing and consumption. While it is difficult to speculate as to the economic impacts that would accrue through solving the problem of PPD, the demand for its resolution from producers and processors alike, confirms that it would be welcome.

Several practical solutions to PPD have been tried or are available, while these have useful specific applications they do not provide universal solutions for a variety of reasons and have not been adopted extensively. Processing involves conversion of fresh cassava roots shortly after harvesting into a more stable product. These include traditional food products such as gari or farinha, or industrial intermediate or final products such as dried cassava chips for animal feed or starch. Processing can be a successful solution when carried out at a small-scale or village level. At a larger scale, distance and time between harvesting and processing can cause PPD to become a serious problem by varying the input quality to the industrial processes and the ensuing wastages have to be factored into the costs [7]. Exclusion of oxygen significantly reduces or delays PPD [8, 9]. However, recommended methods of storage in plastic bags [10, 11] or beneath tarpaulins [5, 12] have not been adopted, often due to the cost involved and the care that must be put into reducing damage during harvesting and transport [13, 14]. However, farmers on the Atlantic coast of Colombia did change to transporting cassava in polypropylene sacks instead of traditional sisal ones as these enhanced storage life sufficiently to get the roots to market [14]. On the other hand, coating individual roots with paraffin wax has been successfully adopted for

the export of cassava for food to markets or countries where the roots can command a high price. However, this is not a practical solution for the bulk treatment of such a low cost commodity in producing countries. Storage in controlled atmospheres with reduced oxygen content is also only a practical solution when the roots can command a high price. Freezing of fresh or par-boiled roots will preserve the roots for an extensive period. But, again, it is only a practical solution for high-price markets. Pruning cassava stems 10 – 20 cm above the ground two weeks before harvest does significantly reduce susceptibility to PPD [15, 16]. However, pruning negatively impacts on eating quality and processing, and so has not been adopted as a practical solution [14]. Therefore, processing cassava into chips, starch or traditional products is economically viable but has to accept the additional costs caused by unreliable input qualities and wastage due to PPD. While several mechanical solutions exist for unprocessed cassava, they are costly and largely uneconomical except for markets that are prepared to pay a high price, such as immigrant communities in developed countries.

Research in the 1970's and early 80's by UK and Japanese scientists on post-harvest deterioration in cassava established that the early stage of the response (PPD) is purely physiological, while microbial deterioration sets in subsequently [9, 17]. The visual symptoms of PPD are blue-black discolorations of the vascular tissues that spread from wound sites caused by harvesting or handling [18]. There is a strong fluorescence under ultra violet light of the cassava tissue that precedes this discoloration [19]. Microscopy reveals that coloured occlusions and tyloses block xylem vessels [20]. Accompanying these visual events are increases in respiration [21], changes in lipid composition [22], secondary metabolite accumulation [23-26], the synthesis of the phytohormone ethylene [27], and in the activity of a range of enzymes including phenylalanine ammonia lyase (PAL), acid invertase, catalase, dehydrogenases, peroxidases and polyphenol oxidase [23, 28]. Evidence from cyclohexamide inhibition of protein synthesis [29], from *in vivo* labelling of proteins [30], and cDNA cloning [31], confirm that PPD is an active, rather than a degenerative, process involving changes in gene expression and protein synthesis.

PPD in cassava can be reduced or delayed by pre-stressing the plant (for example by pruning or defoliation) [16, 32], or by excluding oxygen [33], suggesting parallels to the systemic acquired resistance (SAR) induced by pre-exposure to pathogens observed in other plants [34], and a role for oxidative stress responses, reactive oxygen species (ROS) and anti-oxidants during PPD.

Evaluation of this literature suggests that PPD shows strong parallels to wound responses in other more fully studied plants [35]. Plant wounding produces or induces the production of signalling molecules that initiate the wound response; this response includes the production of defensive compounds and enzymes, the preparation of the plant for the potential extension of wounding, and wound repair that is followed by the inhibition of signals [36, 37]. While these aspects of the wound response are present in the harvested cassava root, the wound repair and the resultant down-modulation of the signals are inadequate, which leads to continuous cascades of wound responses that spread throughout the root; it is this that is observed as PPD [38, 39]. It is interesting that wounded roots that remain attached to the plant are capable of normal wound repair [40, 41], suggesting that either efficient wound repair of the detached roots has been lost during the millennia since domestication or that, because the root serves no function once detached from the plant, the trait has been lost during evolution as of no biological purpose.

Project Purpose

To generate the intellectual and practical context and tools necessary for the rational improvement of cassava germplasm with respect to post-harvest physiological deterioration (PPD) via breeding and genetic modification. Essentially, this strategic research consisted of producing a comprehensive understanding of the important biochemical features of PPD within the context of the anatomy and structure of the root, and of the genetics of the crop, in order to identify key features as potential candidates for modulating PPD via either breeding or genetic modification. The achievement of these objectives should enable the CG centres, NARS and other laboratories concerned with the improvement of cassava, to embark on research and development programmes leading to the release of cassava varieties with improved post-harvest storage characteristics. Thereby, providing small and large scale farmers and processors with a more uniform and versatile crop, and enabling them to enhance their cash income, whilst at the same time providing the consumer with a reliable product at a reasonable price.

Research Activities

A brief description of the Activities will be given here, as the results of those Activities and their contribution to achieving the Outputs is more important; these will be elaborated on in the Outputs section. A detail list of these is provided in the LogFrame (Appendix 1).

Preparatory phase:
- Cassava cultivars with differential PPD responses were established in the Bath tropical glasshouse.
- Using initially commercially available cassava roots and subsequently Bath-grown roots extraction and separation methods (HPLC and TLC) for fractionating cassava root secondary metabolites were optimised. Similarly methods for certain enzyme assays were also established. These methods were further optimised during the course of the project.
- Cassava cultivars with high and low PPD responses and with high and low carotenoid content were selected and used as parents in crosses to examine aspects of the hereditability of PPD.
- Light microscopy methods were used to generate an understanding to the anatomy of the cassava root and the changes that it undergoes during PPD. Electron microscopy methods were developed to study the cassava root.
- Family K, the progeny used to construct the molecular genetic map of the cassava genome, was planted in two distinct agro-ecologies in Colomba: CIAT's experimental stations at Palmira and Quilichao for subsequent evaluation of their PPD response and to score for PPD-related quantitative train loci (QTLs).
- Maria X Rodriguez, a Colombian post-graduate, student started research in Bath in January of 1999. It had originally been planned that she spend the first year at CIAT; however, it was decided that from a training point of view it would be better if she started at Bath. She spent one year at Bath, one year at CIAT, six months at Bath and six months at CIAT. Ms Rodriguez received funding for her fees and living expenses at Bath from Colfuturo.

Development phase:
- The biochemical methods established were used to compare deteriorating cassava roots from cultivars, grown at Bath, with contrasting deterioration responses.
- Compounds of interest were identified by comparison to known standards and structurally identified where necessary.
- Biochemical methods were applied to field-grown materials at CIAT.
- Aspects of wound repair were studied in deteriorating cassava roots.
- Material from crosses was harvested and evaluated.

Analytical phase:
- Biochemical data from glasshouse and field grown material was analysed.
- QTL determination of Family K was carried out.
- Analysis of data from cross of high and low PPD and high and low carotenes.
- Results were prepared for publication and dissemination.

Outputs

As a result of the mid-term review of R6983 the original Outputs were slightly modified as follows; these are reflected in the attached LogFrame (Appendix 1):

Original Outputs:

1/ Rigorous, broad-based, yet detailed understanding of PPD from ultrastructural, biochemical and genetic perspectives generated.

2/ Specific enzymes, metabolites or structural features showing a strong association with high and low PPD identified.

3/ Identification of molecular markers linked to PPD-related quantitative trait loci (QTLs) assisted.

4/ Parental material for recombination and selection for increasing cassava storability identified.

5/ Stability of biochemical processes under a range of environmental and agro-ecological conditions determined.

6/ Key candidate genes and metabolic pathways with the potential to be manipulated via genetic modification experiments in order to modulate the PPD response identified.

