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FACULTE DES SCIENCES
DEPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE VEGETALES
****
CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE, SANTE ET ENVIRONNEMENT

REPUBLIC OF CAMEROUN
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UNIVERSITY OF YAOUNDE I
FACULTY OF SCIENCE
DEPARTMENT OF PLANT BIOLOGY
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CENTRE FOR RESEARCH AND DOCTORAL TRAINING IN GRADUATE STUDIES IN LIFE SCIENCES, HEALTH AND ENVIRONMENT

Cassava postharvest physiological deterioration: a complex phenomenon involving calcium signaling, reactive oxygen species and proteome regulation

THESIS
Presented in partial fulfillment of the requirements
for Doctorate/PhD in Plant Biology

Par : **DJABOU MOUAFI ASTRIDE STEPHANIE**
Master with thesis (DEA) in Plant Biotechnologies

Sous la direction de
BOUDJEKO Thaddee
Associate Professor
OMOKOLO NDOUMOU Denis
Professor

Année Académique : 2018

UNIVERSITÉ DE YAOUNDÉ I
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FACULTÉ DES SCIENCES
FACULTY OF SCIENCE

DÉPARTEMENT DE BIOLOGIE ET DE PHYSIOLOGIE VÉGÉTALES
DEPARTMENT OF PLANT BIOLOGY

Yaoundé, le 24 SEPT 2018

ATTESTATION DE CORRECTION DE THÈSE DE DOCTORAT/Ph.D

-:-:-:-:-:-:-

Nous soussignés membres du jury de la thèse de Doctorat/Ph.D de Madame **DJABOU MOUAFI Astride Stéphanie**, Matricule **05W063**, soutenance autorisée par la correspondance **N°181436/UYI/VREPDTIC/DAAC/DEPE/SPD/CB-ebo** de Monsieur le Recteur de l'Université de Yaoundé I en date du 16 Août 2018, et soutenue **le 6 Septembre 2018**, sur le thème **« Cassava post-harvest physiological deterioration : a complex phenomenon involving calcium signalling, reactive oxygen species and proteome regulation»**, certifions qu'elle a effectué les corrections conformément aux remarques et recommandations du jury.

En foi de quoi, nous lui délivrons cette attestation de correction pour servir et valoir ce que de droit. /.

Rapporteur

NJOUEFEKO Thaddée, Ph
Maître de Conférence
Université de Yaoundé

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(By Department and by Grade)
DATE OF ACTUALISATION : 10 Mars 2018

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4	KAMGANG Pierre	Professeur	En poste
5	MEDJO EKO Robert	Professeur	*Coseiller Technique/UYII*
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7	NDAM NGOUPAYOU Jules-Remy	Maître de Conférences	En poste
8	NGOS III Simon	Maître de Conférences	DAAC/Uma
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19	MOUNDI Amidou	Maître de Conférences	*CT/ MINIMDT*
20	BEKOA Etienne	Chargé de Cours	*En poste*
21	BISSO Dieudonne	Chargé de Cours	*Directeur/Projet Barrage Memve'ele*
22	ESSONO Jean	Chargé de Cours	*En poste*
23	EKOMANE Emile	Chargé de Cours	*En pste*
24	FUH Calistus Gentry	Chargée de cours	*Sec. D'Etat/MINMIDT*
25	GANNO Sylvestre	Chargé de Cours	En poste
26	LAMILEN BILLA Daniel	Chargé de Cours	En poste
27	MBIDA YEM	Chargé de Cours	*En poste*
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31	NGO BELNOUN Rose Noël	Chargée de Cours	En poste
32	NGO BIDJECK Louise Marie	Chargée de Cours	En poste
33	NGUETCHOUA Gabriel	Chargé de Cours	CEA/MINRESI
34	NYECK Bruno	Chargé de Cours	En poste
35	TCHAKOUNTE J. épse NOUMBEM	Chargée de Cours	*CT / MINRESI*
36	METANG Victor	Chargé de cours	En poste
37	NOMO NEGUE Emmanuel	Chargé de cours	En poste
38	TCHAPTCHET TCHATO De P.	Chargé de cours	En poste
39	TEHNA Nathanaël	Chargé de cours	En poste
40	TEMGA Jean Pierre	Chargé de cours	En poste
41	MBESSE CECILE OLIVE	Chargée de cours	En poste
42	ELISE SABABA	Chargé de cours	En poste
43	EYONG JOHN TAKEM	Assistant	En poste
44	ANABA ONANA Achille Basile	Assistant	En poste

Répartition chiffrée des Enseignants de la Faculté des Sciences de l'Université de Yaoundé I

NOMBRE D'ENSEIGNANTS					
DÉPARTEMENT	**Professeurs**	**Maîtres de Conférences**	**Chargés de Cours**	**Assistants**	**Total**
B.C.	5 (1)	10 (5)	20 (10)	3 (1)	**39 (16)**
B.P.A.	11 (1)	9 (3)	20 (8)	3 (5)	**43 (17)**
B.P.V.	4 (0)	9(2)	10 (2)	4 (4)	**27 (8)**
C.I.	9(1)	8(2)	16 (4)	0 (2)	**33 (9)**
C.O.	8 (0)	13 (3)	8 (2)	1 (0)	**30 (5)**
I.N.	3 (0)	1 (0)	8 (0)	12 (3)	**24 (3)**
M.A.	3 (0)	5 (0)	18 (1)	4 (0)	**30 (1)**
M.B.	2 (0)	5 (2)	6 (2)	0 (0)	**13 (4)**
P.H.	8 (0)	17 (0)	15 (2)	2 (1)	**42 (3)**
S.T.	5 (0)	15 (2)	22 (3)	2 (0)	**44 (5)**
Total	**58 (3)**	**92(19)**	**144 (33)**	**31(16)**	**325(71)**

Soit un total de **325(**71**)** dont :

- Professeurs **58 (3)**
- Maîtres de Conférences **92 (**19**)**
- Chargés de Cours **144 (33)**
- Assistants **31** (16**)**

( ) = Nombre de Femmes

DEDICATION

To

My husband Jean Daniel FONDJO

Our daughter PINGSIE FONDJO Priscila Josepha

My parents MOUAFI Appolinaire and PENE Jeanne

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ABBREVIATIONS

2-DE: Two-dimensional gel electrophoresis
APX: ascorbate peroxidase
BSA: Bovine Serum Albumine
CaM: calmodulin
CAT: catalase
CHAPs: 3[(3-Cholamidopropyl) dimethylammonio]-propanesulfonic acid
CIAT: International Centre of Tropical Agriculture
CSR: cassava storage roots
Cu/ZnSOD: copper/zinc superoxide dismutase
DAH: day after harvest
ddH_2O_2: double distilled water
DNA: Desoxyribonucleic acid
dNTP: desoxyribonucleotide triphosphate
DTT: Dithiothreitol
H_2O_2: Hydrogen peroxide
HSP: Heat shock protein
IEF: Isoelectric focusing
IPG: Immobilized pH gradients
mA: milliamper
MALDI: Matrix-assisted laser desorption/ionization
MAPK: Mitogen activate protein kinase
Mr: relative molecular mass
MS/MS: tandem mass spectrometry
MS: Mass spectrometry
NAD: nicotinamide adenine dinucleotide
NADH: Nicotinamide adenine dinucleotide reduced form
NADPH: Nicotinamide adenine dinucleotide phosphate reduced form
NBT/BCIP: nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
NCBI: National Center of Biotechnology Information
PAGE: Polyacrylamide gel electrophoresis
PCD: Programmed cell death
PMN: Protein Metabolic Network
POD: peroxidase
PPD: Post harvest physiological deterioration
qRT-PCR: quantitative real time polymerase chain reaction
QZ1: Qiong Zhong 1
RanGTPase: Ras-related Nuclear protein guanosine triphosphatase

RNA: Ribonucleic acid
ROS: Reactive oxygen species
SC5: South China 5
SDS: Sodium dodecyl sulphate
SOD: superoxide dismutase
STRING: Search Tool for the Retrieval of Interacting Genes/Proteins
TOF: Time-of-flight
U: Unit
V: Volt
W: Watt

ABSTRACT

The production of cassava (*Manihot esculenta* Crantz), the fifth most important starch crop worldwide after rice, wheat, maize and potato, is limited by many factors among which the short shelf life of its storage roots is a major constraint. This phenomenon known as postharvest physiological deterioration (PPD), is a syndrome attributed to a cascade of signalling events triggered by reactive oxygen species (ROS) at the wounded site of the tubers. The consequence is the rapid deterioration of cassava storage roots 24 – 48 hours after harvest, which renders the roots unpalatable and unmarketable. However, the molecular mechanisms and the interactions between different pathways involved in PPD in cassava are still poorly understood. This thesis focuses on investigating the interactions between Ca^{2+}-calmodulin, ROS and proteome to fine-tune PPD process. An approach to delay PPD through the modulation of calmodulin gene expression and ROS activities using calcium and magnesium fertilizers was also assessed.

Two cassava genotypes, South China 5 (SC5) susceptible and Qiong Zhong 1 (QZ1) tolerant to PPD were selected for this study. The experiment was performed at the Tropical Crops Genetic Resources Institute of Chinese Academy of Tropical Agricultural Science (CATAS) at Danzhou-Hainan (China). The activities of antioxidant enzymes of cassava storage roots during storage in PPD-susceptible (SC5) and PPD-tolerant (QZ1) genotypes were investigated at the time-points 0, 3, 6, 15 and 30 days after harvest (DAH). The analysis of their proteome changes using two-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry (MALDI-TOF-MS/MS) was performed. In addition the effect of fertilizer containing calcium and magnesium fertilizers on antioxidant activities and PPD onset was tested.

The results of the two-dimensional gel electrophoresis (2-DE) analysis demonstrated that there were 106 differentially expressed proteins (DEPs) in the two genotypes in the pairwise comparison of 3DAH/0DAH, 6DAH/0DAH, 15DAH/0DAH and 30DAH/0DAH from which 63 identified in QZ1 and 75 in SC5. The most DEPs were identified by MALDI-TOF-MS/MS as defense proteins (25 % and 23 %), carbohydrate and energy metabolism-associated proteins (19 % and 21 %), chaperones (14 % and 21 %) and antioxidant related proteins (13 % and 7 %) in QZ1 and SC5 respectively. PPD development also induced qualitative and quantitative changes in protein profiles, depending on the genotype. Western blot analysis of some expressed proteins confirmed the results of 2-DE. Antioxidant activities showed that cassava storage roots undergoing PPD are subjected to reactive oxygen species (ROS). High amounts of hydrogen peroxide (615.67 ± 3.41 µmol/g FW) in SC5 compared to

QZ1 (252.38 ± 3.10 µmol/g FW) was observed at 6DAH corresponding to the date of the onset of PPD in SC5 confirming the implication of Hydrogen peroxide in PPD onset. Subsequently, the increased activities of ascorbate peroxidase, catalase (CAT), peroxidase and superoxide dismutase (SOD) were observed in PPD susceptible genotype SC5 at early stage of storage (3 to 6 DAH). The results suggest that SOD in combination with CAT could be the first line of defense against PPD to support PPD-tolerant cassava varieties. In addition, ethylene highly produced in SC5 (44.44 ± 1.80 ng/ml FW and 48.23 ± 1.37 ng/ml FW compared to 33.88 ± 0.42 ng/ml FW and 37.12 ± 0.32 ng/ml FW in QZ1 respectively at 3 and 6DAH) may act alone as an up-stream signal or simultaneously with CAT in response to the accumulation of H_2O_2 since a positive correlation was observed between these two components at a level of 5 %. The detection of calmodulin (CaM) at protein level coupled to the high relative expression level of its gene before the peak of hydrogen peroxide suggest it as a possible trigger of ROS during wound induced PPD in cassava storage roots (CSRs).

The application of calcium and magnesium fertilizers delayed PPD onset for more than 10 days in the susceptible genotype (SC5); but magnesium fertilizer elevated PPD in the tolerant genotype by early onset at 6DAH in roots from plant treated with magnesium. In addition, Ca^{2+} fertilization led to a significant increase of H_2O_2 content (275 ± 0.6 µmol/g FW and 255.4 ± 0.01 µmol/g FW respectively in QZ1 and SC5 in roots from plant treated with calcium compared to the controls 245.8 ± 0.03 µmol/g FW and 263.6 ± 1.16 µmol/g FW for SC5 and QZ1 respetively. This increase of H_2O_2 was followed by an elevation of the activities of catalase and peroxidase in both genotypes, especially at harvest. Our findings suggest that soil fertilization with fertilizers containing calcium and magnesium may play a distinct role in PPD delay in cassava storage roots.

These data further extend our knowledge about the chronology of the cascade of events occurring during PPD and the interaction between ROS production, calmodulin and proteome during PPD process. The results also provide new methodology to enhance PPD tolerance in cassava susceptible genotypes and reduce postharvest losses. Moreover, the results confirm that PPD is an active and complex process involving crosstalk between different pathways.

Key words: *Manihot esculenta*, postharvest physiological deterioration, Ca^{2+}-calmodulin, ROS, proteome, fertilization.

RESUME

La production du manioc (*Manihot esculenta* Crantz), la cinquième culture riche en amidon après le riz, le blé, le maïs et la pomme de terre, est limitée par plusieurs contraintes, dont la principale est la courte durée de conservation de ses racines. Ce phénomène connu sous le nom de détérioration physiologique post-récolte (DPP), est un syndrome attribué à une cascade d'événements déclenchés par les espèces réactives d'oxygène (ERO) au niveau du site de la blessure après la récolte. Il en résulte une détérioration rapide des racines 24 à 48 heures après leur récolte, les rendant ainsi désagréables et non commercialisables. Cependant, les mécanismes moléculaires et les interactions entre les différentes voies impliquées dans la DPP du manioc sont encore mal compris. Ce travail avait pour objectif principal l'étude des interactions entre le Ca^{2+} - calmoduline, les ERO et le protéome pour mieux comprendre la chronologie de la cascade des évènements qui se produident au cours de la DPP. En outre, une approche pour retarder la DPP à travers la modulation de l'expression de la calmoduline et des activités antioxydantes à travers l'utilisation des fertilisants riches en calcium et en magnésium a également été présentée.

Deux génotypes de manioc South China 5 (SC5) sensible et Qiong Zhong 1 (QZ1) tolérant à la DPP ont été sélectionnés pour cette étude. L'expérience a été réalisée à l'Institut des ressources génétiques des cultures tropicales de l'Académie chinoise des sciences agricoles tropicales de la ville de Hainan-Chine. Les activités antioxydantes des racines de manioc ont été évaluées les 0, 3, 6, 15 et 30^{e} jours après la récolte (JAR). Les profils protéiques ont été évalués par électrophorèse bidimensionnelle (2-DE) en combinaison avec spectrometrie de masse.

Les analyses protéomiques ont permis d'identifier 106 protéines différentiellement exprimées chez les deux génotypes à la suite des comparaisons par paires des jours 3JAR/0JAR, 6JAR/0JAR, 15JAR/0JAR et 30JAR/0JAR. Parmi ces protéines, 63 protéines ont été identifiées chez le génotype QZ1 et 75 protéines chez le génotype SC5. La majorité des protéines différentiellement exprimées étaient les protéines de défense (25 % and 23 %), des protéines associées au métabolisme des hydrates de carbone (19 % and 21 %), les protéines de chocs (14 % and 21 %) et les protéines liées aux activités antioxydantes (13 % and 7 %) respectivement chez QZ1 et SC5. La mise en place de la DPP induit également des changements qualitatifs et quantitatifs dans les profils protéiques en fonction du génotype. L'analyse par Western blot a confirmé les résultats des électrophorèses bidimensionnelles. Des teneurs élevées en péroxyde d'hydrogène (615,67 ± 3,41 µmol/g MF) chez SC5 comparée à QZ1 (252,38 ± 3,10 µmol/g MF) le 6^{e} jour de conservation correspondant au jour de l'apparition de la deterioration chez SC5, confirmant ainsi l'implication du péroxyde

d'hydrogène dans la mise en place de la DPP. Subséquemment, des activités antioxydantes accrues d'ascorbate péroxydase, catalase, péroxydase et superoxyde dismutase ont été observées chez ce génotype sensible les premiers jours de conservation (Jours 3 et 6). Le superoxyde dismutase en combinaison avec la catalase agiraient en première ligne de défense contre la DPP pour soutenir les variétés de manioc tolérantes à la DPP. De plus, l'éthylène produit en grande quantité chez le génotype sensible en début de conservation (44,44 ± 1,80 ng/ml MF et 48,23 ± 1,37 ng/ml MF chez SC5 comparé à 33,88 ± 0,42 ng/ml MF et 37,12 ± 0,32 ng/ml MF chez QZ1 respectivement au 3^{e} et au 6^{e} jours de conservation), agirait seul comme signal en amont ou simultanément avec la catalase en réponse à l'accumulation du péroxyde d'hydrogène puisqu'une corrélation positive au seuil de 5% a été observée entre ces deux composants. La détection de la calmoduline (CaM) au niveau du profil protéique additionnée aux expressions relatives élevées de son gène observées avant le pic de péroxyde d'hydrogène laissent penser qu'elle serait le stimulateur des espèces réactives d'oxygène et subséquemment à l'origine de la mise en place de la DPP au cours de la conservation des racines de manioc.