7/ Results disseminated via reports, scientific meetings and publications to international (CIAT and IITA) and national centres and the wider research community concerned with cassava improvement.

Modified Outputs in light of the Mid-Term Review process:

1/ Rigorous, broad-based, yet detailed understanding of PPD from structural, biochemical and genetic perspectives generated.

2/ Specific enzymes, metabolites or structural features showing a strong association with high and low PPD identified.

3/ Identification of molecular markers linked to PPD-related quantitative trait loci (QTLs) assisted.

4/ Parental material for recombination and selection for increasing cassava storability identified.

5/ Stability of biochemical processes under a range of environmental and agro-ecological conditions determined.

6/ Key candidate genes and metabolic pathways with the potential to be manipulated via genetic modification experiments in order to modulate the PPD response identified.

7/ Results disseminated and Outputs promoted via reports, scientific meetings and publications to international (CIAT and IITA) and national centres and the wider research community concerned with cassava improvement.

1. Rigorous, broad-based, yet detailed understanding of PPD from structural, biochemical and genetic perspectives generated.

Structural survey of cassava roots.

This area had been de-emphasised as a result of the mid-term review process. However, a histological atlas of cassava (Appendix 2) was produced for internal use within the research group as it was felt that an understanding of the anatomy of the plant would provide a useful holistic perspective and context to biochemists and molecular biologists.

There is a dearth of transmission electron microscopic (TEM) ultrastructural studies on cassava roots, which is largely due to the difficulties in preparing the material. High levels of starch, lignin, suberin, tannin, lipid and phenolic materials in this type of tissue present problems in achieving adequate fixation and infiltration of resin into the cells necessary for successful ultramicrotomy and TEM. We devised a protocol for the successful preparation of cassava root material, including fixation with acrolein (acrylaldehyde), extensive vacuum treatments to draw fixative and resin into the cells and vessels, extended resin infiltration times, and a prolonged freezing step or the use of low viscosity resin. In addition, scanning electron microscopy (SEM) was used for obtaining general structural information. These methods permitted the examination of various aspects of the ultrastructure of the cassava roots including parenchymatic tissue containing amyloplasts. Deteriorating storage roots from high and low PPD cultivars were examined; increasing numbers of tyloses and vessel occlusions were observed, but no differences between the roots were apparent. A paper describing these improved methodologies has been submitted for publication to *Microscopy and Analysis* (Appendix 3).

Biochemical analysis of cassava roots.
The analysis of cassava root extracts revealed a high number of compounds belonging to different groups of secondary metabolites. Using a range of assays it was shown that functionally several of these compounds were antioxidants or were anti-microbial. Based on this, the focus of the project was on those compounds that differed during PPD either by their quantity or their quality. In this group of metabolites we could structurally identify four hydroxycoumarins (esculin, esculetin, scopolin and scopoletin) and three flavan-3-ols ((+)-gallocatechin, (+)-catechin and (+)-catechin gallate). The dominant compounds in cassava roots during deterioration are scopolin, scopoletin and (+)-gallocatechin. However, the timing of the accumulation of these compounds differs significantly. Whereas scopolin and scopoletin accumulate shortly after harvest, (+)-gallocatechin accumulates later after four to six days. Therefore, we postulate from this that the coumarins are related to PPD whereas the flavan-3-ols are more related to microbial decay. The main features of this work have been published in two papers [42, 43] (Appendices 4 and 5).

Reactive oxygen species.
By the use of a specific stain sensitive for hydrogen peroxide we showed that all cassava tubers accumulate H_2O_2 in the parenchymatic tissue during PPD. Differences between the cultivars occurred only in the exact timing of accumulation (Fig. 1). Histochemical investigations of the tissue revealed that H_2O_2 occurred in the apoplast of the cells (Fig. 2A). By using a luminol-based method for the quantification of H_2O_2 we were able to perform comparative analysis of different cassava cultivars. It revealed that H_2O_2 accumulation occurred directly after harvest in all cultivars and all over the tuber (Fig. 2B). After four to five days the H_2O_2 content started to decline again. In particular, the decline of H_2O_2 correlated with the decline in scopoletin and suggests that there may be a link between this metabolite.
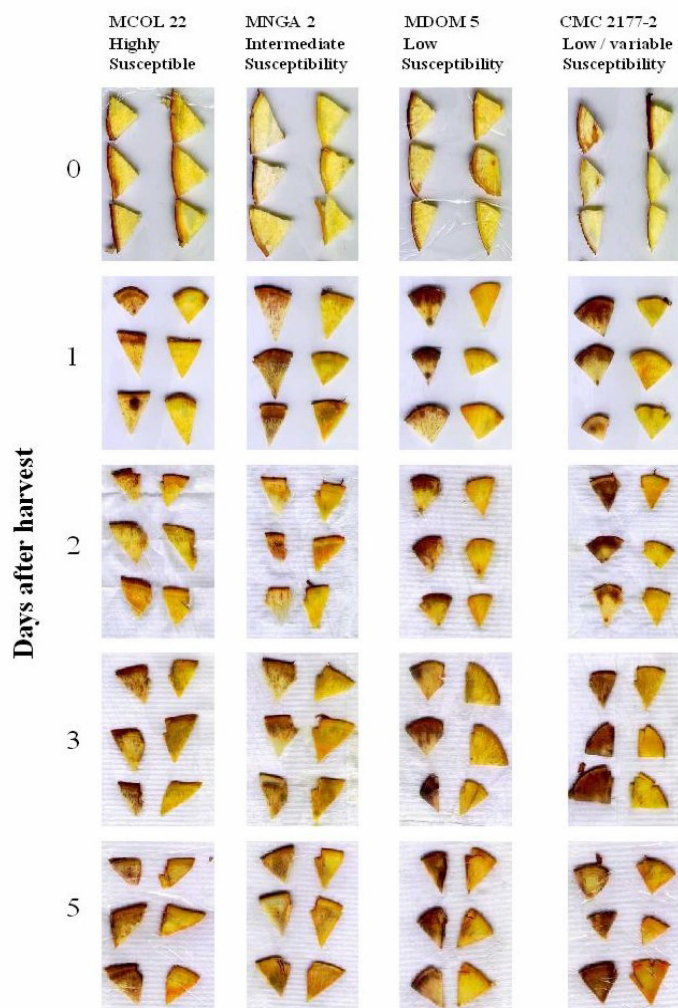
Fig. 1: Detection of H_2O_2 in slices of cassava roots by means of vacuum infiltration with 3,3-diaminobenzidine tetrahydrochloride (DAB). Each column represents a cassava cultivar, each row a different storage time of the roots. In each block the three slices on the left were infiltrated with DAB only, whereas the slices on the right were co-infiltrated with DAB and the antioxidant ascorbate.
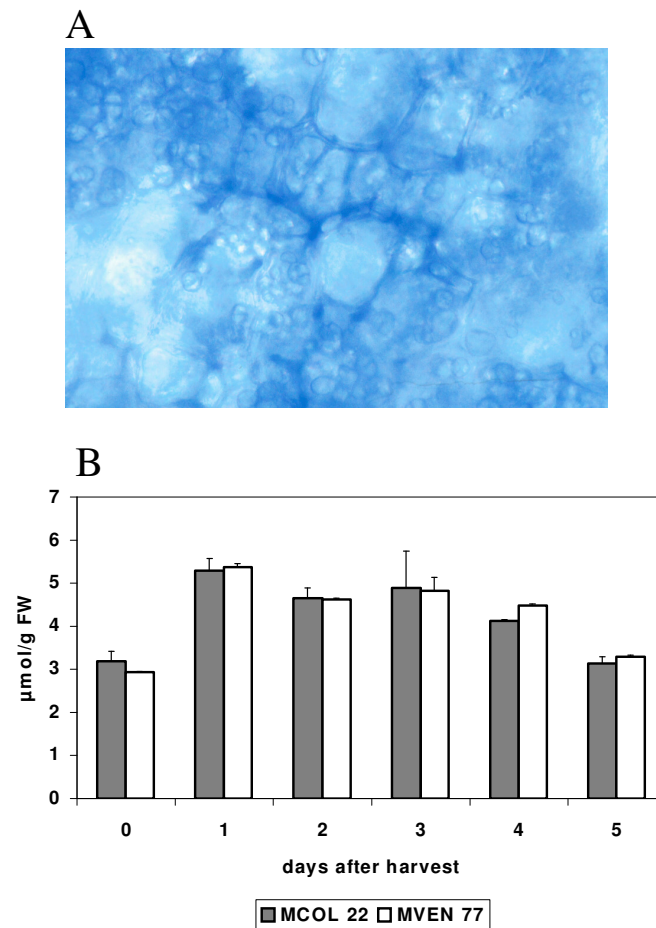
A

B

Fig 2: A) Histochemical localization of H_2O_2 in cassava root tissue by means of staining with iodine. H_2O_2 accumulates rapidly in the apoplast; B: Quantification of free H_2O_2 in cassava root slices of two different cultivars (MCOL 22 [high susceptible] and MVEN 77 [low susceptible]) over a storage period of five days. Each column represents the mean of three different roots per cultivar taken from different plants (mean ± SD).

It was found that, catalysed by anionic peroxidases, scopoletin and hydrogen peroxide formed a blue-black precipitate (Fig. 3). This reaction could explain the parallel decline in scopoletin and H_2O_2 and, at least part, of the blue-black discoloration of the cassava root vascular tissue that is symptomatic of PPD. The detection of other antioxidants in deteriorating cassava roots provides strong evidence for ROS, and compounds and enzymes that modulate ROS, playing a significant role in the PPD response. The finding that carotenoids (strong antioxidants), above a threshold concentration are associated with reduced PPD (see below) provides further support for this view.
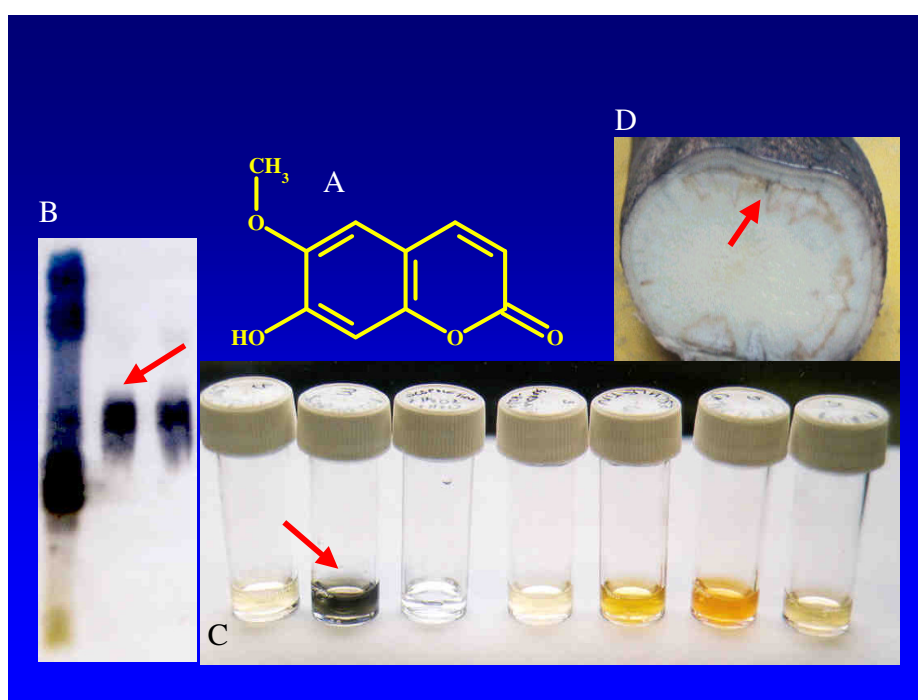

Fig. 3. Scopoletin, H_2O_2 and anionic peroxidases produce a blue-black precipitate. A, Scopoletin; B, isoelectric focusing gel stained with scopoletin and H_2O_2 showing reaction with anionic peroxidase; C, cassava root extract, scopoletin and H_2O_2 producing blue-black precipitate; D, cassava root showing symptoms of PPD.

Wound repair.

The major criterion used to assess wound healing was the suberinisation of specific cells in the parenchyma cells near the wound surface, the potential re-meristematisation of parenchymatic cells and/or the build up of a wound periderm. To investigate these histological changes the tuber tissue near the wound surface were sectioned longitudinal after different storage periods. Light microscopy and histochemical (staining with Sudan III, for suberin, and phloroglucinol, for lignin) investigations of these tissues were performed. In all cases there was dried (white) parenchymatic tissue of 1 to 2 cm underneath the wound surface followed by living parenchyma. In a small area (about 2 to 3 cells thick) at the frontier of dried and vital parenchyma, the cells were brown. However, this coloration of parenchymatic cells was not due to suberinisation (no staining with Sudan III), but to oxidation of phenolic molecules produced by the cells during PPD. In no case there was there any indication of the synthesis of a wound periderm. However, we, and others, have observed wound healing in non-detached roots [41], indicating that some factors that are required for root wound repair may be derived from other parts of the cassava plant.

While the overall wound healing process of the cassava root during PPD was inadequate, other work in the laboratory has shown that components of wound healing were active; for example, a family of extensin genes (including hydroxyproline-rich glycoproteins (HRGPs)) has been isolated. Extensins are insolubilised by H_2O_2 to strengthen the cell wall. Therefore, using an immunological technique, the *in situ* localisation and accumulation of HRGPs on tissue prints was followed over a time course of deterioration in cassava roots with contrasting PPD responses. These results show that HRGPs accumulate throughout the root tissue during the deterioration response and that this accumulation is more extensive in the cultivar with the more rapid deterioration, CM 523-7 (Fig. 5).
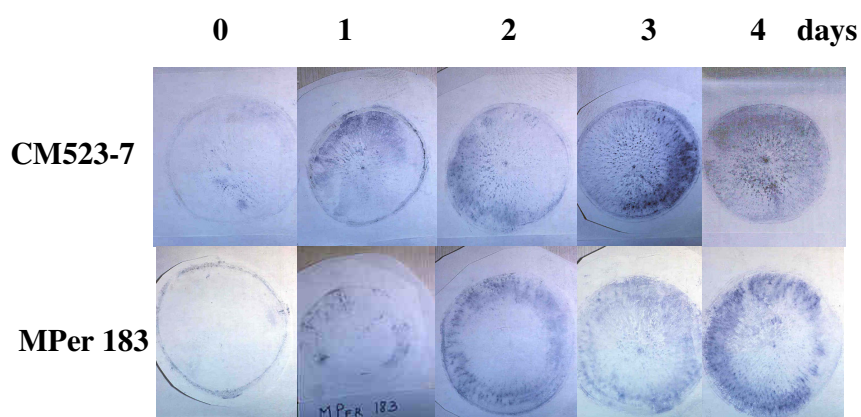

Fig. 5. Accumulation of HRGPs as detected with an anti-HRGP antibody on tissue prints of deteriorating cassava cultivars MPer 183 and CM 523-7.

Genetic perspectives.

Experiments with the mapping population, Family K, and with other crosses, between high and low PPD parents have confirmed conclusively that PPD is a hereditable trait. An ANOVA analysis of the progeny of Family K for its PPD response gave a heritability value of 60%, which indicates that a part of the observed phenotypic variation in PPD has a genetic component. However, they also confirm that the trait is strongly influenced by the environment, which can complicate the interpretation of results. Although PPD is a quantitative variable that is significantly influenced by environment, the genotypic contribution to final character expression is highly significant. However, the environmental influence can be a problem, cultivars, some more than others, can have contrasting PPD scores from one year to the next, and individual roots from the same plant can give different scores. As a result the roots from the one plant produced from a single seed of a cross cannot give reliable date, so a further year is required to give sufficient clonal plants derived from the initial seed for a full evaluation. These problems are probably exacerbated due to the assays being carried out under ambient conditions, outside in the shade. Therefore, the daily differences in temperature and humidity experienced in the tropics will affect the results. Ideally the experiments should be carried out under carefully controlled conditions so as to reduce this variation due to environmental changes during the assay period, but this is impracticable for large numbers of samples. However, the important conclusion from these data is that there is segregation for PPD in the progeny of a cross and that progeny can have more extreme, higher or lower, PPD responses than either of the parents.