L'application de fertilisants à base de calcium et de magnésium a permis de retarder la DPP de plus de 10 jours chez le génotype sensible. De plus, les racines issus des plants fertilisés au calcium ont montré une augmentation significative de leur teneur en péroxyde d'hydrogène (275 ± 0,6 µmol/g MF et 255,4 ± 0,01 µmol/g FW respectivement chez QZ1 et SC5 chez les racines issus des plants traités au calcium comparés aux controls 245,8 ± 0,03 µmol/g MF et 263,6 ± 1,16 µmol/g MF respectivement chez SC5 et QZ1). Cette augmentation de la teneur en peroxide d'hydrogène était suivi d'une élévation des activités catalase et peroxydase chez les deux génotypes, en particulier au moment de la récolte. Ces résultats suggèrent que la fertilisation du sol avec des engrais contenant du calcium et du magnésium pourrait jouer un rôle distinct dans le retard de la mise en place de la DPP chez les racines de manioc.

Ces données apportent d'amples informations sur la chronologie de la cascade d'évènements qui se produisent au cours de la DPP des racines de manioc en ressortant les interactions entre la production des espèces réactives d'oxygène, la calmoduline et le protéome au cours du processus. Ils présentent également une nouvelle approche pour améliorer la tolérance à la DPP de certains génotypes sensibles de manioc à la DPP et de réduire ainsi les pertes post-récolte. En outre, ils confirment que la DPP est un processus actif et complexe impliquant différentes voies métaboliques.

Mots clés: *Manihot esculenta,* détérioration physiologique post-récolte, Ca^{2+}-calmoduline, espèces réactives d'oxygène, protéome, fertilisation

GENERALITIES

I.1.INTRODUCTION

Cassava (*Manihot esculenta*, Crantz) is a staple food for more than 800 million people in Tropical regions and an income generator for many people in developing countries (Kumba et *al.,* 2012). Compared to other tuber crops such as potato (*Solanum tuberosum* L.), sweet potato (*Ipomoea batatas* L. Lam), cocoyam (*Xanthosoma sagittifolium* L. Schott) and yam (*Dioscorea alata* L.), the shelf life of cassava storage roots is very short due to a rapid deterioration after harvest. Postharvest physiological deterioration (PPD) is an inherent constraint for the distribution and processing of cassava. PPD is also a complex phenomenon involving different metabolic pathways such as change in reactive oxygen species (Zidenga et *al.,* 2012; Xu et *al.,* 2013), proteins (Owiti et *al.,* 2011; Vanderschuren et *al.,* 2014) and gene expression (Reilly et *al.,* 2007). In addition, a wide variety of soils, environments and hormones such as ethylene seem to be involved in cassava PPD (Kawano & Rojanaridpiched, 1983; Hirose et *al.,* 1984).

Estimated losses due to PPD reach up to 8 %, 10 % and 29 % in Asia, Latin America and Caribbean and Africa, respectively (Anonymous 1, 2000). Root damage during harvest alters the equilibrium of natural physiological processes of the exposed cells and the whole storage root bursts upon oxidative stress. Changes in response to cell damage after harvest include accumulation of fluorescent compounds and secondary metabolites (Buschmann et *al.,* 2000), decrease of starch content, increase in cell respiration and enzymatic activities including regulation of reactive oxygen soecies (ROS) synthetase (Xu et *al.,* 2013; Zidenga et *al.,* 2012) as well as phenylammonia-lyase.

Even if ROS production isknown as one of the early events observed when plants are submitted to biotic and abiotic stresses, the events that trigger their production are still poorly understood. Recent research has shown the implication of Ca^{2+} in the release of ROS in wound-induced resistance in plants (Sagi & Flur, 2006; Beneloujaephajri et *al.,* 2013). In response to wound induced by *Botrytis cinerea*, it was found that non-wounded leaves of *Arabidopsis thaliana*treated with Ca^{2+} inhibitors were more susceptible to pathogens, suggesting the importance of Ca^{2+} in the induction of basic resistance (Beneloujaephajri et *al.,* 2013). Interestingly, a co-localization of the changes in Ca^{2+} and a burst of ROS were observed after pathogenic or environmental stresses (Ranf et *al.,* 2011). The implication of Ca^{2+}-calmodulin on PPD development in cassava (Owiti et *al*., 2011) as well as the influence of fertilizers containing Ca^{2+}/Mg^{2+} on the delay of fruit ripening (Aghofack-Nguemezi and Dassie, 2007; Park et *al.*, 2005) and stress tolerance (Jiang & Huang, 2001; Jiang et *al.,* 2005)

have been reported in many plants. Ethylene is induced upon a wide range of abiotic stresses such as wounding, flooding, chilling and drought (O'Donnell, 1996; Wang, 2002). Change in ethylene levels in damaged cassava storage roots was reported to be high and continued to increase few hours later (Hirose, 1984).

Cassava storage root is of great interest for starch industries. The problem of PPD induced by inevitable wounds on the tubers at harvest is a great problem for cassava transport and commercialization. Plant wound responses are triggered by signals that initiate different types of reactions. Identification of wound-response elicitors activated in cassava and the genes/proteins response need to be characterized in order to understand the processes involved in cassava physiological deterioration. Proteomics constitute one of the most promising technologies for the functional characterization of multi-protein complexes such as those involved in biochemical damages occurring in plants. The use of proteins to elucidate the biochemical features of postharvest physiological deterioration was previously studied by Owiti et *al.* (2011). Even if this study showed the involvement of different pathways in plant metabolism such as reactive oxygen species turnover, programmed cell death, defense pathway, cell-wall metabolism and signaling pathway, the study did not consider the susceptibility and the tolerance of the genotypes to PPD process. In addition, the chronology of the cascade of the events remains unclear and this affects the strategies put in place to control the phenomenon. In this thesis, we use proteomic data to describe the onset of postharvest physiological deterioration in cassava susceptible and tolerant genotypes by exploring the implication of Ca^{2+}-Calmodulin, ROS and proteins related to programmed cell death. In conjunction with the proteomic analysis, the transcriptome initiative was used to identify genes encoding interest identified proteins.

General objective:

This thesis aims at using significant advances in calcium and calmodulin signaling to highlight cassava postharvest physiological deterioration (PPD) mechanism in tolerant genotype QZ1 and susceptible genotype SC5 using proteomic analysis.

The specific objectives of the research were to:

1- Evaluate the antioxidants activities and the protein dynamics that occur in both genotypes during PPD;
2- Investigate an approach to modulate PPD by increasing tuber calcium content and antioxidant activities through fertilization of cassava plant by calcium/magnesium fertilizer.

I.2. LITERATURE REVIEW

I.2.1. Origin and distribution of cassava

Cassava is a tropical perennial shrub of the family Euphorbiaceae (Alves, 2002) whose main characteristic is the presence of lactifers and the production of latex. This family also includes *Hevea brasiliensis* (rubber), *Ricinus communis* (Castor bean). It was reported that cassava was cultivated in northern Amazonia more than 1000 years ago (Jones, 1959) and was transported to Africa by the early European traders during the 16th and 17th centuries. It was introduced to most of Asia and the Pacific in the late 18th and early 19th centuries (Onwueme, 2002). Then, cassava cultivation has spread to the tropical and subtropical regions where it is grown from sea level up to altitudes of 1800 m within the equatorial belt between 30° north and 30° south of the equator (Alves, 2002).

I.2.2. Taxonomy and diversity

Cassava called “manioc” in French, “tapioca” in India, “yucca” in Spanish and “mandica” in Portuguese, is a perennial shrub grown mainly for its carbohydrate rich tuberous roots. The systematic hierarchy of cassava is presented below (Table I).

Table I.Taxonomic Classification of cassava (CIAT, 1984; Alves, 2002)

Kingdom:	Plantae
Phylum:	Spermaphytes
Sub-phylum:	Angiospermes
Class:	Dicotyledoneae
Sub-class:	Archichlamydeae
Order:	Euphorbiales
Family:	Euphorbiaceae
Tribe:	Manihoteae
Genus:	*Manihot*
Species:	*Manihot esculenta* Crantz

The Euphorbiaceae consists of plants with very different growth habits including trees, shrubs and grasses; and of diverse economic importance. Some produce latex (*Hevea brasiliensis*), others produce oil (*Ricinus communis*) or edible roots (*Manihot spp*.) and some behave as weeds (*Euphorbia spp*.). There are also ornamental and medicinal Euphorbiaceae.

The genus *Manihot* ischaracterized by shrubs adapted to savannah, grassland or desert (Jennings & Iglesias, 2002). It comprises 98 species and all the species studied so far have a chromosome number 2n=36 (Allem, 2002; Nassar, 2002). The wide genetic diversity of cassava results from the easy cross -pollination of the species, its high heterozygosity and its abrupt fruit dehiscence. Thus there are numerous cultivars of the species (Carlos et *al.*, 1984). Natural hybridization can result in polyploid plants such as triploids (2n = 3x = 54 chromosomes) and tetraploids (2n = 4x = 72 chromosomes). The largest cassava germplasm bank is located at the International Centre of Tropical Agriculture (CIAT), Colombia with approximately 6500 accessions, followed by Embrapa collection in Cruz das Almas-Bahia (Brazil) with around 4000 accessions and the International Institute of Tropical Agriculture (IITA), Nigeria with around 3700 Accessions (Allem, 2002).

I.2.3. Cassava botany

Cassava is a perennial shrub of 1 to 3 min height (Fig. 1A) cultivated for its starchy tuberous roots. Many cultivars or varieties of cassava are cultivated in the subtropical and tropical countries. They can be distinguished by their morphological characteristics such as leaf size, color and shape, branching habit, plant height, color of stem and petiole, tuber shape and color, maturity period and yield (IITA, 1990). The cassava plant is made up of a shoot system and root system. The shoot system consists of stem, leaves, and flowers and the root system consists of fibrous roots and storage roots (IITA, 1990). Stems are woody and either non-branching or branched usually with large diameters. Pigmentation of the stem varies from grey to yellow, orange or brown providing one of the most stable characteristics for differentiation of cultivars. Fully developed vegetative leaves have five to nine lobes. However, leaves in association with the inflorescence are invariably reduced in number of lobes (Rogers, 1965). Flowering in cassava is associated with branching, hence, an early branching genotype may start flowering as early as three months after planting while non-branching types do not flower (Hahn et *al.*, 1973). Although cassava is regarded as an allogamous species, considerable selfing may occur, especially in profusely flowering genotypes (Kawano et *al.*, 1978). Two types of roots are observed; fibrous roots and storage roots. The fibrous root has the function of fixing and absorbing nutrients and water, while the tuberous roots store carbohydrate in the form of starch. The storage root (Fig. 1B) develops to a maximum of 0.30 m into the soil, and their ability to absorb nutrients and water decreases considerably (Alves, 2002). Cassava contains two cyanogenic glucosides, mainly linamarin

and a small amount of lotaustralin, which are catalytically hydrolyzed to release toxic hydrogen cyanide (HCN) when the plant tissue is crushed (McMahon et *al.*, 1995). Several varieties of cassava have been identified and grouped into bitter (HCN> 100 mg/kg) and sweet with lower levels of glycoside (Nassar & Ortiz, 2006).The fruit (Fig. 1C) is a dehiscent capsule (when dry) with three locules, each containing a single seed.

Fig. 1. Cassava morphology (Photographs taken by Djabou, 2015).
A=Aerial part of cassava plant; B= cassava storage roots; C= Cassava fruit

I.2.4. Propagation and ecology of cassava

Cassava is propagated vegetatively by stem cuttings. This makes it very advantageous in the sense that in periods of food shortage, the farmer does not have to save a part of the plants edible products for replanting inthe field the next cropping season (Anderson et *al.*, 2000). The crop has a flexible planting and harvesting time which explains its critical role for food security. The cuttings are planted horizontally or inclined on ridges (Keating, 1988). The ideal growth temperature ranges from 24 to 30 °C for optimum growth, production and maximum leaf photosynthesis (El-Sharkawy, 1992). Cassava plants show high heterozygosity and low natural fertility, poor seed set and seed germination. The plants arising from sexual seeds are normally weaker than those obtained from cuttings since they are usually homozygous for recessive and prejudicial genes (Nassar, 2007). The resilience of cassava enables it to grow successfully under a wide range of agro-climatic conditions where few crops could survive without costly external inputs (Puonti-Kaerlas, 1998). The ideal soils for cassava cultivation are light sandy loam with medium fertility (IITA, 1990). Soils with a superficial hard layer or with many stones are not suitable for cassava growth (Alves, 2002; Chavez et *al.*, 2005). Cassava storage roots are a rich source of vitamins and minerals. Cassava storage root can be harvested from 7 to 24 months after planting. This strongly

depends on the variety and the growing locality. However, the tuberous roots can remain under the ground for longer periods until the roots are needed, making it a useful crop to fight against famine (Wenham, 1995; Puonti-Kaerlas, 1998). With these characteristics, cassava is supposed to be a major source of food and an industrial crop in the coming decades. As a matter of fact, it will play a key role in the fight against hunger and malnutrition in the world.

I.2.5. Cassava storage root physiology

There are numerous cassava cultivars with diverse storage root characteristics such as number, bulking time or diameter. Cassava storage roots are derived entirely from root tissue and therefore cannot be considered a true tuber as those derived from stem tissue (Timothy, 2009). As a consequence, the storage roots cannot be used for vegetative propagation as they should in true tuber crops, because of the absence of primordial buds. The cassava storage root is made up of three distinct tissues: bark (periderm), peel (cortex) and the edible parenchyma (Fig. 2). The periderm (3 % of the total weight) is a thin layer thatis partially sloughed off during storage growth. The peel constitutes 11 - 20 % of the fresh root weight with remainder 77 - 86 % accounting for the edible parenchyma (Alves, 2002).

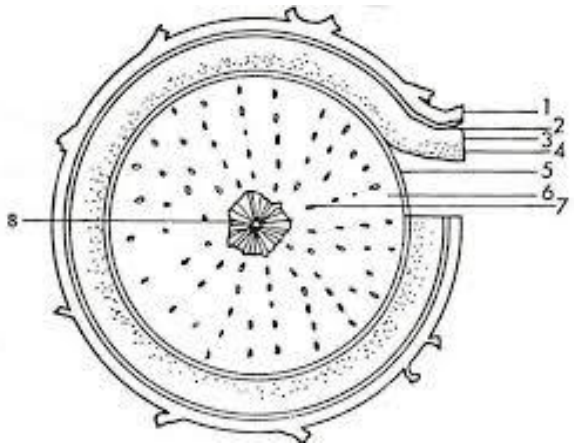

Fig. 2. Transverse section of a cassava storage root (Hunt et *al.*, 1977).

1 = bark/periderm; 2 = sclerenchyma; 3 = cortical parenchyma; 4 = phloem; 2-4 = peel; 5 = cambium; 6 = storage parenchyma; 7 = xylem vessel; 8 = xylem vessels and fibers; 5-8 = edible parenchyma (Hunt et *al.*, 1977).

I.2.6. Production, importance and utilization

I.2.6.1. Production

The total estimated world production of cassava in 2016 was 288.427 million tons of fresh roots according to Anonymous 3 (2016). Almost 60 percent of this production was concentrated in five countries Nigeria, Brazil, Thailand, Indonesia and the Democratic Republic of Congo (Ohimain, 2015). Yields in cassava vary depending on the variety and soil type (El-Sharkawy, 2004). Africa is by far the biggest producer of cassava (more than 50 % of the total World production). Nigeria comes first with 19 % of global market share and its cassava transformation is the most advanced in Africa (Egesi et *al.*, 2006). China has emerged as the leading cassava importer, procuring mostly feed ingredients. In China, 99 % of the cassava growing areas are in the southern provinces of Guangxi, Gouandong, Hainan and Yunnan. The annual planting area in China is about 400 000 hectare, with a total production estimated at 6 000 000 tonnes (Tian et *al.*, 2000). The average world cassava yield is estimated at 12.4 t/ha with African countries having the lowest yields and Asian countries having the highest yields. Since 2000, the world's annual cassava production has increased to an estimated 100 million tons, driven in Asia by demand for dried cassava and starch for use in livestock feed and industrial applications and in Africa by expanding urban markets for cassava food products (Anonymous 3, 2013). Even if cassava has generally not received the deserved attention by governments in the past, it is now being recognized as an important staple crop, a major tool in fighting hunger and poverty and as an export commodity in developing countries.

I.2.6.2. Importance and utilization

According to the FAO (Food and Agriculture Organization of the United Nations), cassava is currently the third most important source of calories in the tropics after rice and corn (Anonymous 2, 2013). More than 800 million people use cassava as a source of food and income in Africa, Asia and Latin America (Alabi et *al.*, 2011). In Africa, about 93 % of the production is used as food (Nweke et *al.*, 2002). Cassava is primarily cultivated for its high carbohydrate storage root content which may lie between 85 – 91 % of the total dry weight (Alves, 2002). However, in addition to providing a small amount of protein (1.76 - 2.68 % fresh weight; 0.77 - 8.31 % fresh weight), cassava storage roots are a rich source of vitamins and minerals. It is an important and cheap source of carbohydrate in tropical regions,

particularly in sub-saharan Africa. As food, cassava is a good source of energy since it has a higher energy density of about 610 KJ/100 g than other crops such as sweet potato with 460 KJ/100 g and taro with 490 KJ/100 g (Bradbury & Holloway, 1988). Both cassava leaves and roots are consumed (kehinde, 2006). Leaves provide an important protein intake (Khampa & Wanapat, 2006) where as roots are a cheap source of carbohydrate (Nwokoro et *al.*, 2005). Cassava is also rich in calcium, vitamin C and contains nutritionally significant quantities of thiamine, riboflavin and nicotinic acid. Bio-fortification has enhanced the vitamin A (beta-carotene) content of improved varieties (Ngozi et *al.*, 2013).