2. Specific enzymes, metabolites or structural features showing a strong correlation with high and low PPD identified.

Biochemical changes during PPD

A wide range of cassava cultivars showing differential PPD responses were assayed for changes in secondary metabolites over the time course of PPD. The only significant differences between cultivars that could be reliably detected were ones of timing or the accumulation or activity of these compounds. In general there was an earlier accumulation in those cultivars with a more rapid PPD response. These data are discussed in Buschmann et al. 2000a and 2000b (Appendices 4 and 5).

Different cassava cultivars with contrasting PPD responses were assayed for their changes in enzymatic activity. Enzymes related with wound responses including phenylalanine ammonia-lyase (PAL), peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO), β-1,3-glucanase and chitinase were tested over a seven days storage time course. Of particular interest were polyphenol oxidase, peroxidase and scopoletin peroxidase, which showed differences between high medium and low PPD response cultivars (Fig 6).
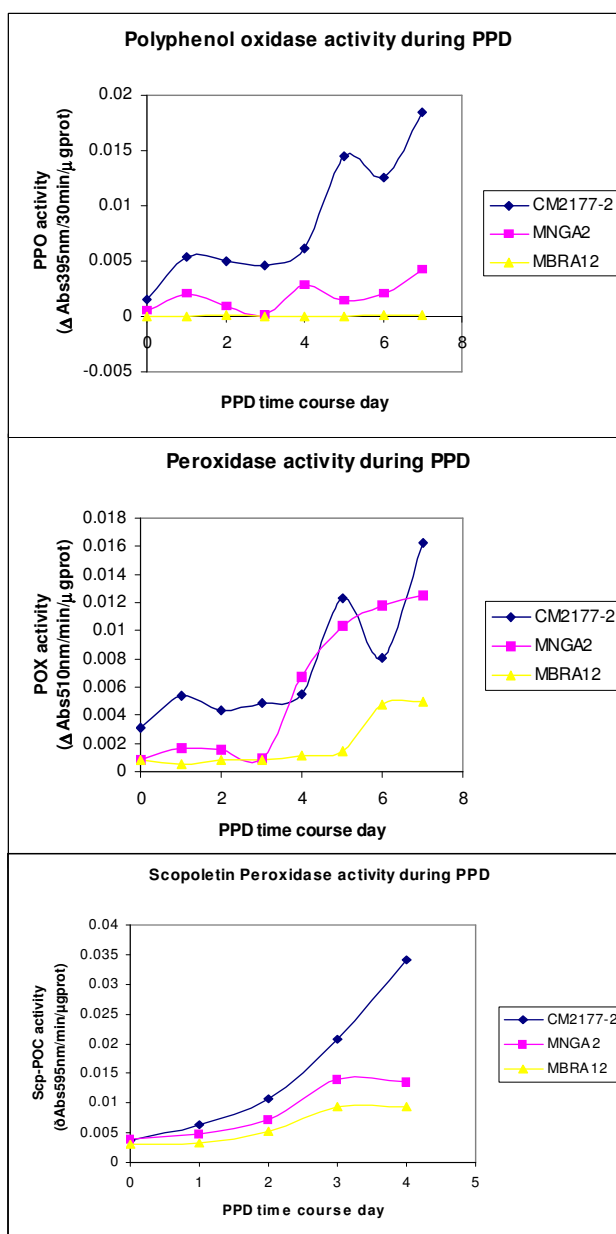
Fig. 6. Polyphenol oxidase, peroxidase and scopoletin peroxidase activity during PPD in three different cassava cultivars: CM 2177-2 – high; MNGA 2 – medium; and MBRA 12 – low PPD response.

PPD markers.
During the analysis of cassava root extracts in 1997 and 1998 two peaks struck were detected that coincided with the onset of PPD in all plants examined. These two peaks were characterized by high retention times in HPLC (RT=47 and 50 min, Fig. 7). In TLC these bands formed one band that migrated the furthest distance (Fig. 8). These compounds seemed to be the ideal

biochemical marker for PPD. Because the UV-spectroscopic data and the retention times of these compounds did not correspond to any of the already isolated compounds we decided to isolate these "PPD markers". But it transpired that these compounds were not present in high concentrations in the fresh prepared extracts from cassava tubers harvested in 1999 and 2000. In HPLC the peaks were hardly detectable and in TLC no band visible. We did not succeed in isolating enough material from these compounds for detailed spectroscopic analysis from material grown in those years and their structural resolution remains to be determined. This environmentally influenced annual variation made these compounds hardly ideal markers. We assume that other factors than / or together with PPD may influence their accumulation.
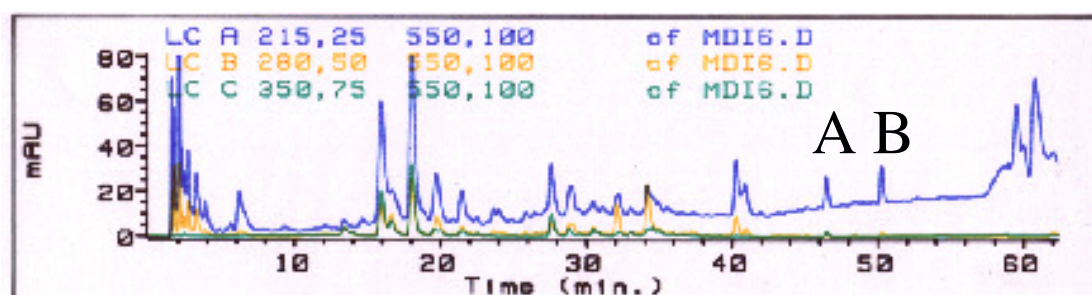

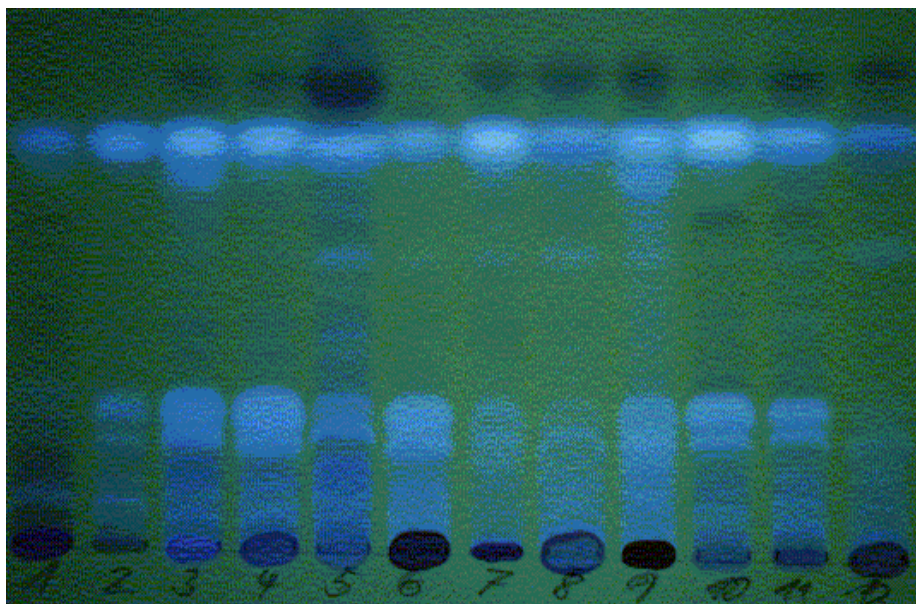Fig. 7. HPLC of cassava extracts showing potential "PPD markers", A and B.

Fig. 8. Thin layer chromatography of cassava root tissues taken over a storage time of 12 days. Blue fluorescence caused by the hydroxycoumarins in the extracts. Upper band caused by an unknown compound that could be used as a marker for PPD.

Carotenoids.