In America, cassava is greatly used for animal feed, accounting for approximately one third of consumption and human food represents only 42 % of the production. Starch also represents an important use of cassava in South America. The situation in Asia is greatly influenced by the export of cassava chips by Thailand to the European community which uses it as animal feed (Westby, 2002). Cassava starch and flour can be used in food industries to produce biscuits, bread and derivatives (Echebiri & Edaba, 2008). With the rising cost of energy, especially in liquid fuel, cassava is seen as a preferred feed stock for ethanol production especially in situations where water availability is limited for the cultivation of sugarcane. In addition, cassava provides unparalled food security due to its drought tolerance, low nutrient requirements, generalized resistance to herbivores and the ability to leave the roots in the soil attached to the plant for up to 3 years.

I.2.7. Research status and developmental trend of cassava in Cameroon

In Cameroon, cassava is the first most cultivated tuber crop and a staple food for about 8 million people. The way it is consumed (processed, prepared and packed) is heterogeneous across cities, ethnic groups, or regions. Its production is of strong importance with regard to basic food intake of the population, representing 20 % of cultivated land and around 46 % of national food crop production. National production was estimated to be around 3.1 millions tons in 2010 and has remained steady over the last years (Tolly, 2013). Cassava products represent 60 percent of the roots and tubers' market share, comprising 40 percent of processed products (fufu, gari, sticks, and waterfufu) and 20 percent of fresh roots. The main production areas for cassava are concentrated in the Southwestern region and another part in the littoral region (Tolly, 2013). The area devoted to its cultivation was estimated at 204.548 hectares with an annual production of 4.8 million tons (Anonymous 3, 2014). Cassava is mainly produced by smallholder farmers, whose farms are usually less than 2 ha, receiving low input

and pooragricultural practices and often subjected to pest attack. The problems of cassava production in Cameroon are the threat of diseases and pests, human resources (cassava is cultivated by women already overloaded with household work), the lack of modern processing techniques (lack of adapted equipment), physiological constraints relating to the consumption of cassava products (goitre), poor knowledge of markets and volatility of prices, the perishable character of harvested fresh tubers worsenby the problem of access to markets (bad state of roads). These favour the process of deterioration which continues to be a huge challenge to the commercialization of cassava in eastern and central Africa. Without this, the production chain of cassava from the field, storage and transportation to the processing centers could benefit from a longer shelf life than the current situation. This aspect has drawn limited research attention in Cameroon. However, many industries in Cameroon need fresh roots of cassava to incorporate, either natural or in the form of modified starch, in their production process (Mouafor et *al.,* 2016). Research on cassava in Cameroon is mainly focused on cassava mosaic disease (Akinbade et *al.,* 2010; Fondong & Chen, 2011) and cassava anthracnose (Ambang et *al.,* 2016). The identification of new clones which can demonstrate a good adaptation in fields with high yield compared to local varieties is not left out. In this case many improved varieties have been released from Nigeria. However, to the best of our knowledge, there is no current study about cassava postharvest deterioration in Cameroon.

I.2.8. Research status and developmental trend of cassava in China

Cassava is an important upland crop in China and plays an important role in many rural areas. China is the fifth cassava producer in Asia with about 4.5 million tons (Anonymous 3, 2014). Guangxi and Hainan provinces account for about 60 % and 7.5 % of both the planted area and production in China respectively. The cassava processing industry is mainly concentrated in Guangxi and Guangdong provinces. Of approximately 525000 tons of cassava based products processed annually in China, 75 % comes from Guangxi, about 20 % from Guangdong, 6 % from Hainan and 2 % from Yunnan (Tian et *al.,* 2000). About 95 % of these products are starch or modified starch (Tian et *al.,* 2000). However, cassava production in China faces some liabilities such as the lack of an effective organization and management system, lack of good varieties and low yields, serious soil erosion and decline in soil fertility and the problem of rapid deterioration of the roots after harvest. Though cassava is a hardy crop, its increased production is hampered by multiple liabilities.

I.2.9. Constraints to cassava production

I.2.9.1. Biotic constraints

In spite of its adaptability to edaphic and climatic factors, cassava is vulnerable to pests and diseases that can cause heavy yield losses. The most important disease of cassava is cassava mosaic virus disease (Fig. 3A). It occurs in all cassava-producing regions in Africa, India and Sri Lanka resulting in annual yield losses estimated at one billion pounds (Fargette et *al.,* 1988). In sub-saharan Africa cassava mosaic disease causes major economic losses between 20 - 80 % of total yields and can result in complete crop failure (Fregene & Puonti-Kaerlas, 2002; Alabi et *al.,* 2011). Another important disease is cassava bacterial blight which (Fig. 3B) has caused more damage to the crop leaves in the last decade, but is now considered to be of minor importance compared to cassava mosaic virus disease because of the development by researchers of integrated control measures which have limited its spread in many cassava-growing areas. Cassava anthracnose disease (Fig. 3C) caused by *Colletotrichum gloeosporioidessp.manihotis* is the most important fungal disease of cassava in the field (Hahn et *al.,* 1989; Ambang et *al.,* 2016). The most outstanding effect of the disease is its ability to cause severe stems damage causing canker on stem, wilting of leaves and diebacks (Owolade et *al.,* 2005). Several pests feed on cassava including cassava whiteflies, cassava green mites (*Mononychellus tanajoa*), cassava mealybugs (*Phenecoccus manihoti*), and nematodes particularly (*Meloidogyne spp*.). Whiteflies are the most damaging insect pests in all cassava producing regions. Although some farmers use insecticides to control them, spraying is usually ineffective. A two-year experiment in Cameroon found out that intercropping cassava with maize and cowpeas was associated with a drop of 50 % in the adult whitefly population and a 20 % reduction in the incidence of cassava mosaic disease (Ambe, 1993). Cassava mites can be controlled using resistant or tolerant varieties and by fertilizing the crop to improve plant vigor.

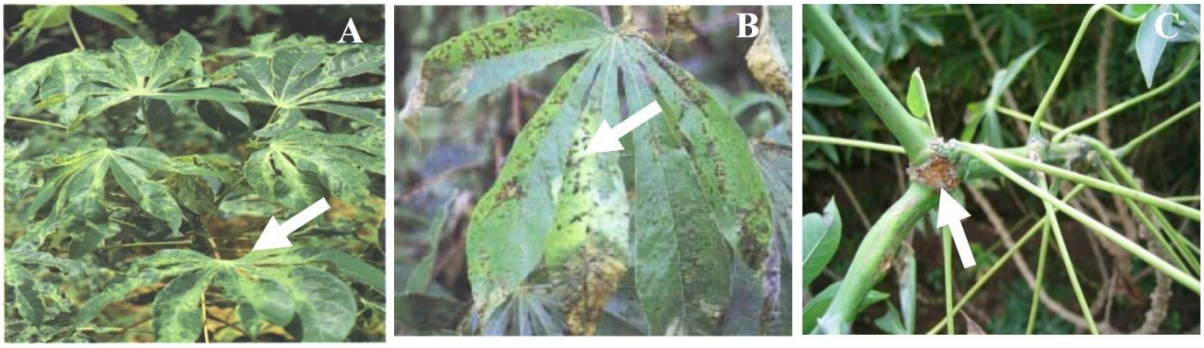

Fig. 3. Morphological observation of some cassava abiotic constraints.

A: cassava mosaic virus, B: Cassava bacterial blight, C: Anthracnose

I.2.9.2. Abiotic constraints

Environmental factors that impact on cassava production include soil erosion, low soil fertility, infestation of the fields with weeds and poor climatic conditions. Poor soil fertility was identified as the most important abiotic constraint despite the general perception that cassava is tolerant to poor soil fertility (Howeler, 2002; Fermont et *al.*, 2009). Low soil fertility affects many cassava-growing areas because the fallow periods are shorter as the pressure on arable land near homesteads is increasing. However the growing of cassava continuously for many years without adequate fertilization in soils that tend to be of low fertility and susceptible to erosion contribute to a further decline in soil fertility (Anonymous 1, 2000). Low winter temperature mainly in southern China and North Vietnam was also identified to have a negative impact in cassava production; however cassava's relatively poor adaptation to cool temperature is compensated by its tolerance to poor soils in these areas (Anonymous 1, 2000). Cassava is also quite susceptible to competition of weeds especially at the early growth stage. Failure of timely weeding can cause a total loss of harvest.

I.2.10.Cassava post-harvest deterioration

I.2.10.1. Definition and impacts

Cassava storage roots are far more perishable than other staple food crops. Subsistence and commercial utilization of cassava are affected by its short shelf-life due to a rapid postharvest physiological deterioration process (PPD). PPD is a process of deterioration which takes place on the site of damage, causing general discoloration of the vascular parenchyma throughout the root. The duration of cassava shelf life depends on the cultivars, harvest practices and handling, and storage conditions. However, PPD commonly occurs

within 72 hours after harvest and renders the root unpalatable (Buschmann et *al.,* 2000; Iyer et *al.,* 2010). The consequence is the prompt consumption or processing of the cassava roots after harvesting. The process of deterioration is divided into two phases' namely primary deterioration and secondary deterioration (Buschmann et *al.,* 2000; Reilly et *al.,* 2004; Reilly et *al.,* 2007). The primary deterioration is initiated by mechanical damage which occurs during harvesting. This is known as postharvest physiological deterioration (Fig. 4B). The visible signs are black - blue to black discoloration or vascular streaking which begins at the broken or cut surfaces and subsequently spreads to the adjacent storage parenchyma and the stored starch undergoes structural changes. This primary deterioration is characterized by physiological and biochemical changes, and does not involve microorganisms (Noon & Both, 1977). The secondary deterioration (Fig. 4C) is due to the infection with microorganisms leading to fermentation and softening of the root tissue (Plumbey & Rickard, 1991; Wenham, 1995). PPD has a significant impact than secondary deterioration since; the coloration of the roots is used as a marker for its cooking quality in the market.

Traditionally, PPD has not been a significant problem as cassava has been grown and consumed or processed locally immediately after harvesting. However, with increasing urbanization in less developed countries, the entry of rural farmers into cash economy and processing on a larger industrial scale (Westby, 2002), the times and distances between field and consumer or processor has increased, and PPD has become a major constraint to the development of cassava for farmers, processors and consumers (Beeching, 2002). Estimated losses due to PPD reach up to 10 % and 8 % in Latin America and Caribbean and in Asia respectively, whereas in Africa they reach 29 % (Anonymous 1, 2000). The short shelf-life severely limits the marketing options because it increases the likelihood of losses, marketing costs and access to urban markets is limited to those close to the production sites. For FAO, the increase of the storage life of cassava root to a minimum of two weeks could have a substantial effect on cassava utilization (Van Oirschot et *al.,* 2000).

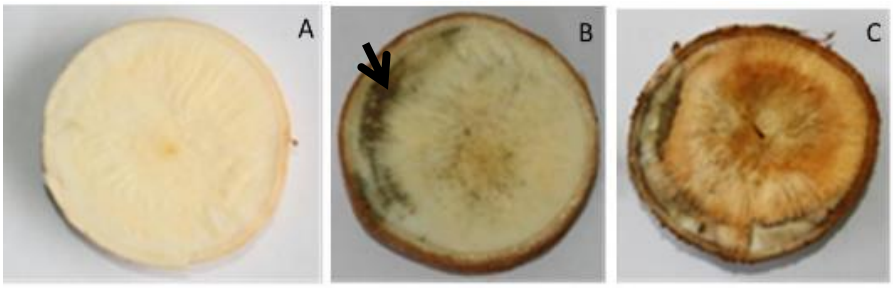

Fig. 4. Cross sections of cassava storage roots (Djabou et *al.*, 2017).

Freshly harvested storage roots (A); roots three days after harvest under PPD process marked by black arrow (B); roots at ten days after harvest under secondary deterioration (C).

I.2.10.2. Method of evaluation of postharvest physiological deterioration (PPD)

The method of evaluating PPD in cassava roots is a key step leading to a better understanding of events taking place during PPD and subsequently, the identification of varieties with delayed PPD. Several methods have been used to evaluate the susceptibility of cassava roots to PPD.

These are:

- Subjective visual scoring using entire roots (Booth, 1977; Pino, 1979);
- Subjective analysis of biochemical and physiological changes in transverse sections of roots under ultraviolet (UV) light (Uritani et *al.*, 1983) ;
- Use of the severity of discoloration during PPD (Uritani et *al.*, 1983);
- Scoring of transverse sections of the root by visual inspection of the root pith during PPD (Wheatley, 1982).

All these strategies are based on subjective evaluations and there is the need to implement an objective quantitative and systematic phenotypic evaluation of PPD (Han et *al.*, 2001). Buschmann et *al.* (2000) and Oirschot et *al.* (2000) have suggested the measuring of UV fluorescent compounds (hydroxycoumarins) as biochemical markers to assess PPD susceptibility. Significant differences of hydroxycoumarin have been found among cassava varieties using High Presure Liquid Chromatography (HPLC). However, these differences do not correlate with PPD susceptibility (Buschmann et *al.*, 2000; Oirschot et *al.*, 2000; Salcedo et *al.*, 2010). Another alternative to evaluate PPD is the measurement of the sugar/starch ratio (Oirschot et *al.*, 2000) where there is a strong correlation between them. However, it would be difficult for this method to be widely accepted since sugar/starch ratio in cassava is strongly affected by environmental and geographical conditions. In the present study, PPD evaluation

was performed by cross section of the middle of the entire roots (Booth, 1977 & Wheatley, 1982).

I.2.10.3. Physiology of postharvest physiological deterioration

The process of PPD is considered to resemble a typical wounding response in which the healing process is inadequate (Wenham, 1995; Beeching et *al.,* 1998). In fact, common wound responses in plants involve several events which begin by the production of signals that act firstly on the wound site through the production of some components like Jasmonic acid, abscissic acid, salicylic acid and hydrogen peroxide. Secondly, it is followed by the production of defensive enzymes and molecules for the protection against pathogens. The effects of wounding can also include lytic enzymes (glucanase and chitinase) that attack components or affect the synthesis of suberin and ligninin from phenolic components, the insolublisation of hydroxyproline-rich glycoproteins by hydrogen peroxide (H_2O_2) and the formation of a wound meristem (Beeching et *al.*, 1994; Wenham, 1995; Yuanhuai et *al.,* 2001). This repair leads to the sealing of the wound, the inhibition of the production of signals triggering the wound response and a return of the plant to normal development (Uritani, 1999).

In other tuber crops such as sweet potato (*Ipomoea batatas*), yam (*Dioscorea spp.*) and cocoyam (*Xanthosoma sagittifolium*), postharvest loss is caused by an increase in respiration leading to a reduction in weight and loss of marketability (Ravindran & Wanasundera, 1992). The formation of lignin in the mechanically damaged cassava is poor under ambient conditions (Uritani, 1999). The sealing of wounds in yam via curing, suppresses the respiration rate and water loss, allowing storage for several months. The curing of yams is dependent on temperature, relative humidity (Passam et *al.,* 1976) and possibly light irradiation. Sweet potato can be stored for approximately 2 - 3 weeks or several months if the storage roots are handled and stored correctly; deterioration is heavily influenced by mechanical damage sustained during harvesting, transport or storage. The discoloration observed during storage is only linked to microbial infection and not to any plant phenolic reactions like in cassava. Cassava wound repair can occur if the root remains attached to the plant (Plumbey & Rickard, 1991; Reilly et *al.,* 2004); thus, the problem begins once the root is detached. Although the wound response is present, the healing process and the subsequent down-regulation of the signals are insufficient or too low (Salcedo & Siritunga, 2011). It is thought that at some point during evolution, cassava roots lost their efficiency in wound repair

(Reillyet *al.,* 2004). Thus the studies of the biochemical features of PPD are important to understand the process (Hirose, 1986; Buschmann, 2000ab).

I.2.10.4. Biochemical features of postharvest physiological deterioration

Several studies have been performed to investigate the biochemicalfeatures and molecular events related to postharvest physiological deterioration (PPD) syndrome (Reilly et *al.,* 2004; Reilly et *al.,* 2007; Owiti et *al.,* 2010). Changes in response to cell damages after harvest included accumulation of fluorescent compounds and secondary metabolites (Buschmann et *al.,* 2000ab), decrease of starch content in the profit of sugar (Sanchez et *al.,* 2013); the main soluble sugars found by high performancy light chromatography were raffinose, sucrose, fructose, and glucose (Uarrota et *al.*, 2015); increase in cell respiration and enzymatic activities including regulation of reactive oxygen species (ROS) synthetase (Xu et *al.,* 2013; Zidenga et *al.,* 2012) as well as phenylammonia-lyase. PPD has been found to be correlated to the content of β-carotene. Morante et *al.* (2010) observed a less susceptibility to PPD for the genotypes with high level of β-carotene compared to those with less level of β-carotene. PPD development was also associated to change in gene expression where many genes get altered during the process (Huang et *al.,* 2001; Cortes et *al.,* 2002). Zidenga et *al.* (2012) suggested that mechanical damage that occurs during harvesting initiated cyanogenesis by bringing linamarin and linamarase in contact and subsequently the release of cyanide. The cyanide (HCN) released inhibits mitochondrial respiration by inhibiting complex IV in the mitochondrial electron transfer chain. Inhibition of complex IV causes a burst of reactive oxygen species (ROS) production at complexes I and III. It is this oxidative burst that causes PPD.

Recent studies about metabolome analyses showed increases in carotenoids, flavonoids, anthocyanins, phenolics, reactive scavenging species, and enzymes (superoxide dismutase family, hydrogen peroxide, and catalase) during PPD (Uarrota et *al.*, 2014). In the same study, a positive correlation was observed between PPD and anthocyanins and flavonoids while a negative correlation was observed with phenolic compounds and carotenoids. Several proteins were up- or down-regulated during the process (Owiti et *al.,* 2011). Plants respond to various stresses such as pathogen attacks, harsh growing conditions and wounding by inducing the expression of a large number of genes that encode diverse proteins. The response of plant tissues to wounding has been studied for a very long time and more recently it has been demonstrated that several genes are wound-inducible. The

proteomic approach is a very powerful tool to study the protein patterns that result from differential gene expressions as well as from post-translational modifications (Gray & Health 2005; Lee et *al.*, 2007; Timpero et *al.*, 2008). Proteome profiles of cassava storage root (CSR) at harvest and during PPD onset revealed 300 proteins showing significant abundance regulation during PPD (Vandershuren et *al.,* 2014). The identified proteins were mostly associated with oxidative stress, phenylpropanoid biosynthesis (including scopoletin), the glutathione cycle, fatty acid-oxidation, folate transformation, and the sulfate reduction II pathway in which glutathione peroxidase was identified as a possible candidate for reducing PPD. All these information clearly show that PPD is an active and complex phenomenon involving different pathways. Therefore, more understanding towards PPD or the control of PPD should include the investigation of different pathways involved in such process as well as the possible interaction/crosstalk between the pathways.

I.2.10.5. Mechanisms of ROS scavenging in plants

Plants and other living organisms in the oxidizing environment constantly produce reactive oxygen species (ROS) from chloroplasts, mitochondria, peroxisomes and other subcellular organelles because of biological metabolic processes such as photosynthesis and respiration. Overproduction of ROS is triggered by a pathogen attack and stress exerted by environmental conditions. ROS or active oxygen species (AOS) or reactive oxygen intermediates (ROI) include the superoxide radical ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2), alkoxy radical (RO^{-}), peroxy radical ($ROO^{\cdot}$) and singlet oxygen ($^1O_2$) (Vellosillo et *al.*, 2010). Plants have either enzymatic or non-enzymatic defense systems to scavenge ROS toxicity and protect against oxidative damage (Vranova & Inze, 2002). The major scavenging system includes superoxide dismutase (SOD), enzymes and metabolites from the ascorbate glutathione cycle, and catalase (CAT). The organism's equilibrium is determined by the ROS homeostasis, including ROS productionin combination with its scavenging. Contact with invading microbes as well as wounding in many plants elevates plant plasma membrane-bound NADPH activity, leading to the rapid accumulation of ROS including hydrogen peroxide (H_2O_2), which diffuses into cells and activates defenses (Apel & Hirt, 2004). Increasing evidence indicates that ROS has a dual role as cytotoxic damage, and as a signaling molecule involved in the regulation of response during pathogen attack or various physiological processes (Foyer & Noctor, 2005; Mittler et *al.*, 2004). The accumulation of ROS at the site of infection during hypersensitive response

may contribute to limit the spread of the pathogens or induce signals for establishment of further defense (Mur et *al.,* 2008). Feed-back, or feed-forward interaction between ROS and many hormones such as ethylene, jasmonic acid, abscisic acid, gibberellic acid and salicylic acid in response to biotic and abiotic stresses has also been presented (Mittler et *al.,* 2004; Mur et *al.,* 2008). Since ROS are ideally suited to act as signaling molecules because of their small sizes and ability to diffuse over short distances (Mittler et *al.,* 2004; Pei et *al.,* 2000; Overmyer et *al.,* 2000), it is not surprising thatseveral researchers paid close attention to the oxidative burst in damaged cassava storage roots.

I.2.11. Enzymatic scavenging of reactive oxygen species (ROS) in plants

I.2.11.1. Superoxide dismutase (SOD)

Superoxide dismutases are a group of metallo-proteins that catalyze the dismutation of superoxide (O^{2-}) to molecular oxygen and hydrogen peroxide (H_2O_2). In response to environmental stresses, plants produce increased levels of ROS and SOD provides the first line of defense and is thus important in plant stress tolerance. O^{2-} is removed by dismutating one O^{2-} and reducing it to H_2O_2 while another is oxidized to molecular oxygen (O_2). These metalloenzymes are classified into three known types by their metal cofactors. They are copper/zinc (Cu/Zn-SOD), manganese (MnSOD), iron (FeSOD) and localized in different cellular compartments (Mittler, 2002). MnSOD is found in peroxisomes and mitochondria; Cu/Zn - SOD isozymes are located in higher plant chloroplasts and cytosolic fractions (del Rio et *al.,* 2003). Although FeSOD isozymes are rarely detected in plants, they are linked to the chloroplasts when available (Alscher et *al.,* 2002).

I.2.11.2. Ascorbate peroxidase (APX)

Ascorbate peroxidase is the most important antioxidant enzyme in the chloroplast. It uses ascorbate as the reductant to scavenge H_2O_2 in a process that forms water as a by-product and dehydroascorbate (DHA) as the final product (Moller, 2001). A certain level of ascorbate has to be maintained in the cell and this is done by dehydroascorbate reductase (DHAR) via recycling of deshydrogenoascorbate (DHA) back into ascorbate by oxidizing reduced glutathione (GSH) (Moller, 2001). APX scavenges ROS and protects cells in algae, higher plants, euglena and other organisms (Gill & Tuteja, 2010). The APX family consists of five known isoforms, such as, thylakoid and glyoxisome membrane forms, chloroplast stromal

soluble form and the cytosolic form (Noctor & Foyer, 1998). In comparison to catalase and peroxidase, APX has a higher affinity for H_2O_2 (µM range) and could have an important role in ROS management during stress.

I.2.11.3. Catalase (CAT)

Catalases are vital for ROS detoxification during stress conditions. The CAT-mediated detoxification process is characterized by the direct dismutation of H_2O_2 into water and molecular oxygen (Garg & Manchanda, 2009). This group of enzymes has been identified in plants and is very heterogeneous. One CAT molecule is able to convert about 6 million molecules of H_2O_2 to H_2O and O_2 per minute. CAT is essential in removing H_2O_2 generated in peroxisomes using oxidases involved in β-oxidation of fatty acids, photorespiration and purine catabolism

I.2.11.4. Glutathione peroxidase (GPX)

Glutathione peroxidases are a large family of isozymes that protect plant cells from oxidative stress by using GSH to reduce H_2O_2 and organic and lipid hydro peroxides (Noctor & Gomez, 2002). GPXs are a family of multiple isozymes involved in scavenging hydrogen peroxide enzymes and characterized by the presence of three conserved cysteine residues in the coding region (Rouhier & Jacqout, 2005). The GPX enzymes found in plants are both structurally and functionally different from the GPXs found in animals. Animal GPX uses glutathione only as a reductant while the plant GPX shows an alternative pathway. This is evidenced by glutathione peroxidase proteins identified in *Arabidopsis thaliana* and Chinese cabbage that had glutathione - dependent peroxidase activity and showed high affinity to thioredoxin as a reductant (Mittler, 2002). These enzymes are localized in all the plant parts and different cell compartments (Rouhier & Jacqout, 2005; Ashraf, 2009). Plant GPXs are responsive to ROS accumulation induced by both biotic and abiotic stress.

I.2.11.5. Glutathione reductase

Glutathione reductase belongs to a group of flavoenzymes and contains an essential disulfide group; where, one mole of NADPH is required to reduce glutathione (GSSG) to oxidative form (GSH). Glutathione is a flavor-protein oxidoreductase that is harbored in prokaryotes and eukaryotes (Romero-Puertas et *al.*, 2006). GR plays an important role in ROS

detoxification, GSH regeneration and confers abiotic stress tolerance in plants (Hasanuzzman et *al.*, 2010; Hasanuzzaman et *al.*, 2012). It is found in mitochondria, cytosol and chloroplasts. Increased glutathione reductase activity confers stress tolerance and has the ability to alter the redox state of important components of the electron transport chain. The major involvement of GR in conferring stress tolerance is the recycling of GSH and the maintenance of GSH/GSSG ratio in plant cells (Hasanuzzman et *al.*, 2010).

I.2.12. Non-enzymatic scavenging of ROS in plants

I.2.12.1. Ascorbic acid

Ascorbic acid is a potent, ubiquitous and water soluble antioxidant laboring to prevent or minimize ROS mediated damage in plants (Smirnoff, 2000; Athar et *al.*, 2008). It is found in all plant tissues, although its quantities are more in photosynthetic cells and meristems. Additionally, it has been found to be high in concentration in mature leaves that have high chlorophyll and fully developed chloroplasts. Under homeostatic conditions, ascorbic acid (ASC) is available in its reduced form in the leaves and chloroplast (Smirnoff, 2000). Stromal concentration of ascorbate is in the region of 50 mM and approximately 30 – 40 % of total ascorbate is located in chloroplast (Foyer, 2005). Plant mitochondria produce ASC, metabolize and regenerate its oxidized forms (Szarka et *al.*, 2007). ASC is regarded as a potent ROS scavenger due to its ability to donate electrons in enzymatic and non-enzymatic reactions. It protects membranes by directly scavenging O^{2-} and OH^{-}.

I.2.12.2. Glutathione

Glutathione is considered as one of the significant metabolites in plants and is crucial in intracellular defense against ROS induced oxidative damage. It is found ubiquitously in its reduced form (GSH) in plant tissues and in all cell compartments (Mittler & Zilinskass, 1992; Herna et *al.*, 1998). Additionally, it plays an important role in a number of physiological processes, such as sulfate transport regulation, signal transduction, metabolite conjugation, the expression of stress responsive genes (Xiang et *al.*, 2001; Mullineaux & Rausch, 2005). GSH plays a pivotal role in plant growth and development processes, like cell differentiation, senescence and death, pathogen resistance and enzymatic regulation (Rausch & Wachter, 2014). GSH is crucial for the maintenance of a normal reduced cell state thereby counteracting the inhibitory effects of ROS induced oxidative stress (Meyer, 2008). It

scavenges O^{2-}, H_2O_2 and OH^-. It has been shown that GSH concentrations decline and redox state becomes more oxidized when stress levels are elevated, causing a deterioration of the system (Tausz et *al.*, 2004).

I.2.12.3. Proline

Proline is an antioxidant, protein stabilizer, metal chelator and OH^- and O^{2-} scavenger and potential inhibitor of programmed cell death (PCD). Proline is now known as a nonenzymatic antioxidant that lessens the deleterious effects of ROS needed by animals, microbes and plants (Chen & Dickman, 2005). Plants under stress contain approximately 5 % of proline among other free amino acids (Soshinkova et *al.*, 2013). Proline has been shown to scavenge both hydroxyl and superoxide radicals (Shevyakova et *al.*, 2009). Proline improves stress tolerance through up-regulation of some proteins, decreasing lipid peroxidation and preventing photo-inhibition (Islam et *al.*, 2009).

I.2.13. Features of ROS associated to postharvest physiological deterioration

Cassava storage root is inevitablypredisposed to cell damage due to mechanical injury caused by harvest. Therefore, constitutive defense mechanisms are activated upon harvest in intact plants subjected to abiotic stress. The response of cell damage produces ROS including superoxide anion and hydrogen peroxide with both localand systemic actions. In the case of cassava storage root (CSR), studies have been focused on the ROS production and their scavenging induced by cell damage during PPD syndrome (Xu et *al.*, 2013; Zidenga et *al.*, 2012). A burst of superoxide anion and the increased activities of ROS scavenging enzymes such as superoxide dismutase and catalase were observed after oxidative burst caused by harvest damage (Iyer et *al.*, 2010; Reilly et *al.*, 2007). The shortage of scavengers for the overproduced ROS results in an acceleration of the onset of PPD. Zidenga et *al.* (2012) suggested that the cyanide which is produced immediately when cassava is mechanically damaged may trigger the oxidative burst responsible of PPD onset. In fact cassava produces potentially toxic levels of cyanogenic glucosides which break down to release cyanide following cellular disruption and release cyanogens from the vacuole (Siritunga & Sayre 2003; Siritunga et *al.*, 2004). Therefore an increase in the production of ROS scavengers is presented as a key regulator of PPD. The induction of the overexpression of mitochondrial alternative oxidase (AOX) in transgenic cassava was followed by the extension of the shelf-life of storage roots for two weeks (Zidenga et *al.*, 2012). Another experiment showed that the

co-overexpression of Me/Cu/Zn-SOD and MeCAT1 induced in transgenic cassava could also enhance scavenging ROS in CSR after tissue damage (Xu et *al.,* 2013). In addition, the regulation of the activity of glutathione-associated enzymes, including glutathione reductases, glutaredoxins, and glutathione S-transferases have been reported as potential modulator for the onset of PPD (Vandershuren et *al.,* 2014). Taken together, these observations strongly support the implication of ROS during PPD process but the molecule that trigger their production is still poorly study.

I.2.14. Calcium signaling and cassava postharvest physiological deterioration

I.2.14.1. Calcium signaling and abiotic stress

Plant cells have developed an elaborate system for perceiving their endogenous and exogenous environments and eventually producing proper physiological responses. In plants, calcium ions (Ca^{2+}) are the second messengers coupling physiological responses to external and developmental signals (Reddy & Reddy, 2004). Calcium plays a key role in the integrity of the cell wall and the membrane system and has been shown to act as an intracellular regulator in many aspects of plant growth and development including stress responses (Reddy, 2001; White & Breadly, 2003). Changes in cytosolic free calcium ion concentration were observed by many authors during transduction of abiotic stimuli including light, low and high temperature, touch hyperosmotic and oxidative stresses and also in the case of biotic stimuli including fungal elicitors and nodulation factors (Rudd & Franklin-Tong, 2001; Sanders et *al.,* 2002). These calcium signatures are decoded by several types of Ca^{2+} sensor proteins. Ca^{2+}-binding sensory proteins include calmodulins (CaMs), calmodulin-like proteins, calcineurin B-like proteins (CBL), and Ca^{2+}-dependent protein kinases (CDPKs) (Sanders et *al.,* 2002, Snedden & Fromm, 2001).The cascade of events involving calcium signaling and ROS during abiotic and biotic stress is presented in Fig. 5 (Rejeb et *al.,* 2014). These authors suggested that stimuli coming from both biotic and abiotic stress are first sensed by the plant cell, and then the information is transduced to appropriate downstream-located pathway(s). Sensors as well as signal transducers might be shared by both types of stressors. Reactive oxygen species (ROS) and Ca^{2+} are known among others to play a prominent role as transducers and mitogen-activated protein kinases (MAPK) cascades have been shown to be used by both types of stresses. MAPKs are centrally positioned in Ca^{2+}-ROS crosstalk as well as in the signal output after exposure to a specific stress. The

importance of ROS has repeatedly been described for both types of stresses too, and, therefore, ROS might represent crucial elements in the integration of both stresses during cross-tolerance. Plant hormone signaling is of utter importance for stress adaptation. While abscisic acid (ABA) is predominantly involved in abiotic stress adaptation, salicylic acid (SA) and jasmonate/ethylene (JA/ET) are more responsible for the plant's reaction to biotic stress. However, there is a tremendous amount of crosstalk taking place between the various hormonal pathways, and the exact nature of this crosstalk during simultaneous biotic and abiotic stress remains to be investigated. ABA signaling contributes positively to pre-invasion defense and is responsible for enhancing callose deposition. ABA presents a positive interaction with JA/ET signaling. The activation of SA signaling by pathogen challenge can attenuate ABA responses. ABA signaling negatively affects signals that trigger systemic acquired resistance, enhancing pathogen spread from the initial site of infection. The interaction of SA, JA, and ET signaling results in increased resistance to pathogens. Hormones, secondary metabolites, priming agents, and further chemicals located in the cytoplasm finally up-regulate transcription factors (TF), pathogenesis related (PR) and defense genes, heat shock protein (Hsp) genes, and othergenes involved in protection against stress and thus lead to the phenotypic expression known as cross-tolerance.