Cassava cultivars contain varying amounts of carotenoids; this can be observed in the range of pigmentation of the root tissues from white to yellow (Fig. 9). Carotenoids, as well as being important micronutrients for human health are strong antioxidants and, as such, could possible play a role in the PPD response. Therefore, the germplasm collection a CIAT was screened for its total carotenoid content and PPD response (Fig. 10). While overall there was no correlation between the carotenoid content of the roots and the PPD response, if one examines the data for those cultivars with over 50 mg/100 g fresh weight, these all have reduced PPD response suggesting that there is a threshold value beyond which this antioxidant plays a role in modulating the PPD response. Therefore, the role of carotenoids in PPD merits further study.

Fig. 9. Cassava varieties with a range of carotenoid content and pigmentation from very yellow to white.
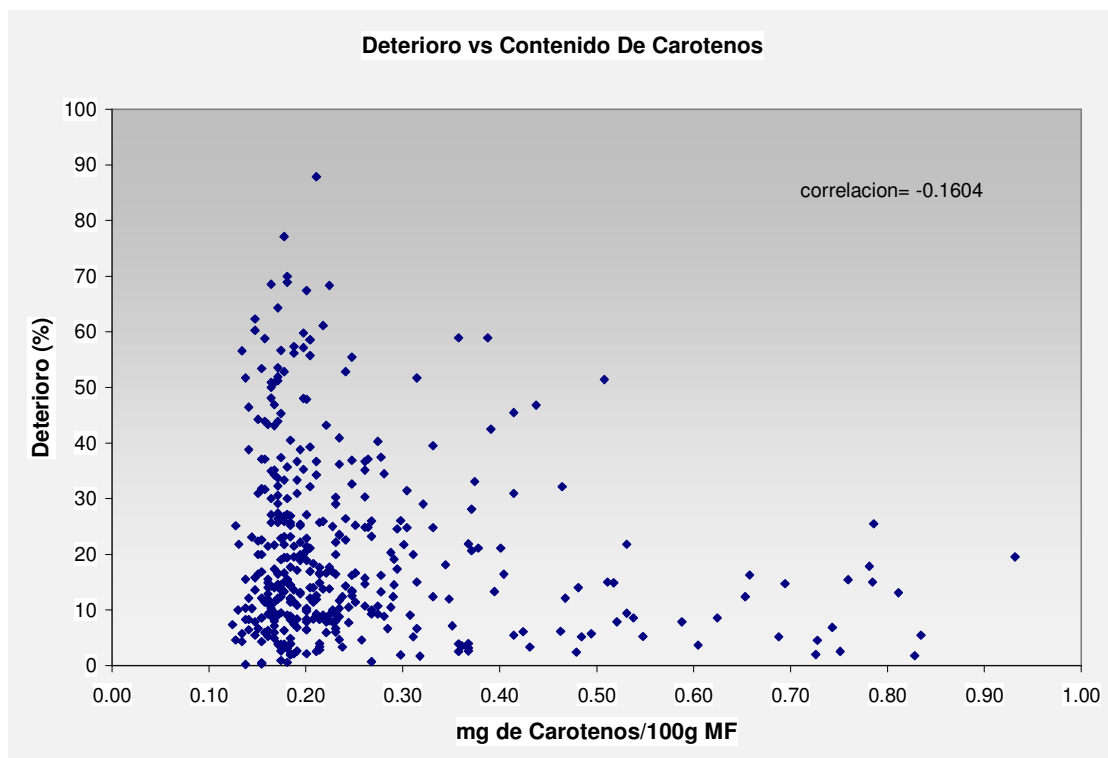

Fig. 10. Plot of % PPD against carotenoid content in the CIAT germplasm collection.

3. Identification of molecular markers linked to PPD-related quantitative trait loci (QTLs) assisted.

The Family K mapping population was assayed twice for its PPD response (the first assay was deemed unreliable for several reasons, including *El Niño* induced climatic changes). Family K was used to generate the molecular map of cassava, and as a result it has been recommended that it, and its parents, TMS 30572 (female) and CM 2177-2 (male), should be the experimental material of choice for genetic and other experiments. While this is appropriate for African cassava mosaic virus (ACMV) or cassava bacterial blight resistance, these experiments demonstrated that it was not particularly appropriate for research on PPD as the parents did not possess strongly contrasting PPD responses and segregation was not altogether clear cut in the progeny.

The QTL analysis based on genetic markers was used as an approach to study PPD in the Family K mapping population in two different agro-ecologies, at Quilichao and Palmira. The genetic map of cassava was based on 240 RFLPs, 100 RAPDs, 5 isoenzymes and 85 SSRs [44] was used to identify the QTLs involved in PPD. Phenotypic evaluation of PPD data was regressed on the genotypic classes of the molecular markers, using the single-point and interval analyses of the QGene program [45]. The significant QTLs identified were located in the linkage groups Nga U, Nga P, Nga X, Nga G, Nga M, Nga J, Nga L and Nga E in the female map and Cm C, Cm L, Cm A, and Cm E in the male map (Table 1).

From the simple linear regression, markers in eight linkage groups of the female map and five linkage groups of the male map were associated with QTLs for PPD at the significance level of α = 0.01. When α = 0.001, only two (female map) and one (male map) linkage groups contained markers linked to putative QTLs.

Thus, while QTLs associated with the PPD response were identified, the correlations were not strong. Therefore, there is now recognition of the need for new crosses between parents with distinctly contrasting PPD responses and the generation of new molecular maps.

Marker	Location	Linkage group	Parent source	R^2	Probability	Additivity
GY138	Quilichao	NgU	Nga2	0.12	0	5.36
rGY164	Quilichao	NgU	Nga2	0.09	0.0004	4.97
rNI1.C2	Quilichao	CmC	Nga2	0.13	0.0008	4.94
rSSR83	Palmira	NgP	Nga2	0.09	0.0012	11.18
CDY131	Palmira	NgX	Nga2	0.13	0.0025	13.15
rK16d	Quilichao	NgG	CM 2177-2	0.11	0.0026	-5.47
GY120	Palmira	CmL	CM 2177-2	0.11	0.0028	-11.91
SSR6	Quilichao	NgU	Nga2	0.08	0.0032	4.11
SSR6	Palmira	NgU	Nga2	0.07	0.0045	9.29
GY202	Palmira	NgL	Nga2	0.05	0.0057	8.02
rM5a	Palmira	NgX	Nga2	0.10	0.0070	11.73
rGY22-1	Palmira	CmA	CM 2177-2	0.09	0.0078	-11.20
AC-1	Palmira	NgM	CM 2177-2	0.10	0.0082	-10.95
rGY26	Quilichao	CmC	Nga2	0.09	0.0082	3.89
rE14b	Palmira	NgJ	CM 2177-2	0.10	0.0091	-11.76
rHRGP	Palmira	NgE	CM 2177-2	0.05	0.0094	-7.83
CDY123a	Quilichao	CmE	CM 2177-2	0.09	0.0094	-5.10

Table 1. QTL markers associated with PPD as identified by Qgene.

4.   Parental material for recombination and selection for increasing cassava storability identified.

This Output has only been partially achieved, largely due to the unsuspected inadequacy of Family K for PPD analysis. However, several crosses were made between cultivars with high and low PPD responses and high and low carotenoid content, which were analysed for their PPD response and other relevant factors. These were speculative crosses set up at the beginning of the project; the seeds were harvested and planted out in the second year. From these plants stakes were taken and planted so as to give several clones derived from each seed, each of which would provide several roots, these were harvested at the end of the final year of the project and analysed. In this way it was it hoped to have sufficient replicates to reduce the error. The crosses were:

- Family CM9726 (MDOM 5, female, low PPD x CM523-7, male, high PPD) with 29 progeny
- Family CM9679 (SM1551-18, female, white x CM8371-7, male, yellow) with 33 progeny.
- Family CM9680 (HMC1, female, white x CM8371-7, male, yellow) with 49 progeny.
- Family CM9681 (CM8371-7, female, yellow x MPER 183, male, white, low PPD) with 21 progeny.

The roots were scored for:
- Colour of the peel
- Colour of the flesh
- Thickness of the peel
- Resistance to penetration of the peel
- Length of the root
- Diameter of the root
- PPD
- Dry matter content
- Carotenoid content.