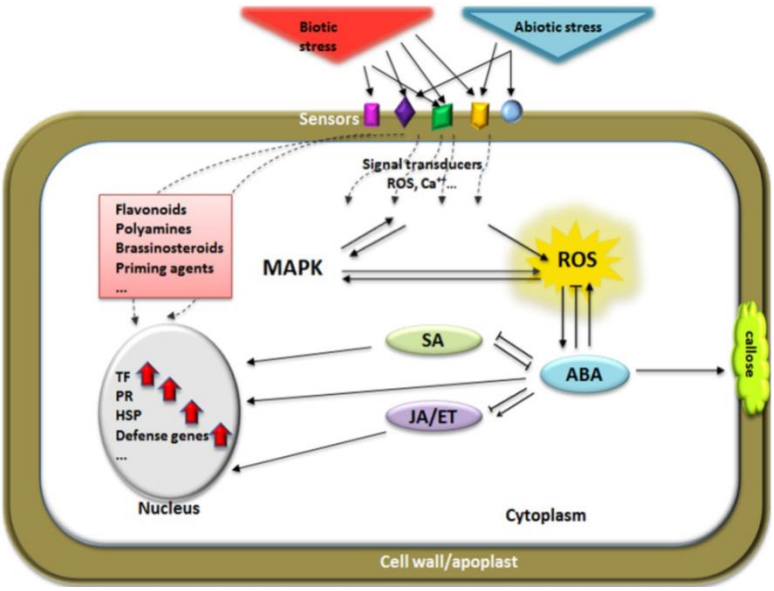

Fig. 5. Elements possibly involved in cross-tolerance between biotic and abiotic stress (Rejeb et *al.*, 2014). Arrows: induction; flat-ended lines: repression

I.2.14.2. Calcium signal and postharvest physiological deterioration process

There is ample evidence to show the involvement of Ca^{2+} signaling in abiotic stress responses. Intracellular changes of Ca^{2+} levels were reported as the first response to diverse

abiotic signals and biotic stresses (Beneloujaephajri et *al.,* 2013; Ranty et *al.,* 2006). Numerous data supported that Ca^{2+} and its sensor proteins; calmodulin (CaM), calcium dependent protein kinases (CDPKs) and calcineurin B-like protein (CBLs) and downstream elements played an important role in plant adaptation to abiotic stress. CaM is one of the most studied eukaryotic proteins and has been shown to interact with and modulate the activity of numerous target proteins (Snedden and Fromm, 2001). Several studies indicated that Ca^{2+}-CaM complex triggered the activation of target proteins to produce cellular physiological responses (Bouché et *al.*, 2005; Nookaraju et *al.,* 2011). An example was found in tobacco stressed by wounding, in which three CaM isoforms at different Ca^{2+} concentrations activated the target enzymes NO synthase and NAD kinase (Karita et *al.,* 2004). Similar effects of Ca^{2+} on NO or nicotinamide adenine dinucleotide (NAD) kinase during PPD can not be ruled out.

In the case of cell injuries such as that occurring in PPD syndrome of cassava storage root, a significant up-regulation of CaM observed atthe early events of PPD (Owiti et *al.,* 2011) may be associated with a rapid increase in Ca^{2+} which resulted in the oxidative burst as observed in *Arabidopsis thaliana* (Kaplan et *al.,* 2006). This phenomenon was similar to the expression of heat shock proteins under heat stress attributed to the accumulation of CaM in plants (Liu et *al.,* 2003; Zhang et *al.,* 2009). Ca^{2+} seems to be involved in signaling transduction to trigger the activation of programmed cell death (PCD) (Levine et *al.,* 1996; Hoeberichts & Woltering, 2002; Zhang et *al.,* 2009). It was suggested that CDPKs, in response to elevated cytosolic Ca^{2+} levels, could induce NADPH oxidase activity which is one of the key points of an oxidative burst and PCD process. In roots of *Arabidopsis thaliana,* mechanical stimulation triggeredthe rapid and transient increase of cytoplasmic Ca^{2+} concentration; this mechanical stimulation likewise elicits apoplastic ROS production with the same kinetics (Monshausen et *al.,* 2009).

I.2.14.3. Calcium/magnesium fertilization and plant growth

A wide variety of factors affect plant development. These include soil, environment and hormones. Among the soil factors, calcium (Ca) and magnesium (Mg) are two cationic mineral nutrients which have either structural, physiological or biochemical functions in growth and stress tolerance in plants. Ca is an essential component of the plant cell wall giving mechanical strength for normal transport and retention of other elements. Furthermore, Ca has been shown to influence protein phosphorylation in plants (Budde & Chollet, 1988) by affecting the calcium binding modulator proteins such as calmodulin and protein kinases.

When plants are exposed to a stress situation, a quick rise in cytosolic Ca^{2+} takes place, which is a key factor in the expression of stress-responsive genes and physiological responses of plant cells to stress conditions, such as extreme temperatures, drought, salinity and pathogenic attack. The changes in cytosolic Ca^{2+} concentrations are often closely related to the severity of the stress. A co-localization of the changes in Ca^{2+} and a burst of reactive oxygen species (ROS) were observed after pathogenic or environmental stress (Ranf et *al.*, 2011). The implication of Ca^{2+}-calmodulin on PPD in cassava (Owiti et *al*., 2011) as well as the influence of fertilizers containing Ca^{2+}/Mg^{2+} on the delay of fruit ripening (Aghofack-Nguemezi & Dassie, 2007; Park et *al.*, 2005) and stress tolerance (Jiang & Huang, 2001; Jiang et *al.*, 2005) was recently reported in many plants. Tuber calcim concentration may increase through fertilization. Experiments on potato have shown that calcium application can increase tuber calcium concentration and reduce internal brown spots (Collier et *al.*, 1978). An effective postharvest control of bacterial soft rot in potato was suggested to be achieved by increasing tuber calcium concentration through fertilization and postharvest vacuum infiltration with calcium sulphate or calcium nitrate (Conway et *al.*, 1992; Miles et *al.*, 2009). In addition, calcium in combination with calmodulin participates in several metabolic processes (Snedden & Fromm, 2003; Zhang & Lu, 2003).

On the other hand, magnesium (Mg) is one of the most important nutrients involved in plant growth and development. Mg holds a fundamental role in phloem export of photosynthetic substances from photosynthetic organs to the roots (Esfandiari et *al.*, 2010). Plants under low Mg supply are very sensitive to high light intensity, heat stress and easily becoming chlorotic and necrotic, probably due to extensive production of reactive oxygen species (Kumar et *al.*, 2006; Cakmak & Yazici, 2010; Hermans et *al.*, 2010; Kobayashi et *al.*, 2013). In plants, when the intracellular Ca concentration is submicromolar, the concentration of closely related divalent Mg cation is millimolar (Aghofack-Nguemezi & Tatchago, 2010). Despite this difference, cellular processes remain very selective for Ca (Helper, 2005). Niu et *al.* (2014) showed that ROS and cytosolic Ca^{2+} are signaling factors which indicate magnesium deficiency. This is in line with a previous hypothesis that antioxidation is an early response to magnesium induced by ROS (Cakmak & Kirkby, 2008; Hermans et *al.*, 2010). In addition, Mg^{2+} also plays an important role in activating some enzymes such as RuBP-carboxylase, ATPases and Mg chelatase (Esfandiari et *al.*, 2010). It has been suggested that plant cells compensate for low Ca^{2+} by increasing Mg^{2+} transport activity, while high Ca^{2+} inhibits Mg^{2+} availability to plants (Hermans et *al.*, 2010). A positive response in cassava by

the increase in yield through inorganic fertilization has been reported by many authors (Howeler, 2002; Kamaraj et *al.,* 2008; Asare et *al.,* 2009). Few studies have been conducted on the evaluation of the influence of fertilization on cassava, and its impact on antioxidant status and gene expression on cassava storage roots undergoing PPD.

I.2.15. Proteome

I.2.15.1. Definition and application

Proteome is a set of expressed proteins in a given type of cell, organ or organism at a given time, under defined conditions. Proteomic is the systematical analysis of protein profiles in a given extract. It is also a direct measurement of proteins in terms of their presence and relative abundance (Wilkins et *al.*, 1996). This analysis is made by two-dimensional electrophoresis which consists of a separation of proteins in the first dimension by isoelectric focusing (IEF) according to its isoelectric point (IP) and in the second dimension, perpendicularly to the first dimension according to its molecular weight. The excellent resolving power of this technique can result in the separation of thousands of the major proteins in a tissue or subcellular fraction. Visualization of protein spots is achievable by the use of visible stains such as silver, coomassie and fluorescent stains. Proteins within spots of interest are then identified by digestion to peptides, typically trypsin and subsequently analysed using mass spectrometric methods. The main step of protein separation by two dimensional gel electrophoresis is presented in Fig. 6. The ability to compare dynamic changes in the proteome is an exciting new addition to the research programs of many plants. The proteome of an organism is subjected to spatial and temporal alterations; consequently, proteomics has become a vital tool in studying comparisons between diseased and healthy, stressed and unstressed systems. Proteomics facilitates the analysis of post-translational modifications as well as a comprehensive view of the protein-protein interaction in plants. The continuing enhancements in protein resolution, loading capacity, staining methods and image analysis software, 2-D gel electrophoresis were applied for the analysis of plant proteins with great success. Nevertheless, it should be noted that 2-D gel electrophoresis has some limitations. In contrast to gene profiling, where thousands of transcripts can be analyzed at once, even high-resolution 2-D gels can resolve about 1,000 proteins and these are exclusively the high abundant protein species in a crude mixture. Furthermore, extremely acidic and basic proteins are not amenable to 2-D gel electrophoresis using conventional pH

gradients ranging from 3 to 10. In addition, 2D analysis is time consuming and labor intensive. A single experiment spans over two days and one sample can be electrophoresed per gel.

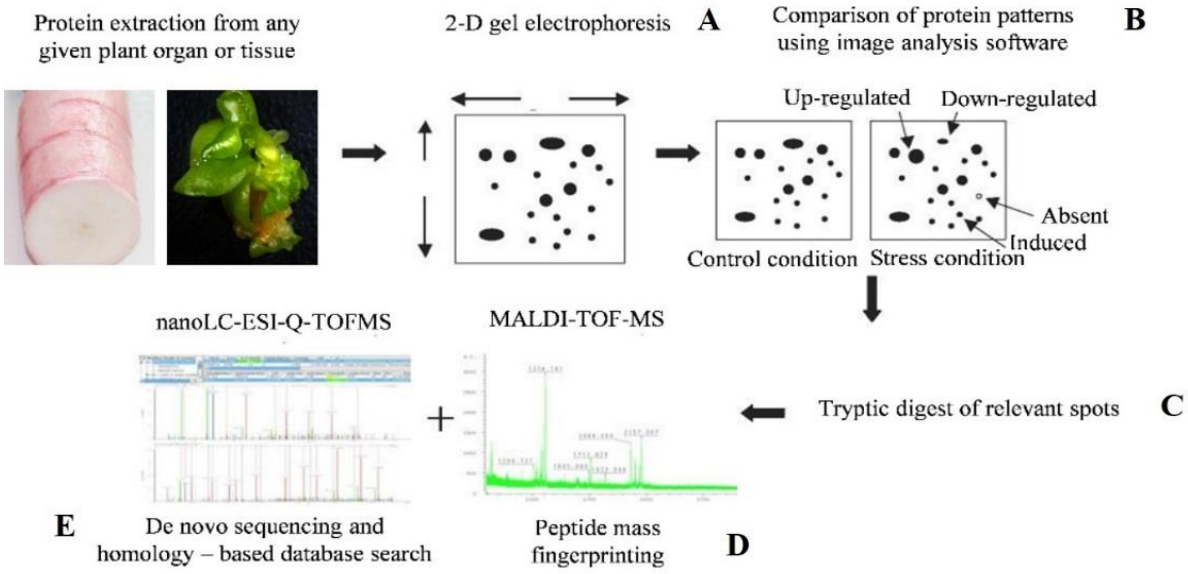

Fig. 6. Two-dimensional gel electrophoresis for protein separation and quantitation coupled with mass spectrometry for protein identification (modified from Witzel, 2008).

Protein extracts are separated in the first dimension according to the isoelectric point using immobilised pH gradients and in the second dimension according to the molecular weight (MW) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (A). The resulting gel patterns are compared and spot volumina quantified by image analysis software (B). Spots with alterations in expression are collected from the 2-D gel and digested with trypsin. The tryptic peptides are analysed by mass spectrometry (C). For the identification via peptide mass fingerprinting, the acquired peptide masses are compared with theoretical digests of proteins annotated in public databases (D). Using tandem mass spectrometry, the amino acid sequences of the tryptic peptides are determined and subjected to homology-based database search (E)

I.2.15.2. Proteomics as a tool to study stress responses in plants

Abiotic stresses that yield cellular dehydration like freezing, salt and water stress have a cross talk in regard to signaling pathways and have similar changes in plant gene expression and metabolism (Kreps et *al.,* 2002; Rabbani et *al.,* 2003; Cook et *al.,* 2004; Sung et *al.,* 2001). Since the amount of mRNA produced is inversely proportional to that of cellular proteins; taking into account post translational modification of proteins, this makes proteomics the choice of study (Futcher et *al.,* 1999; Gygi et *al.,* 1999). Although numerous genomes have been sequenced, the information supplied is insufficient to provide details regarding plants ability to adapt to stress, regulatory biology and gene function. It is therefore important to use tools that tackle both quantitative and qualitative analysis (Timpero et *al.,* 2008). In addition, changes in proteins are rapid within cells/tissues as influenced by certain

modifications like cell cycle, external stimuli, physiological states and tissue studied. Owing to these, proteomics is thus the tool of choice when analyzing biochemical pathways and the complex response of plants to environmental stimuli. Using proteomics to examine plants comparatively in regard to stress response, a link between proteome, transcriptome and metabolome is established (Cook et *al.,* 2004; Gray & Heath, 2005). A study investigating the response of rice leaves to high temperature stress obtained 48 differentially expressed proteins, from samples obtained after 12 or 24 hours exposure.From these, 18 were heat shocked proteins (Lee et *al.,* 2007). A 2D comparative drought stress and recovery study was done using tall wheatgrass at its vegetative stage.

I.2.15.3. Proteome and cassava postharvest physiological deterioration

Plants respond to various stresses such as pathogen attacks, harsh growing conditions, wounding by inducing the expression of a large number of genes that encode diverse proteins. The response of plant tissues to wounding has been studied for a very long time and more recently it has been demonstrated that several genes are wound-inducible. The proteomic approach is a very powerful tool to study the protein patterns that result from differential gene expressions as well as from post-translational modifications. Owiti et *al.* (2011) earlier reported a total of 60 proteins in which 33 proteins were shown to be up-regulated and 27 proteins were down-regulated relative to the 0 h control and 12 h after harvest of cassava tubers. The up-regulation of several key proteins early and later after cassava harvesting has been observed by Owiti et *al.* (2011) confirming that postharvest physiological deterioration is an active process involving signal pathway, changes in genes expression and proteins synthesis, together with the accumulation and oxidation of phenolic compounds as previously suggested by Reilly et *al.* (2004). It seems to be a highly regulated complex involving proteins that can be potential candidates for biotechnology approaches to understand the mechanism of the onset of such deterioration and provides strategies to reduce such deterioration. Vanderschuren et *al.* (2014) report about 300 proteins showing significant abundance regulation during PPD in cassava. Most of these proteins were associated to oxidative stress, glutathione cycle and phenylpropanoid biosynthesis. Here we are reporting our study set up towards the application of proteomics in an attempt to understand the molecular basis of PPD in cassava tolerant and susceptible genotypes to such process.

I.2.16. Programmed cell death

I.2.16.1. Definition and characteristics

Programmed cell death (PCD) is a process aimed at eliminating redundant or harmful cells from healthy tissues during the life cycle of a multicellular organism. It is a highly regulated cellular suicide process essential for growth and survival in eukaryotes. This phenomenon is characterized by biochemical and physiological features such as compaction and shrinkage of the cytoplasm and nucleus, DNA and nuclear fragmentation into large (50 to 300 kb) and subsequently small fragment (200 bp), nucleosomal fragment (DNA laddering), and fragmentation of the cell into membrane -confined vesicles called apoptotic-bodies (Wang et *al.,* 1996; De Jong et *al.,* 2000). PCD has been observed in many plant processes including reproductive development such as embryogenesis, flower petal senescence and vegetative development such as xylogenesis, parenchyma formation (Pennel & Lamb, 1997; Gray, 2004). DNA ladders have been reported during death induced by different stresses such as cold (Koukalova et *al.,* 1997), nutrient deprivation (Callard et *al.,* 1996), pathogen or pathogen toxin (Navarre & Wolpert, 1999; Ryerson et *al.,* 1996; Wang et *al.,* 1996).

The core component of the apoptotic machinery is a proteolytic cascade involving a family of cysteine proteases named caspases which specifically cleave after the aspartic residues of many proteins. They cleave proteins involved in the attachment of the cell to their neighbors, thereby helping the dying cell to detach and round up making it easy to ingest. Even if no similarities were found between endonucleases involved in mammalian apoptosis and the ones nominated for plant PCD, the existence of caspase-like proteins (CLPs) in plant is proposed based on different observations (Woltering et *al.,* 2002). The existence of cell death related plant proteases that recognize and process synthetic peptide inhibitors and fluorogenic substrates that mimic the caspase substrate recognition site was reported (He et *al.,* 2008; Woltering et *al.,* 2002). Then caspases aspases-like activity may play a pivotal role in plant PCD. This is supported by the identification of plant proteases that recognize and process the natural substrate poly ADP-ribose polymerase (PARP) at a caspase recognition site; the functionality of macromolecular caspase inhibitors (IAPs and in particular p35) in plants and the observation of the caspase recognition site p35 which abolishes its effect (Woltering et *al.,* 2002).

Based on this observation, two families of caspase-like proteins were identified; designed paracaspases (humans and *Caenorhabditis elegans*) and metacaspases (fungi and plants) (Uren et *al.*, 2000; Koonin & Aravind, 2002). In plants, two types of metacaspases have been reported to be represented by nine genes (AtMC1-9) in *Arabidopsis* (Vercammen et *al.*, 2004). Type I metacaspases, including AtMC1-3, are characterized by the presence of proline-rich repeat and zinc-finger motif containing a prodomain and a short linker/loop region. Type II metacaspases (only in plant), no prodomain and contain a longer linker/loop region (Uren et *al.*, 2000). However, plant metacaspases were unable to cleave caspase substrates and thus didn't possess caspase-like activity (He et *al.*, 2008; Bonneau et *al.*, 2008). Nevertheless, some reports show that they have been implicated in PCD in *Arabidopsis* and yeast. In fact, transgenic overexpression of some arabidopsis metacaspases (AtMC4, AtMC5) have increased the level of cell death induction upon treatment with ROS inducing agents and the loss of those genes resulted in a decrease or delay of cell death (Lam & Zhang, 2012). Madeo et *al.* (2002) also found caspase-like activity in yeast when stimulated by H_2O_2 to undergo apoptosis.

Following the experimental data obtained from a broad variety undergoing PCD, Hoeberichts & Woltering (2002) proposed a possible model of plant PCD (Fig. 7). In this model, ROS are presented as playing a central role in the process. NADPH oxidase complex may be stimulated by caspases (-like) activity. Upon activatation, they catalyse the reduction of oxygen to $^{\cdot}O_2^-$ followed by dismutation of $^{\cdot}O_2^-$ to H_2O_2. Subsequently, caspase-inhibitors act to prevent cell death and the preceding accumulation of ROS. Mitochondria provide an additional source of elevated ROS levels and at the same time strikingly, salicylic acid-dependant formation of ROS both trigger an increase in cytosolic Ca^{2+} (Kawano et *al.*, 1998) and inhibits mitochondrial function (Xie & Chen, 1999).

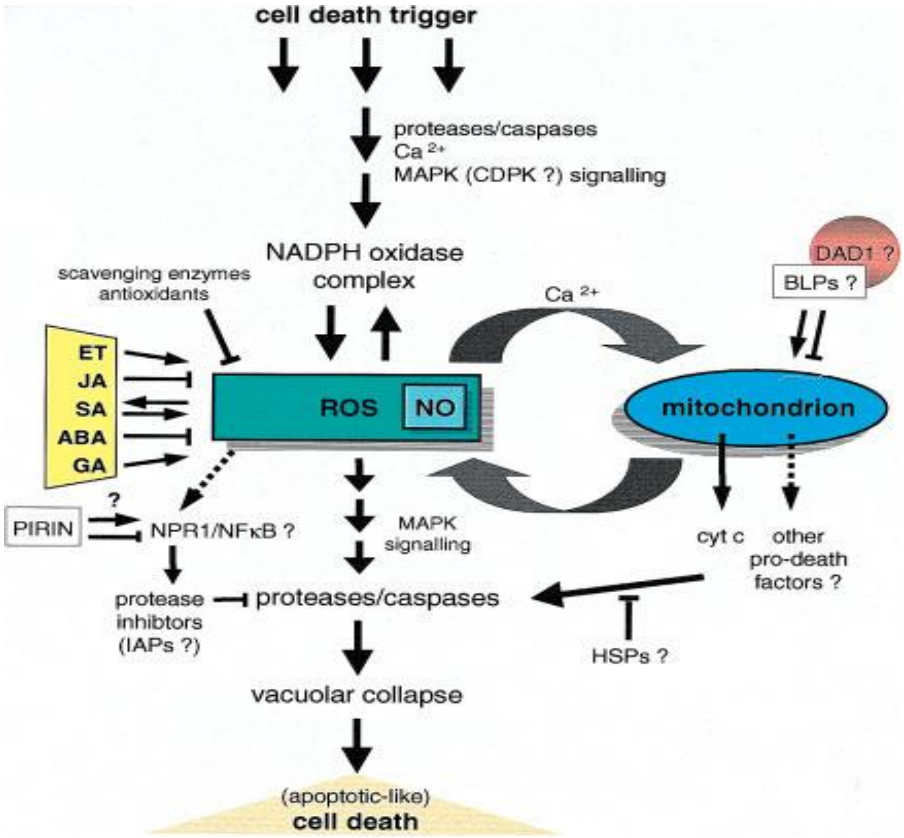

Fig. 7. Model of plant programmed cell death (Hoeberichts & Woltering, 2002).

I.2.16.2. Programmed cell death and postharvest deterioration in cassava

Programmed cell death following oxidative burst has been demonstrated in many plants (Woltering et *al.*, 2002; Vacca et *al.*, 2006; Danon et *al.*, 2000). Several calcium-binding proteins were induced in response to the stresses. Calcium dependent protein kinases (CDPKs), in response to elevated cytosolic Ca^{2+} level, can induce NADPH oxidase, which catalyses the production of ROS such as superoxides (Sagi & Flur, 2006). Even if the oxidative burst induced by wound in cassava storage roots after harvest has been studied, the linkages between oxidative burst and PCD are poorly understood.

Regarding cassava storage root PPD syndrome, down-regulation response of cysteine protease may enhance protease activity leading to PCD (Owiti et *al.*, 2011). The down regulation of many peptides of the phospholipase D α-group of the enzymes during early and late PPD, were also presented (Owiti et *al.*, 2011). However, further studies need to be carried out to describe how this pathway works in a PPD process. Biochemical detection of caspase-3-like activity and its inhibitors in plant PCD can be useful to elucidate the mechanism of PPD. Apart from this further functional characterization, understanding of the mechanism on

which this pathway interacts with other pathways such as Ca^{2+} and ROS as mentioned in other plants during stress, can increase the knowledge about the PPD process in cassava.

I.2.17. Strategies to overcome postharvest physiological deterioration

I.2.17.1. Pruning

Pruning consists in removing leaves from the plant two weeks prior to the harvest of tubers. This operation can delay PPD for 2-3 weeks on tubers after harvest, but at the expense of the reduction in dry matter content of the root (Van Oirschot et *al.*, 2000). Even if the exact mechanism at work after pruning is not well known, it results in a decrease in the respiration and weight loss (Tanaka et *al.*, 1983). Pruning may also negatively affect the organoleptic quality of cassava storage roots and reduce the starch content (Reilly et *al.*, 2004).

I.2.17.2. Traditional techniques

The simplest means of preserving cassava tuberous roots for long is to delay harvesting until the crop is needed. However this method decreases the quality of the storage roots which become more fibrous and woody and prevents the use of the land for other agricultural activities. In addition, it leaves the cassava plants susceptible to a wide array of pests and diseases that can detract from the storage root quality (Westby, 2002). After harvesting, PPD can also be minimized by the exclusion of oxygen by storing and transporting the roots in plastic bags or coating individual roots with paraffin wax. The treatment of cassava tuberous roots with fungicide before packing into plastic or ethylene bags can also delay the onset of PPD (Wheatley, 1989). Wrapping roots in plastic, dipping in paraffin wax or storage in controlled environments can lead to a 4 week shelf-life but these are high-cost methods and therefore suitable only for storage root export to developed countries since cassava isa low-cost commodity in the countries of origin (Rickard & Coursey, 1981; Reilly et *al.*, 2004).

I.2.17.3. Processing

There are various processing methods used to produce different food products, depending on locally available processing resources, local customs and preferences (Hillocks, 2002). Cassava processing improves palatability, increases shelf life, facilitates transport and, most importantly, detoxifies cassava roots by removal of cyanogens (Nweke, 2002).

Processing into more stable traditional or industrial products avoids the problem of PPD. Therefore processing facilities at/or near the site of production can be a good way to reduce postharvest losses.

I.2.17.4. Breeding

The use of conventional breeding to produce resistant cultivars to physiological deterioration is a good alternative to overcome the problem of PPD. However this technology is faced with low cassava seed set, high heterozygosity (Zhang, 2002), the difficulty to incorporate the trait into different cultivars without altering the characteristics of the parent genotypes (Westby, 2002), the lack of genetic variability for resistance to physiological deterioration (Ceballos et *al.*, 2004) and the inverse relationship between postharvest physiological deterioration and cassava root dry matter content (Estavao, 2007). There is a strong genetic link between PPD and the advantageous trait of dry matter content (Jennings & Iglesias, 2002) making delaying PPD through breeding challenging. Another strategy that could facilitate or accelerate a breeding program for delaying deterioration is marker-assisted selection (MAS). While attempts have been made to isolate quantitative trait loci for PPD, few markers linked to major loci have been identified (Cortés et *al.*, 2002). Varieties with low levels of phenyl amonialyse (PAL) expression or high levels of post-harvest antioxidant gene expression could be identified and used as parents in crosses. However, the PPD phenotype exhibits a high "genetic x environment" interaction that makes scoring for minor differences difficult (Rodríguez, 2001).

I.2.17.5. Biotechnology

Genetic modification seems to be appropriate to solve the problem since the technology can transfer new traits to cassava varieties without altering other desired traits. Genetic engineering of crops in Africa and other developing countries for the benefit of the farmer and consumer is generally seen as a potential positive influence on food availability as opposed to the prevalent attitude in wealthier nations, especially in Europe. The genetic modification of cassava for improved agronomic and other characteristics are in its infancy, mainly due to the cost involved, the necessary infrastructure and expertise required and the lack of importance of this crop to developed nations. The modification of cassava for enhanced starch production by enhancing storage root ADP-glucose pyrophosphorylase activity, which led to a higher yield through an increase in root size and number (Ihemere et

al., 2006); the over-expression of hydroxynitrile lyase in cassava plants, which led to a reduction in the content of the harmful acetone cyanohydrin (Siritunga et *al.,* 2004); the overexpression in cassava transgenic plants of cyanide intensive mitochondrial enzyme alternative oxidase of *Arabidopsis* resulting in the delay of postharvest physiological deterioration in plants exhibiting for approximately three weeks (Sayre et *al.,* 2010); the use of cDNA amplified fragment lenght polymorphism (AFLP) approach to isolate transcript derived fragments (TDFs) related with the regulation of gene expression during postharvest deterioration (Huang et *al.,* 2001), is an encouragement for research in biotechnology. There is currently a wide array of active cassava genetic engineering projects with the aim of generating material with improved nutritional value for agricultural use in cassava-producing countries (Sayre et *al.,* 2011; Telengech et *al.,* 2015). The BioCassava Plus project is seeking to enhance nutrient content, increase virus resistance, reduce cyanogenesis and delay post-harvest deterioration in cassava storage roots through genetic engineering (Sayre et *al.,* 2011; Telengech et *al.,* 2015). However, new studies need to be carried out in order to provide more information on genes and proteins involved in key pathways to develop potential strategies for PPD control.

Previous studies have associated ROS-mediated process and ethylene biosynthesis in wound induced PPD (Hirose et *al.,* 1986; Buschmann et *al.,* 2000; Reilley et *al.,* 2007; Xu et *al.,* 2013) in cassava storage roots. In this research project, we have undertaken a proteomics profiling approach to analyze the change in total proteins in cassava susceptible and tolerant genotypes by investigating the implication of calcium signalling in the release of ROS burst during cassava postharvest physiological deterioration. The functional role of some of the identified proteins have been characterized through enzyme assays, western blot and their regulation at the transcript level validated through quantitative real time polymerase chain reaction analysis. In addition, an approach to delay PPD onset through soil fertilization by fertilizer containing calcium and magnesium is also proposed.

MATERIAL AND METHODS

II.1. MATERIAL

II.1.1. Study area

The study was realized in Hainan – China. Hainan is an island situated in the South of China (Fig. 8.). The climate is tropical with two distinct seasons the wet and dry seasons. The dry season (spring and winter) is warm and windy whereas the wet season (summer and autumn) is hot and humid. The climate permits the cultivation of long growing crops like cassava. The average annual temperature ranges from 24.5 to 25.28 °C and the average annual rainfall is about 2000 mm or less than 1000 mm (Gao et *al.*, 1988). Hainan is much hotter than some tropical countries at the same latitude, such as America and Mexico, while it is cooler than some southeast countries like Thailand and Singapore. Precipitation varies greatly, with distinct dry and wet seasons; 90 % of the rain falls from May to October, and little rain from November to April. The total population of Hainan Island is approximately 8.6 million. The natural conditions are very good for agricultural production. The traditional agricultural productions are rubber, cassava, vegetables, and tropical fruits (coconut, banana, mango and pineapple). In Hainan, soils are also more fertile compared to other cassava producing provinces in China (Guandong, Guangxi and Yunnan). The cassava area is 30000 ha with about 450000 tons of fresh roots. Hainan province accounts for about 7.5 % of cassava planted area and production in China. All the experiments were made in the experimental field of Chinese Academy of Tropical Agricultural Science (CATAS) in Danzhou city.

Fig. 8. Localization of Hainan in China and Hainan cities (Lu et *al.*, 2012).

II.1.2. Plant material

Two cassava genotypes South China 5 (SC5) and Qiong Zhong 1 (QZ1) renamed SC14 were selected for this study. SC5 and QZ1 are considered as susceptible and tolerant to PPD respectively. After six months screening of different genotypes these two were selected. The tolerance of QZ1 to PPD compared to SC5 during the six months of screening as well as the lack of research efforts on this genotype justifiedour choice. The genotype SC5 was breed in the farmer participatory crop improvement program from SC8013 X ZM8625 hybrid (Howeler, 2001). SC5 is well used in industries due to its high yield and high starch production while QZ1 was collected from germplasm of Qiong Zhong town. It is well used for cooking, silkworm feed though little research work has been performed on it.

II.1.3. General stock solutions and buffers

II.1.3.1. Solutions for pH adjustment

The solutions for pH adjustment were constituted by hydrochloric acid and sodium hydroxide. Hydrochloric acid was performed with 36 – 38 % hydrochloric acid (43.64 ml) slowly added to a graduated beaker containing 50 ml of ddH_2O and the volume was adjusted to 100 ml with ddH_2O and stored at room temperature. For sodium hydroxide, NaOH pellets (20 g) were added to 80 ml of ddH_2O and dissolved using a magnetic stirrer. The volume was adjusted to 100 ml with ddH_2O and the mixture was stored at room temperature.

II.1.3.2. Solution for protein extraction and solubilization

The protein extraction buffer was performed using 0.1 M of Tris-HCl (1.2114 g) in 60 ml of ddH_2O, pH adjusted to 8 with hydrochloric acid. Thereafter 5 % (w/v) sucrose (5 g), 2 % (w/v) sodium dodecyl sulphate (SDS) 2 g, 50 mM DL-Dithiothreitol (DTT) 0.77 g, 2 ml of complete inhibitor were added to the previous homogenized solution and the volume brought to 100 ml with ddH_2O. The complete inhibitor solution was prepared using one tablet of Roche molecular Biochemical dissolved in 2 ml of ddH_2O and stirred using a micropipette. The ammomium acetate was prepared using ammonium acetate powder (3.854 g) solubilized in 500 ml of methanol and homogenized using the magnetic stirrer. The mixture was stored at -20 °C. Acetone (400 ml) was added to a container and brought to 500 ml with ddH_2O and mixed by stirring andstored at -20 °C. For protein solubilization, a protein solubilization buffer was prepared using 9.5 M Urea (5.706 g), 2 M Thiourea (1.523 g), 4 % (w/v) 3-(3-cholamidopropyl dimethylammonio -1- propanesufonate (CHAPS) 0.4 g, 1 % (w/v) DTT (0.1 g), 2.5 mM EDTA (0.007 g) and 2.5 mM EGTA solubilized in 5 ml of ddH_2O. After complete solubilization, the volume was adjusted to 10 ml with ddH_2O and stored in 2 ml aliquots at -20 °C. BSA 2 mg/ml in ddH_2O was used to make a standard curve and the quantification of the protein was performed using a Thermo Scientific™ coomassie (Bradford) protein assay kit stored at 4 °C.

II.1.3.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) solutions

10 % of sodium dodecyl sulphate (SDS) (w/v) was prepared using SDS (10 g) dissolved in 100 ml of ddH_2O and the mixture was stored at room temperature. The preparation of 1.5 M Tris buffer, pH 8.8 was performed by dissolving a Tris-HCl (90.85 g) in 400 ml of ddH_2O and the volume was brought to 500 ml with ddH_2O. The mixture was stored at 4 °C. For 1 M of Tris buffer, pH 8.8, Tris-HCl (60.57 g) was dissolved in 400 ml of ddH_2O and the final volume was brought to 500 ml with ddH_2O and the solution wasstored at 4 °C. The preparation of 1 M of Tris buffer, pH 6.8 was performed using Tris-HCl (60.57 g) dissolved in 400 ml of ddH_2O and, the final volume was brought to 500 ml with ddH_2O. The solution was stored at 4 °C. A protogel (National Diagnostic, Dublin, Ireland) of 30 % of acrylamide and protogel (National Diagnostic, Dublin, Ireland) of 40 % of bisacrylamide were used for solidification. 10 % ammonium persulphate (APS) and N, N, N', N'-tetramethylethylenediamine T8133 were used for polymerization. 1 % of bromophenol blue solution was used to mark the line of migration. The running solution was performed with Tris-HCl (3.03 g), glycine (14.4 g), SDS (1 g) dissolved in 800 ml of ddH_2O and the final volume was adjusted to 1 L with ddH_2O. The solution was stored at room temperature.