The means of these data were used for analysis. These demonstrated the segregation of these traits in the progenies. However, there were no significant correlation between any of the traits except between colour of the flesh and carotenoid content and between PPD and dry matter content (Fig. 11. There was no correlation between PPD and carotenoid content (Fig. 12). However, it is important to point out that none of the progeny, or their parents, had carotenoid contents above 50 mg/100 g fresh weight, which has been identified as a threshold value above which there exists an association between carotenoid content and reduced PPD. These crosses had been set up before this association was discovered. Therefore, the conclusion is that further crosses are required between parents with higher carotenoid content. It would also be desirable to produce larger progenies from these crosses, but this is hampered by the poor fertility of the parents.
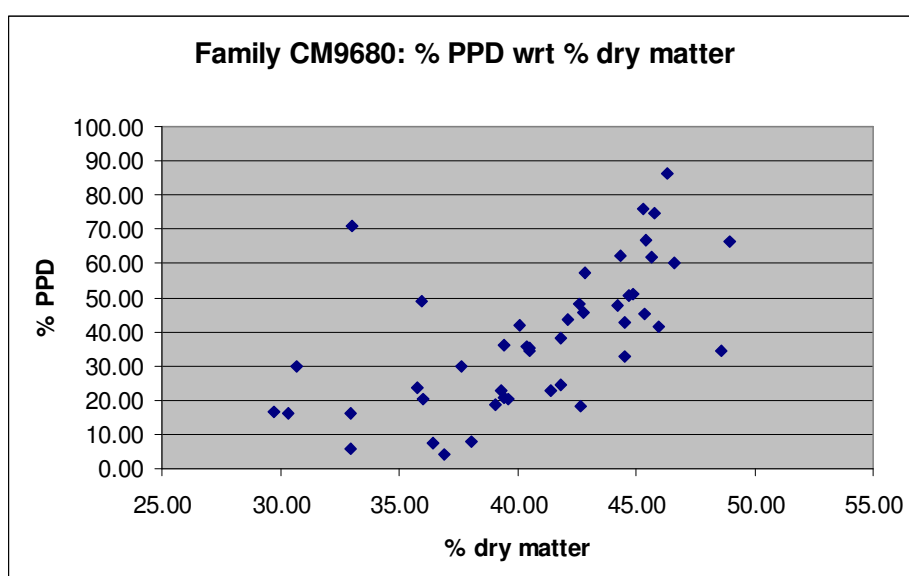

Figure 11. Relationship between PPD and dry matter content in Family CM9680.
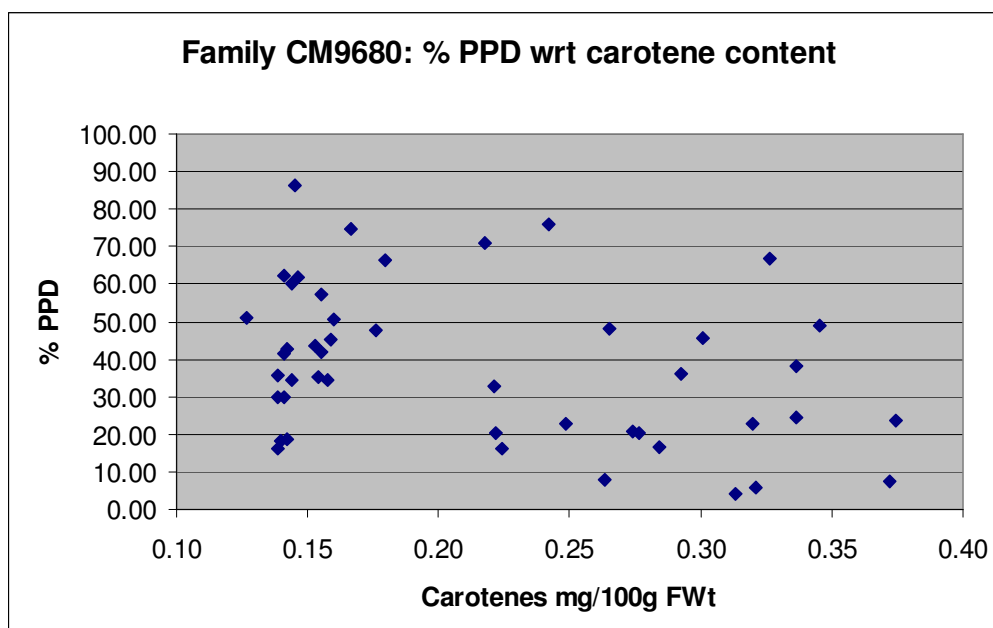
21

Figure 12. Relationship between PPD and carotenoid content in Family CM9680.

While these crosses provided useful understanding of the genetics of the problem that will assist future breeding experiments, they have not so far produced breeding material as such. The close association between dry matter (a desirable characteristic) and the PPD response, together with the environmentally induced variability of the PPD response (see 5, below), suggest the intractability of breeding for delayed PPD. In addition the high heterozygosity of cassava makes improving farmers' preferred varieties via breeding for most traits a complex and unattractive prospect. Therefore, while breeding and genetic experiments can provide invaluable data to assist potential biotechnological solutions, breeding itself will, in turn, require substantial biotechnological input (e.g. marker assisted selection) to be able to make any progress in this area.

5. Stability of biochemical processes under a range of environmental and agro-ecological conditions determined.

The Family K analyses were the most detailed and thorough analyses of the PPD response to date in genetically related or identical plants grown in different agro-ecologies. These conclusively confirmed that the PPD response, though hereditable, is significantly affected by environmental factors. Great individual variation was measured between roots of the same plant and between those of genetically identical plants, superimposed upon which were climatic and agro-ecological induced effects. This variation greatly complicated the collection of good data.

The Family K mapping population was grown simultaneously in two different and contrasting agro-ecological locations, Palmira and Quilichao, in 1998. In Quilichao the observed distribution of the PPD response was asymmetric to the left, presenting a high frequency of low values between 0% and 5%, while in Palmira the population had a normal distribution with respect to the same trait (Fig. 13). The

principal reason to explain the differential genotypic expression between these two environments is the genotype by environment interaction (GxE). Significant differences between the two locations for rainfall, pH, salinity, content of organic material, concentration of ions and permeability exist. These differences could explain the differential genotypic expression, but it was not possible to establish a correlation between a particular environmental variable and the differential gene expression. The subsequent year, 1999, the same plants were grown again in the two locations and assessed for their PPD response. Similar contrasting distributions of PPD responses were observed as previously. However, surprisingly the approximately normal distribution observed at Palmira in 1998 was moved to the left in 1999 (Figure 14). Again, this reinforces the importance of the strong G x E interaction.
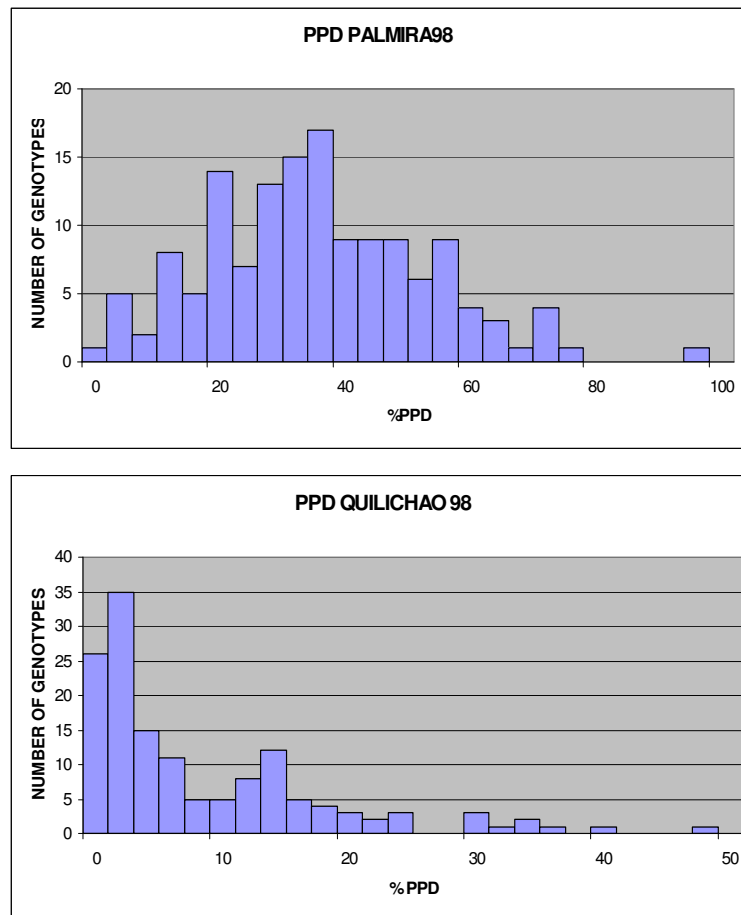