The rehydration buffer was performed with 7M Urea (4.204 g), 2M Thiourea (1.523 g), 3 % CHAPS (0.3 g), 0.5 % Triton x- 100 (50 µl) dissolved in ddH_2O up to 10 ml andstored at -20 °C. The equilibration buffer was performed with 50 mM Tris-HCl pH 8.8 (10 ml), 6 M Urea (72.02 g), 30 % glycerol (60 ml), 2 % SDS (4 g) brought to 200 ml with ddH_2O and stored in 10 ml aliquots at -20 °C. The agarose sealing solution was performed with 0.006 % (w/v) agarose (0.6 g) preapred in 100 ml of running buffer. The mixture was homogenized by little warming. After complete solubilization, 1 % (w/v) bromophenol blue (200 µl) was added to the mixture and the solution wasstored at room temperature.The fixing solution was prepared with 100 % ethanol (250 ml) and phosphoric acid (10 ml) and 240 ml of ddH_2O.The coomassie blue staning solution was prepared using 7 % (w/v) ammonium sulphate (85 g), 34 % (v/v) methanol (170 ml), 2 % (v/v) phosphoric acid and 0.5 g brilliant blue G-250. The final volume was made up to 500 ml with ddH_2O and stored at room temperature.For the preparation of 4 mini-gels, 12 % SDS-PAGE resolving gel was performed with 30 % acrylamide (10 ml), 1.5 M Tris-HCl pH 8.8 (6.3 ml), ddH_2O (8.2 ml), 10 % ammo,ium persulfate (APS) (250 µl), 10 % SDS (250 µl), Temed (10µl). The total volume was about 25

ml.The 12 % of staking gel for 4 mini-gels was performed using 30 % acrylamide (830 µl), 1M Tris-HCl pH 6.8 (630 µl), ddH_2O (3.4 ml), 10 % APS (50 µl), 10 % SDS (50 µl), Temed (5 µl) for a total volume of 5 ml. The preparation of 4 gels of 12 % SDS-PAGE for two dimension gel electrophoresis was performed with 30 % of acrylamide 30 % (6.5 ml), 0.8 % of bisacrylamide 40 % (52 ml), 1M Tris-HCl pH 8 (56.3 ml), ddH_2O (32.25 ml), 10 % APS (1.5 ml), 10 % SDS (1.5 ml), Temed (60 µl) for a total volume of about 150 ml.

II.1.3.4. Western blot solutions

Differents solutions were used western blot analysis. The 2 x SDS extraction protein buffer prepared with 1 M Tris-HCl, pH 6.8, 200 mM DTT, 4 % SDS and 20 % glycerol; the 1 x SDS buffer for protein solubilisation prepared with 2 x SDS extraction buffer in ddH_2O in proportion (1:1). The phosphate buffer saline (PBS) tween was prepared by dissolving one tablet of PBS in 200 ml of ddH_2O and 200 µl of tween-20. The mixture was stored at 4 °C. 5 % of blocking solution was prepared with 5 % (w/v) Elite fat free instant milk powder (5 g) in 100 ml of PBS and 1 % of blocking solution was prepared with 1 % (w/v) Elite fat free instant milk powder (1 g) in 100 ml of PBS. The western blot staining solution was prepared with 0.1 M Tris-HCl (6.057 g), pH 9.5 dissolved in 200 ml ddH_2O where 0.1 M NaCl (2.922 g), 0.05 M $MgCl_2$ (10.165 g) were added and the final volume made up to 500 ml with ddH_2O. The staining solution was prepared by adding at 10 ml of this solution, 200 µl p-nitrotetrazolium-blue and bromo-chloro-indolyl phosphate solution (NBT/BCIP system, GIBCO/BRL).

II.2. METHODS

II.2.1. Production of tubers in the field

The trial was established at the experimental field of Tropical Crops Genetic Resources Institute of CATAS over the 2013/2014 growing season. The soil chemical composition is presented in Table II. The experimental design was constituted by two blocks (10 m x 7 m / Block) spaced at 1 m. Stem cuttings (Fig. 9) of about 15 - 20 cm length, 20 mm diameter with five buds taken from 12 months old plants were horizontally planted at a distance of 1 m x 1 m between and within rows. During the experiment, no fertilizer was applied and weeds were constantly controlled. Ten months later cassava storage roots were harvested, washed and kept in controlled environment chamber at 25 °C, 60 - 80 % humidity for PDD evaluation and biochemical analysis.

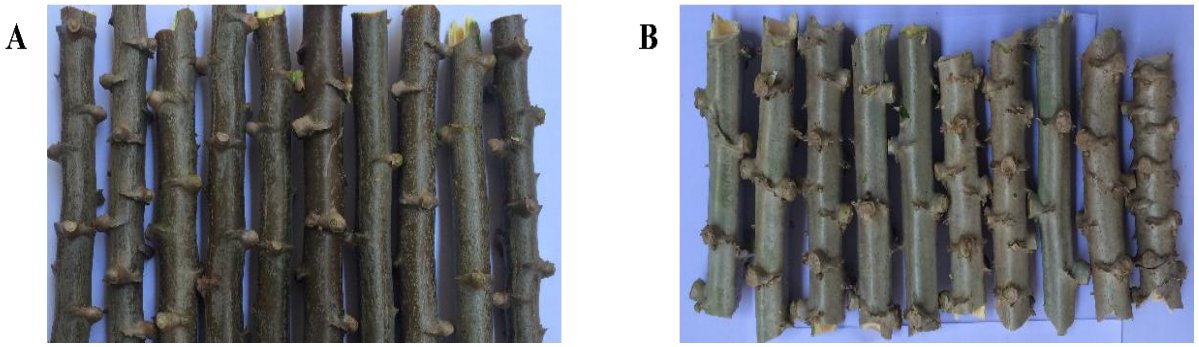

Fig. 9. Twelve months old stem cuttings of QZ1 (A) and SC5 (B).

Table II. Soil chemical composition.

soil properties	**Composition**
pH	7.26
C (%)	19.44
K (mg/Kg)	256.5
P (mg/kg)	43.38
Ca (cmol/kg)	1.62
Mg (cmol/kg)	1.07
bacteria (CFU/g dry soil)	8.57
fungi (CFU/g dry soil)	6.11

II.2.2. Production of tubers in pots and treatments

The experiment was conducted at the Cassava Germplasm Pool, Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences (CATAS) from July 2014 to February 2015. Stem cuttings of 15 cm length, and 20 mm width with five buds taken from cassava genotypes SC5 and QZ1 were used in the experiment. The stem cuttings were planted in 20 L (30 cm diameter) plastic pots containing a composite soil (Table II) collected from the cassava experiment field of CATAS. The experiment was carried out in the open air. The plants were treated with two different fertilizers containing calcium and magnesium from Sinochem Shandong fertilizer co., LTD. Treatment 1 consisted of supplying 6.6 g of calcium fertilizer (CaO 23.5 %, N 11.7 %), and treatment 2 consisted of supplying 22.47 g of magnesium fertilizer (Mg 8.9 %, B+Fe 0.6 %). The control consisted of growing

the plants without any of the calcium and magnesium fertilizers. The fertilizers were applied three months after planting, based on the recommendation of the International Centre of Tropical Agriculture (CIAT) (Howeler, 2002). Each treatment consisted of five plants, in three repetitions with one plant per pot. Eight months after planting, cassava storage roots (CSRs) were harvested, washed and kept in controlled environment chamber at 25 °C, 60 - 80 % humidity for PDD evaluation and biochemical analysis at 0, 3, 6, 15 and 30 days after harvest (DAH). Slices of flesh sample were snap frozen in liquid nitrogen and kept at -80 °C for future biochemical analysis.

II.2.3. PPD Evaluation and sampling procedure

PPD evaluation was made by cross section of the middle of at least three CSRs at 0, 3, 6, 15 and 30 days. A sliced section of about 2 cm thickness was photographed for visual observation of PPD development. PPD onset was determined by the observation of blue/black/brownish discoloration on parenchyma as characteristic of PPD (Wheatley, 1982; Salcedo et *al.,* 2010). At each time point, harvested tubers were manually peeled with broad bladed stainless steel knives. Thereafter, samples were used to make slices with a knife for flesh samples of different time points, snap-frozen in liquid nitrogen and stored at -80 °C for later biochemical analysis (Fig. 10).

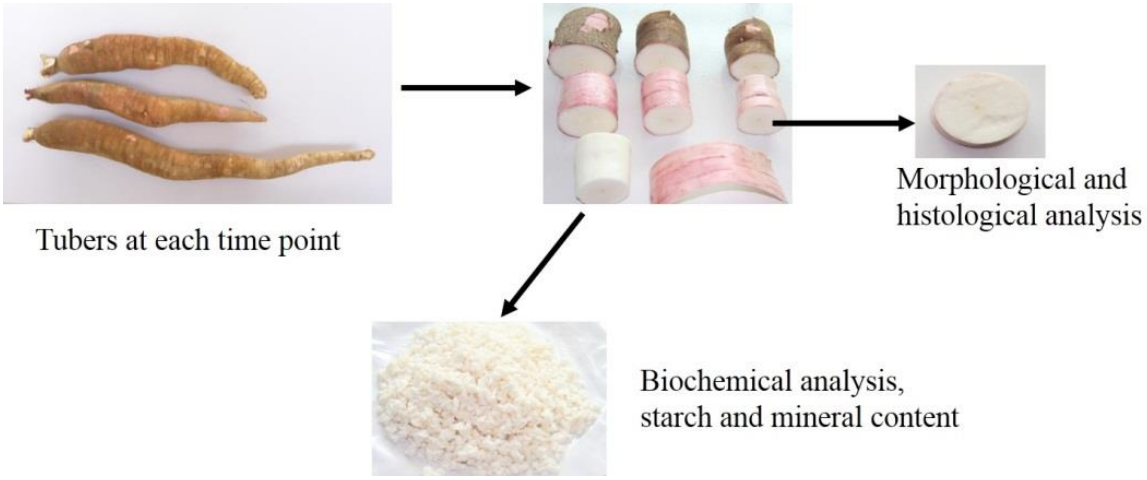

Fig. 10. Sampling for morphological and biochemical analysis. At least three tubers for each genotype were selected.

II.2.4. Histological analysis

Approximately 0.7 x 0.5 x 0.3 cm^3 small cassava pieces were excised from flesh roots using a razor blade and subjected to a vacuum for 5 min to remove air from the samples, then

fixed in a fresh solution of formalin acid-alcohol for 24 h. The samples were dehydrated in a grade of ethanol series followed by n-butanol, then, embedded directly in paraffin wax, serially sectioned at 10 µm thickness (microtome Leica, RM 2245) and mounted on glass slides. These slides were deparaffinized in xylene (twice for 15 min each) followed by xylene/ethanol (1:1) for 1 min, then treated with different grades of ethanol for 1 min each before staining with safranin-O (1 % in 50 % ethanol) and washed with a different grade of ethanol for 1 min each before staining with fast green (0.2 % in 95 % ethanol) according to Johansen (1940). In addition, cutting of samples were stained in astrablue/safranin (9:1) and subjected to a series of different grades of ethanol and xylene for dehydration. Examination was made under light microscope (Zeiss Imager M2, Germany) with a camera (Zeiss) using Axio vision (version 4.8).

II.2.5. Determination of starch content

Precisely 0.1 g of dry flesh was used for the determination of starch content, according to the International Association for Cereal Science and Technology (ICC) method 122 (1994). Ten milliliters of 70 % ethanol were added to the sample; the mixture was heated at 60 °C for 20 min and centrifuged at 5000 g for 5 min. The supernatant was discarded and the pellet was collected. The operation was repeated three times. The remaining starch was dissolved in a boiling solution of calcium chloride at 119 °C for 20 min. After cooling at room temperature 1 ml of 15 % K_4[Fe (CN)6]•$3H_2O$ and 1 ml of 30 % $ZnSO_4$•$7H_2O$ were added to each sample and the mixture was transferred into a 100 ml volumetric flask. The final volume was adjusted to 100 ml with distilled water. Thereafter, the solution was then filtered using a whatman filter paper. The optical activity of the filtrate was measured using a polarimeter (WZZ1, Sanghai-China) and the percentage of starch content was calculated using the following equation.

$$Starch\ content\ (\%) = \frac{(A \times 100) \times 100}{(2 \times 184) \times m}$$

Where *A* is the absorbance of the solute, *2* is the length of the polarimeter tube in dm, 184 is the specific rotation of cassava starch and *m* is the weight of the sample.

II.2.6. Evaluation of mineral contents of cassava storage roots from the field and pots

Mineral analysis was performed on the samples immediately after harvest in order to determine the mineral levels of the tubers. Dried sample (0.4 g) was subjected to various heating grades as follows: 150 °C for 30 min until it ceased to smoke; 300 °C for 30 min and

550 °C for 3 h, were applied to obtain a whitish or grayish ash. The ash was dissolved in 5 ml of HCl 37 % and heated for 30 min at 140 °C. The mixture was transferred into a 100 ml volumetric flask and the volume was made up to 100 ml with distilled water. This constituted the basic solution. The mineral content was determined by atomic absorption spectrophotometry according to García & Báez (2012) with a varian atomic absorption spectrophotometer (AA-7000, Shimadzu -China) with an air-acetylene flame; the wavelengths were set to 422.7, 285.2, 766.5 and 589 nm for calcium, magnesium, potassium and sodium respectively. The samples were aspirated into the flame and the corresponding absorption of characteristic radiation by the atomic vapour of the element was recorded. Calcium carbonate, magnesium sulphate, potassium nitrate and sodium chloride were used as standards for calcium, magnesium, potassium and sodium respectively. For sodium and potassium determination, 1 ml of the basic solution was dissolved in 9 ml of distilled water and for calcium and magnesium determination, 1 ml of the basic solution was dissolved in 3.5 ml of distilled water. To eliminate phosphorus interference, 1 % of lanthanium was added to the sample ash solution of calcium and the standard solution of calcium carbonate. For each ash solutions, the analyses were performed in triplicates and the averages calculated.

II.2.7. Biochemical assays

II.2.7.1. Determination of H_2O_2 content

Cassava storage root flesh was ground into powder using liquid nitrogen in a mortar with pestle. About 100 mg of the frozen powder was homogenized with 900 µl of 15 mM of ice-cold physiological saline solution (0.9 %). The homogenate was centrifuged at 10000 rpm for 10 min at 25 °C and the supernatant was collected. The supernatant (500 µl) was used for the determination of H_2O_2 content using a kit (Nanjing Jiancheng Bioengeneering Institute, China). The fluorescence was quantified using Ultraspec 2100 *pro* (Amersham, healthcare Biosciences, Sweden) at 405 nm. The samples were analysed in triplicates.

II.2.7.2. Determination of Ethylene (ETH) content

Samples were ground into powder using liquid nitrogen. 1 g of the powder was homogenized in 1 ml of phosphate buffer saline (PBS), pH 7.4. The homogenate was centrifuged at 2500 rpm, 4 °C for 20 min. The ethylene quantification was made using Plant ETH Elisa kit, DRE97035. 10 µl of sample were diluted in 40 µl of sample dilution solution

and the mixture was incubated at 37 °C for 30 min. After washing 5 times with washing buffer (from the kit), the plate was dried and 50 µl of HRP-conjugate reagent were added in each well and the plate was incubated at 37 °C for 30 min. This operation was followed by a second 5 times washing with washing buffer and the drying of the plate. Thereafter, 50 µl of chromogen solution A and chromogen solution B were added in each well and the plate was incubated at 37 °C for 15 min. 50 µl of stop solution (from the kit) was added and the blue color of the mixture changed to yellow. The absorbance in the wells was measured at 450 nm. The concentration of ethylene was determined using a standard curve obtained from the kit standard.

II.2.7.3. Antioxidant enzymes activities

II.2.7.3.1. Assay of superoxide dismutase (SOD) and peroxidase (POD) activities

Cassava storage root flesh was ground into powder using liquid nitrogen. About 100 mg of the frozen powder was homogenized in 900 µl or 400 µl of phosphate saline buffer (PBS) 0.1 M, pH 7.4 for POD and SOD evaluation respectively. The homogenate was centrifuged at 3500 rpm for 10 min at 25 °C. The supernatant was collected to determine the activities of each enzyme using spectrophotometric diagnostic kits (Nanjing Jiancheng Bioengeneering Institute, China) in three replicates according to the manufacturer's instruction (Hu et *al.*, 2016). SOD activity was detected using the xanthine oxidase method, based on its ability to inhibit the oxidation of hydroxylamine by xanthine oxidase. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50 % of the rate of the hydroxylamine measured at 550 nm. POD activity was evaluated based on its ability to catalyse hydrogen peroxide. The absorbance was measured at 420 nm.

II.2.7.3.2. Assay of catalase (CAT) activity

Cassava storage root flesh was ground into powder using liquid nitrogen. About 100 mg of the frozen powder was homogenized with 900 µl of 15 mM of ice cold physiological saline solution (0.9 %). The homogenate was centrifuged at 2500 rpm for 10 min at 25 °C. The supernatant was collected to evaluate the activity of the enzyme using kits (Nanjing Jiancheng Bioengeneering Institute, China). The CAT activity was determined according to the ammonium molybdate spectrophotometric method; based on the action of ammonium molybdate which can rapidly stop the H_2O_2 degradation reaction catalysed by CAT and which

reacts with the residual H_2O_2 to generate a yellow complex. The absorbance was read at 405 nm.