Fig. 13. Distribution of the PPD response amongst the Family K mapping population in two contrasting agro-ecologies in Colombia in 1998.
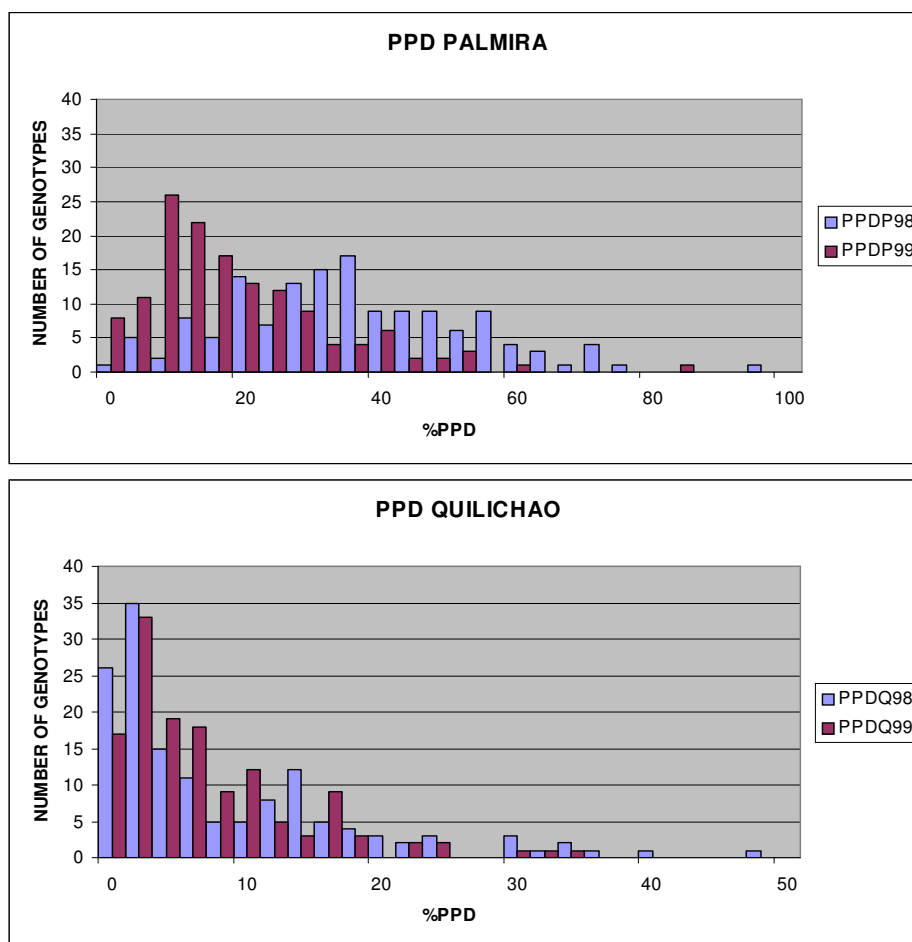
Fig. 14. Comparison of distributions of the PPD response amongst the Family K mapping population in two contrasting agro-ecologies in Colombia in 1998 and 1999.

Traditionally, a visual scoring system has been used to measure PPD that has been suspected as being subjective and unreliable; a more objective tool would be welcome by researchers. The biochemical analytical methods (secondary metabolites and enzymes) developed in this project provided more objective and precise measures of key components of the PPD response. However, they were subject to the same inter-root variability as the visual method. Therefore, the conclusion was that both the visual and the biochemical methods are measuring closely related aspects of the same trait, PPD, and that the extreme variation detected was due to environmental effects on that trait and not to the particular analytical method used. The recommendation is that, unless a rapid biochemical assay can be devised, the conventional, visual method should be used. However, it is important that the staff involved receive adequate training in the method.

6. Key candidate genes and metabolic pathways with the potential to be manipulated via genetic modification experiments in order to modulate the PPD response identified.

This project has highlighted the role that ROS, and the enzymes and compounds that modulate them, including carotenoids, play in PPD. Genes for the biosynthesis of carotenoids and other anti-oxidants are good candidates for cloning and analysis in transgenic cassava plants; in order to precisely dissect their contribution to delayed PPD. Therefore, the cloning of the four key genes for the biosynthesis of carotenoids from a high carotenoid cultivar followed by their expression a low carotenoid variety is recommended as a test for the exact role of these compounds in the PPD response. In addition, the effect of other anti-oxidants, including vitamin C that is present in cassava, should be examined. The accumulation of these could also be modulated via genetic modification.

The synergistic interaction between this and other Bath-CIAT PPD research has also lead to the identification of senescence genes, phytohormones and signalling compounds as playing key roles in the deterioration response, and as meriting further analysis via physiological and genetic modification experiments. The precise roles of cytokinins, ethylene, absicisic acid, jasmonates and salicylic acid need to be determined, especially as the genes for the biosynthesis of several of these are available, in some instances from cassava itself have been isolated and are available. In order to carry out many of these experiments promoters of PPD-related and –specific genes are required; therefore, a rigorous search for these is called for.

7. Results disseminated and Outputs promoted via reports, scientific meetings and publications to international (CIAT and IITA) and national centres and the wider research community concerned with cassava improvement.

Dissemination of the results and Outputs of this project has been extensive and varied; this have included: CPHP reports, peer-reviewed publications, conference presentations, seminars at international and national research institutes, a radio broadcast, a contribution to a Science Museum exhibition, and informal discussions with colleagues around the world. The public dissemination activities are listed below:

Refereed papers.
BEECHING, J.R., HAN, Y., GOMEZ-VASQUEZ, R., DAY, R., COOPER, R.M. (1998) Wound and defense responses in cassava as related to post-harvest physiological deterioration. *Recent Advances in Phytochemistry* **32**: 231-248.
BEECHING, J.R., BUSCHMANN, H., GOMEZ-VASQUEZ, R., HAN, Y., IGLESIAS, C., LI, H., REILLY, K. and RODRIGUEZ, M.X. (1999) An abiotic stress response in cassava: Post-harvest physiological deterioration. pp. 205-210. In: *Plant Responses to Environmental Stress*. Smallwood, M.F., Calvert, C.M. and Bowles, D.J. (Eds.) BIOS Scientific Publishers, Oxford.
BUSCHMANN, H., REILLY, K., RODRIGUEZ, M.X., TOHME, J., BEECHING, J.R. (2000) Hydrogen peroxide and flavan-3-ols in storage roots of cassava (*Manihot esculenta* Crantz) during postharvest deterioration. *Journal of Agricultural and Food Chemistry*. **48**: 5522-5529.

BUSCHMANN, H., RODRIGUEZ, M.X., TOHME, J., BEECHING, J.R. (2000) Accumulation of hydroxycoumarins during post-harvest deterioration of tuberous roots of cassava (*Manihot esculenta* Crantz). *Annals of Botany*. **86**: 1153-1160.

Other disseminations.

BEECHING, J.R. (1997) Stress responses in cassava. University of Antwerp, Belguim. 18 November. (Seminar).

BEECHING, J.R. (1998) The problem of physiological deterioration in cassava. Corpoica, Bogota, Colombia. 21 January. (Seminar).

BEECHING, J.R. (1998) Stress responses in cassava. CIAT, Colombia. 29, January. (Seminar).

During the autumn of 1997 the Science Museum in London had an exhibition entitled "Future Foods" on the actual and potential contribution of genetic modification to the world's food. JR Beeching advised on an interactive computer display that concerned the genetic modification of cassava with respect to African cassava mosaic virus (ACMV) and PPD.

BUSCHMANN, H., RODRIGUEZ, M.X., GOMEZ-VASQUEZ, R., HAN, Y., IGLESIAS, C., LI, H., REILLY, K., IGLESIAS, C., BEECHING, J.R. (1998) Understanding post-harvest physiological deterioration in cassava (*Manihot esculenta* Crantz). Annual German Botanical Society meeting, 30th August – 6th September, Bremen. (Poster).

RODRIGUEZ, M.X., BUSCHMANN, H., IGLESIAS, C., LI, H., REILLY, K., BEECHING, J.R. (1998) Biochemical changes in cassava (*Manihot esculenta* Crantz) roots during post-harvest physiological deterioration. 4th Dutch-German Workshop on Regulation of Secondary Metabolism. Bad Herrenalb, Germany, 27-29 September. (Poster).

BEECHING, J.R., BUSCHMANN, H., GOMEZ-VASQUEZ, R., HAN, Y., IGLESIAS, C., LI, H., REILLY, K., RODRIGUEZ, M.X. (1998) An Abiotic Stress Response in Cassava: Post-Harvest Physiological Deterioration. Annual Symposium of the Plant Protein Club at York, "Plant Proteins in Abiotic Stress Responses" York, Sept. 27 –Oct. 1. (Oral presentation).

BEECHING, J.R., LI, H., HAN, Y., COOPER, R.M., BUSCHMANN, H., GOMEZ-VASQUEZ, R., TOHME, J., REILLY, K., RODRIGUEZ, M.X. (1998) Post-harvest physiological deterioration in cassava. IVth Scientific Meeting of Cassava Biotechnology Network, Salvador, Brazil, Nov, 5-13. (Oral presentation).

BUSCHMANN, H., RODRIGUEZ, M.X., TOHME, J., BEECHING, J.R. (1998) Qualitative and quantitative changes of phenolic components of cassava (*Manihot esculenta* Crantz). IVth Scientific Meeting of Cassava Biotechnology Network, Salvador, Brazil, Nov, 5-13. (Poster).

RODRIGUEZ, M.X., BUSCHMANN, H., TOHME, J., BEECHING, J.R. (1998) Production of phytoalexins in cassava (*Manihot esculenta* Crantz) root during post-harvest physiological deterioration. IVth Scientific Meeting of Cassava Biotechnology Network, Salvador, Brazil, Nov, 5-13. (Poster).

BUSCHMANN, H., RODRIGUEZ, M.X., TOHME, J., BEECHING, J.R. (1998) Qualitative and quantitative analysis of secondary metabolites in cassava roots during post-harvest physiological deterioration. Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela, 9 November. (Seminar).

BEECHING, J.R., HAN, Y., LI, H., REILLY, K., BUSCHMANN, H., GOMEZ-VASQUEZ, R., RODRIGUEZ, M.X., CORTES, D.F., TZIMAS, G., COOPER, R.M. and TOHME, J. (1999) Improving a tropical crop: Cassava. *Molecular Biology's Role in Enhancing Agricultural Productivity*. Amsterdam, Holland. 14-16 March. (Poster).

BEECHING, J.R. (1999) Cassava. *"Who'd Have Thought It?" BBC World Service.* April. 30 minutes. (Radio interview).

BUSCHMANN, H. (1999) Biochemical insights into cassava post-harvest physiological deterioration. CIAT, Colombia. 6 August. (Seminar).

BEECHING, J.R. (2000) Post-harvest physiological deterioration in cassava. CIAT, Colombia. 20 January. (Seminar).

BEECHING, J.R., (2000) Post-harvest physiological deterioration of cassava. Global Cassava Development Strategy: Validation Forum. FAO, Rome. 26-28 April. (Poster).

BEECHING, J.R., (2000) Post-harvest physiological deterioration in cassava. International Society for Tropical Root Crops Symposium. Tsukuba, Japan. September 11-15. (Oral presentation).

BEECHING, J.R., (2000) Post-harvest physiological deterioration of cassava. Central Tuber Crops Research Institute, Trivandrum, India. 26 September. (Seminar).

BEECHING, J.R., (2000) Post-harvest physiological deterioration of cassava. Central Food Technological Research Institute, Mysore, India. 4 October. (Seminar).

BEECHING, J.R., (2000) Post-harvest physiological deterioration of cassava. IITA, Nigeria. November. (Seminar).

BEECHING, J.R., (2001) Deterioracion fisiologica post-cosecha en yuca. Colombian Biotechnology Programme in Agriculture – International Seminar. Cartagena, Colombia. 21 – 23 February. (Invited plenary speaker).

So far uptake of Outputs can be seen in 1/ the stimulation of non-DFID funded PPD research at CIAT; 2/ a Rockefeller Foundation-funded collaborative project between Bath and ILTAB, USA, using transgenic cassava to dissect the role of phenylalanine ammonia lyase (PAL) in PPD. PAL is an important enzyme in many aspects of the deterioration response; 3/ an agreement by IITA for them to screen their germplasm collection for its PPD response; and 4/ an agreement by INIVIT, Cuba, to screen their germplasm collection for its PPD response (a preliminary screening has already been carried out at INIVIT).

Contribution to Outputs

The Outputs of the project have been successfully achieved. While in some cases the details have not always been those anticipated, the depth and diversity of the data generated has enabled the identification of potentially productive ways forward towards the modulation and ultimate control of PPD. In addition this research has stimulated related research in international and national institutes. Research into the understanding and control of PPD in cassava is now firmly established on the map and is recognised not only as a priority but also as a researchable and solvable constraint by relevant institutions such as CIAT, IITA, INIVIT and the Danforth Center. New knowledge on PPD has been generated through the combined application biochemical and genetic approaches; this has led to the production of new understanding of the deterioration process and the identification of new tools (e.g. anti-oxidant biosynthesis genes, phytohormones and signalling compounds) for tackling the problem. Breeding and genetic experiments have provided some invaluable insights into PPD that will assist future research towards its control; at the same time these experiments have revealed certain weaknesses in this approach. While new conceptual and practical tools have been developed, other existing tools, such as Family K, have been discarded as inappropriate for the

purpose in hand. As a result of this work it is now possible to narrow the field of potential ways forward to tackle PPD.

This was a strategic project, which, while its ultimate goal is the poverty elimination through developing aspects of sustainable livelihoods, it never set out to solve the problem of PPD within its lifespan. Rather its aim was to generate knowledge and tools for the understanding of the problem, to point the way forward towards likely avenues for the modulation of PPD, and to stimulate the uptake of its Outputs via PPD-related research at CG centres and NARS. In all these areas the project has succeeded. PPD has always been seen as a major constraint to the development of cassava, now it is recognised as a problem that can be tacked and ultimately solved through collaborative international research, and as a result is high on the agenda in proposals being developed by the centres mentioned above. There is a need to maintain the momentum that has been generated; funding for the next stages should come from a variety of bodies including DFID. It is particularly encouraging that a planned Global Cassava Project proposal linking appropriate CG centres, NARS and laboratories in the US and Europe considers PPD amongst its four priority themes.

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Appendices

APPENDIX 1

Logical Framework for R6983

APPENDIX 2
istological Atlas of Cassava
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PPENDIX 3
Draft of paper to Microscopy and Analysis

APPENDIX 4
Buschmann H, Rodriguez MX, Tohme J, Beeching JR. (2000) Accumulation of hydroxycoumarins during post-harvest deterioration of tuberous roots of cassava (*Manihot esculenta* Crantz). *Annals of Botany.* 86: 1153-1160.

APPENDIX 5
Buschmann H, Reilly K, Rodriguez MX, Tohme J, Beeching JR. (2000) Hydrogen peroxide and flavan-3-ols in storage roots of cassava (*Manihot esculenta* Crantz) during postharvest deterioration. *Journal of Agricultural and Food Chemistry.* 48: 5522-5529.

APPENDIX 6
Beeching JR, Han Y, Gómez-Vásquez R, Day RC, Cooper RM. (1998) Wound and defense responses in cassava as related to post-harvest physiological deterioration. *Recent Advances in Phytochemistry.* 32: 231-248.