II.2.7.3.3. Assay of ascorbate peroxidase (APX) activity

Ascorbate peroxidase activity was measured using the commercial assay kit (Beijing SolarbioTechnology Company, China) according to the supplier's protocol. One unit of APX activity was described as 1 mg tissue catalysing 1 µmol ascorbate at 290 nm between 10 s and 130 s.

II.2.8. Proteomic analysis

II.2.8.1. Protein extraction

Proteins were extracted by phenol precipitation according to Chen et *al.* (2009). About 5 g of each frozen sample was ground in a pre-cooled mortar with liquid nitrogen to a fine powder and dissolved in the extraction buffer. The supernatant was retained, then, 5 ml of phenol were added and the tubes were kept on ice under the shaker for 10 min. After the incubation, the tubes were centrifuged at 10 000 rpm, 4 °C for 20 min. The lower fraction was removed and transferred into a new tube. Five ml of extraction buffer were added in the retained sample and the mixture was subjected to incubation on ice under the shaker for 10 min. The tubes were centrifuged after incubation at 10 000 rpm, 4 °C for 20 min. The lower fraction was recorded and transferred into new tubes with 5 volumes of 0.1 M ice cold ammonium acetate in methanol overnight at -20 °C for protein precipitation.

The protein pellet recorded was washed thrice with 0.1 M ice cold ammonium acetate and finally with 100 % ice cold acetone and 80 % of ice cold acetone. The pellet obtained was dried for 30 min in the hood and the dry protein pellet was re-suspended in an appropriate volume of SDS-soluble buffer. The tubes were vortexed for 30 seconds and later incubated at room temperature to fully dissolve. The mixture was then centrifuged at 13000 rpm for 5 min to remove any insoluble material. The supernatant was collected in small eppendorf tubes of 100 µl and stored at -20 °C.

II.2.8.2. Determination of protein concentration using Bradford

The total protein concentration was quantified according to the principles and methods of Bio-Rad protein assay (Catalog 500-0006, Bio-Rad laboratories, Hercules, CA, USA) using

bovine serum albumin (BSA) standards. BSA protein standard concentration in the range of 0 to 25 µg of proteins and 5 µl of proteins to be analysed were transferred to a plate supply. 5 µl of SDS-soluble buffer were added to each hole and water was added to complete the volume up to 150 µl. 150 µl of the reagent were added to each sample and the final volume brought to 300 µl. The mixture was incubated for 10 min at room temperature. Absorbance of the samples was then measured at 630 nm. A standard curve of absorbance versus BSA concentration was plotted and the concentration of proteins in the samples was determined from the curve.

II.2.8.3. Two-dimensional polyacrylamide gel electrophoresis (2DE-PAGE)

II.2.8.3.1. Rehydration of the strip and first dimensional electrophoresis

Protein separation was done according to An et *al.* (2014). The electrophoresis was performed using 13 cm long Immoboline DryStrip gels with linear pH range 4-7 (GE Healthcare Bio-Sciences AB). The strips were prepared by removing the protective cover using forceps starting at the acidic end. A total of 300 µg of protein were used to perform 2-DE electrophoresis. Preceding the isoelectric focusing (IEF), samples were diluted in the rehydration buffer in which 1 % Immobilized pH gradient (IPG) buffer 4-7, 0.25 % of DL-Dithiothreitol (DTT), and bromophenol blue were added. The total volume was about 312 µl. Then, samples were pipetted into the slots of the re-swelling tray and the strips were gently positioned in the solution in the re-swelling tray using forceps in the gel side down into the holder on top excluding all bubbles. The strips were then overlaid with 3 ml mineral oil to prevent evaporation of the sample and allowed to passively rehydrate overnight at room temperature (10 - 15 h). After rehydration, the strips were removed from the re-swelling tray then placed in the IPGphor cup loading strip holder with the positive end on the right, and gel side up. Filter papers of 0.5 x 20 cm long were cut and dampened with 1 % of DTT 1 M. The damp filter paper wicks were placed at each end of the strip, sticking out slightly. The electrodes were placed over the wicks and the loading cup placed over the strip at the positive end next to the electrode. Then, the strips were covered with cover fluid. The IEF representing the first separation of the proteins according to their isoelectric potential (IP) was performed at 20 °C using a Multiphor II system (GE Healthcare®, Bio-sciences AB, Sweden). Isoelectric focalization parameters used for the strips are presented in table III.

Table III. Isoelectrofocusing parameters used for 13 cm IPG strips.

Steps	Voltage	Type	Intensity	Time	Power
1	300 V	Gradient	380 mA	0:10 h	150 W
2	300 V	Step	380 mA	0:20 h	150 W
3	3500 V	Gradient	380 mA	1:30 h	180 W
4	3500 V	Step	380 mA	4:40 h	180 W
5	300 V	Gradient	380 mA	0:20 h	150 W
6	300 V	Step	380 mA	3:00 h	150 W

II.2.8.3.2. Second dimensional electrophoresis

After isoelectric focusing, the strips were equilibrated gel side up in re-swelling tray channels at room temperature for 15 min in an equilibration buffer with 1 % DTT and subsequently another 15 min in the same buffer, but with 2.5 % (w/v) iodoacetamide instead of DTT. After equilibration, the isoelectric focused proteins were ready for separation on second dimension. This involved separation of the proteins containing the strip using 12 % SDS-PAGE gel on SE 600 series Vertical Slab Gel Unit (Hoefer Scientific Instruments, San Francisco, CA, USA) with a vertical electrophoresis unit (Amersham Biosciences) system. Resolving 12 % gels were gently poured between two glass plates according to the manufacturer's manual (Bio-Rad). The liquid gel was overlaid with isopropanol and allowed to polymerize for about 30 min. When this time had elapsed, the isopropanol was decanted and the plates rinsed with ddH_2O. Each strip was then placed on top of the SDS-PAGE and overlaid with melted sealing agarose gel. The agarose was allowed to settle before placing the gels into the buffer tank. Gels were electrophoresed at 150 V for 5 h 30 min, until the bromophenol blue dye (indicator dye) reached the bottom of the gel.

II.2.8.3.3. Coomassie brilliant blue (CBB) staining

The gels were carefully removed from the plates and placed into storage boxes. The gels were fixed in 50 % ethanol (v/v) and 2 % phosphoric acid (v/v) for at least 2 h. The gels were then rinsed with Milli-Q water three times for 15 min each. Thereafter, the 2D gels were stained with Coomassie blue G-250 (Neuhoff et *al.,* 1988). The gels showing a much higher degree of variation among the three independent biological replications were selected for each time point.

II.2.8.4. Image scanner and data analysis

Gel matching for protein quantification was performed by Image scanner III (Ge healthcare for Delta 2D (DECODON GmbH, Greifswald, Germany) software, and spot pairs were confirmed visually. The differentially expressed spots were determined by using Scheffe's test $P<0.05$. The abundance of each protein spot was estimated by percentage volume (% Vol). Only those with the significant and reproducible changes were considered to be differentially accumulated proteins.

II.2.8.5. Protein identification and analysis

Tryptic in-gel digestion and protein identification were performed according to An et *al.* (2014). The differentially expressed proteins were identified by Liquid Chromatography Mass spectrometry (LC-MS/MS) at Beijing Genome Institute (Shenzhen). The mass spectra were acquired on a LTQ Orbitrap Velos (Thermo Fisher, USA). Peptide mass tolerance was set as 20 ppm and mass spectrometry (MS/MS) ion mass tolerance was set at 0.1 Da; one missed cleavage was allowed, carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine as a variable modification. Routine protein identification required sequence-confirmed data for a minimum of two peptides with recognition as the top ranking match in the Mascot Standard scoring system (An et *al.*, 2014). Amino acid sequences (minimum of six) from the top ranking match in the Mascot Standard scoring (http://www.matrixscience.com) were used for protein identification at the database of Manihot_esculenta_NR.fasta (37724 sequences) from UniProt (http://www.uniprot.org/), NCBI (http://www.ncbi.nlm.nih.gov), Ensembl (http://www.ensembl.org/index.html) and cassava proteome data base at Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html#!search), classification analysis at gene ontology (http://phytozome.jgi.doe.gov/pz/portal.html#!search).

II.2.8.5. Generation of protein-protein interaction (PPI) networks

Differential expressed proteins (DEPs) in QZ1 and SC5 were submitted to search tool for the retrieval of interacting genes (STRING). All interactions in STRING were provided with a probabilistic combined score >0.4. The protein - protein interaction (PPI) network was generated at STRING (http://string-db.org/newstring_cgi/show_input_page.pl) and metabolically function at Protein Metabolic Network (PMN)

(http://pmn.plantcyc.org/CASSAVA/NEW-IMAGE?type=GENE&object=G2Z-8222&redirect=T). The proteins in the protein-protein interaction network considered as nodes and the degree of a node corresponded to the number of interaction with other proteins. The proteins with high degrees were considered as hub nodes.

II.2.9. Western blot analysis

Protein samples for western blot analysis were extracted from 3 g of cassava storage roots flesh ground to a fine powder under liquid nitrogen. Then 3 ml of ice cold western blot extraction protein buffer were added and the homogenate was centrifuged at 10 000 rpm for 15 min at 4 °C. After centrifugation, the supernatant was recorded and three volumes of 100 % of ice cold acetone were added to the extract. Samples were incubated on ice for 1 h and then centrifuged at 10 000 rpm for 15 min at 4 °C. The pellet was washed with 80 % of ice cold acetone by centrifugation at 10 000 rpm for 10 min at 4 °C. The dry pellet was solubilized in 1 x SDS solubilization buffer and heated at 100 °C for 10 min. The protein amount was determined using Bradford method and separated by 12 % of sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolving gel and 5 % stacking gel according to Laemmli (1970). Proteins were blotted into a nitrocellulose membrane (N80 17-5EA, Sigma-Aldrich) and detected by immune-staining with anti-APX AS08368 for APX, anti-Cu/ZnSOD AS06170 for Cu/ZnSOD and anti-HSP70 AS08348 for HSP70 all from Agrisera. Western blots were developed according to the method of nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) from Roche (11681451001). The integrated density of each protein during storage was assessed using Image J software (http://rsb.info.nih.gov/ij/,NIH,MD, USA).

II.2.10. Evaluation of calmodulin (CaM), RanGTPase (RAS), Aspartic proteinase (ASP) and NADH ubiquinone-oxidoreductase (NADH) expression levels

Cassava flesh root was ground into powder in liquid nitrogen with a mortar and a pestle. About 100 mg of the frozen powder was transferred to a 1.5 ml eppendorf. Total RNA was extracted using the RNAprep Pure Plant Kit (Tiangen Biotech®, CO, LTD DP 140916, Beijing, China). Samples were analyzed in triplicates. The RNA quality was determined by running an agarose gel with GelStain (TransGen, Biotech, Code: GS101-01) staining. The RNA concentration was determined with NanoVue™Plus ultramicro spectrophotometer (GE Healthcare).

Reverse transcription was performed according to the manufacturer's protocol (TransGen, Biotech, Code: AT311-02). Each cDNA sample was diluted 10 times in sterile ddH_2O, and 1 µl of this dilution was used as a template for real-time polymerase chain reaction (RT-PCR). In fact, the RNA sample was digested with DNase I and the first strand cDNA was synthetized from 2 µg total RNA of each sample in which 1 µl of oligo (dT_{18}) primer (0.5 µg/µl), 1 µl of random primer (0.1 µg/µl), 1 µl of DNTP mixture (10 mM), 1 µl of prime script^{TM} II reverse transcriptase (200 U), 4 µl of 5x prime script II buffer and 1 µl of RNase inhibitor (20 U) were added (Takara, Japan). The mixture was incubated at 42 °C for 45 min and 70 °C for 15 min. The cDNA reaction mixture was stored at -80 °C. The RT-PCR reactions were performed in a 10 µl volume containing 5 µl of 2 x SYBR1 Premix Ex TaqTM II (Tli RNaseH Plus) (TaKaRa, Code: RR820A), 1 µl (100 ng/µl) cDNA, and 0.8 µl (10 µM of each primer) primers in a Thermo Scientific PikoREAL thermocycler. PCR amplification was conducted by 7-min preincubation step at 95 °C followed by 40 cycles of 95 °C for 5s and 60 °C for 30 s. The primers used are presented in table IV. Quantification was performed by sample of target genes to beta-actin gene using the comparative Ct method. The ΔCt was calculated by subtracting the average Ct of each treatment stage from the average Ct of beta-actin. The ΔΔCt was calculated by subtracting the ΔCt of each treatment stage from the ΔCt of the 0 DAH. The formula 2-ΔΔCt was used to calculate the relative fold change between the treatment stages (Livak & Schmittgen, 2001). All of the samples were measured in triplicate, and the experiments were performed on three biological replicates.

Table IV. Primers used for real time PCR.

Reference gene	**Forward primer**	**Reverse primer**
β-actin	5'- TGATGAGTCTGGTCCATCCA- 3'	5'-CCTCCTACGACCCAATCTCA-3'
Target DNA		
Calmodulin	5'- GGCAGAGCTCCAGGACATGA-3	5'- GGCAGCAGCAGAAATAAAACCA-3
Aspartic proteinase	5'- CCCGTTTCCTGTGACGCTGCTAA-3	5'-CGGTTGTCTTGGCGTCTTCCTCTGT -3'
RanGTPase	5' CACAGCATCCGCTAAACCAGAGT	5'- GGTGGAGAAACGGCAGTAATCG -3'
NADH ubiquinone-oxidoreductase	5'- CTGATGGCATAATGACAACAGTGAC -3'	5'- GCAACCAGTAACAAGAACAATAACC -3'

II.2.11. Data analysis

The data from the field were expressed as mean ± standard error, unless otherwise stated. Analysis of variance (ANOVA) was used to determine the differences amongst the evaluated parameters within each genotype for the tubers from the field and within each

treatment for the tubers from the pots. The pots experiments were arranged following a complete randomized design. All the experiments were performed in triplicates.The Duncan test was applied for multiple comparisons where it was necessary in the field and Tukey's test was used to compare treatment means. Significance was determined at $p<0.05$. The results were expressed as mean of three biological replicates. All statistical analyses were performed with the SPSS software V20.

RESULTS AND DISCUSSION

III.1. RESULTS

III.1.1. Evaluation of agronomic parameters at harvest

III.1.1.1. Evaluation of agronomic parameters in the field

Ten months after planting, tubers were harvested in the field and the average numbers of tubers as well as the average weights were recorded. The aspect of the tubers at harvest from the field is presented in Fig. 11. The average number of tubers per plant was 6.8 ± 1.04 and 6.3 ± 1.12 for QZ1 and SC5 respectively. The average weight of storage root per plant was 0.7 ± 0.17 (kg) and 0.99 ± 0.14 (kg) for QZ1 and SC5 respectively.

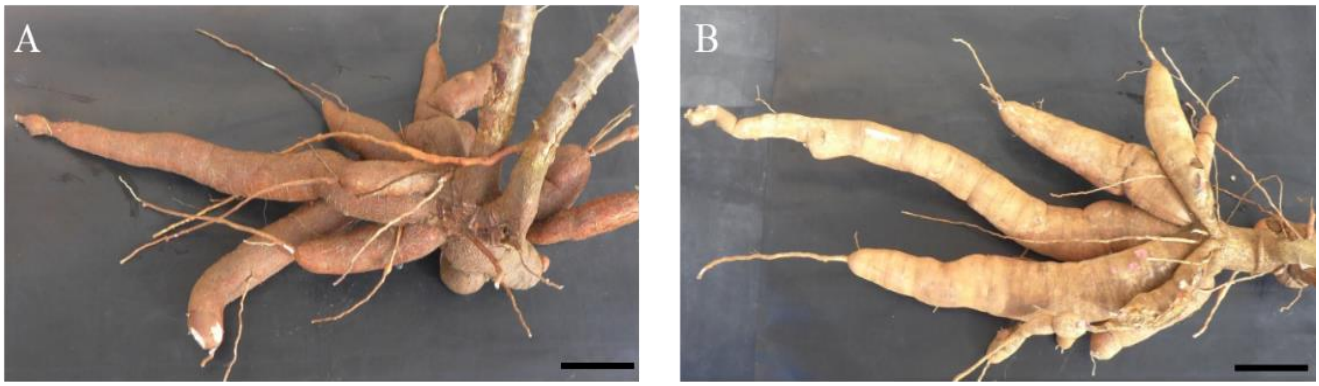

Fig. 11. Tubers aspect at harvest in the field; QZ1 (A) and SC5 (B). Bar= 20 cm.

III.1.1.2. Effect of calcium and magnesium fertilization on the number and fresh weight of tubers at harvest

Eight months after planting, the average number of tubers and the average weight of storage root were recorded. The aspect of the tubers at harvest is presented on Fig. 12. In SC5, the highest number of tubers was observed in plants from the control, followed by the plants from the treatment with Mg, while in QZ1, the number of tubers per plant was increased by the Ca^{2+} and Mg^{2+} application (Fig. 13A). In both genotypes, the highest tuber weight was observed in the treatment with Ca^{2+} (Fig. 13B). The lowest tuber weight was observed in the treatment with Mg^{2+} fertilizer and in the control of both genotypes.

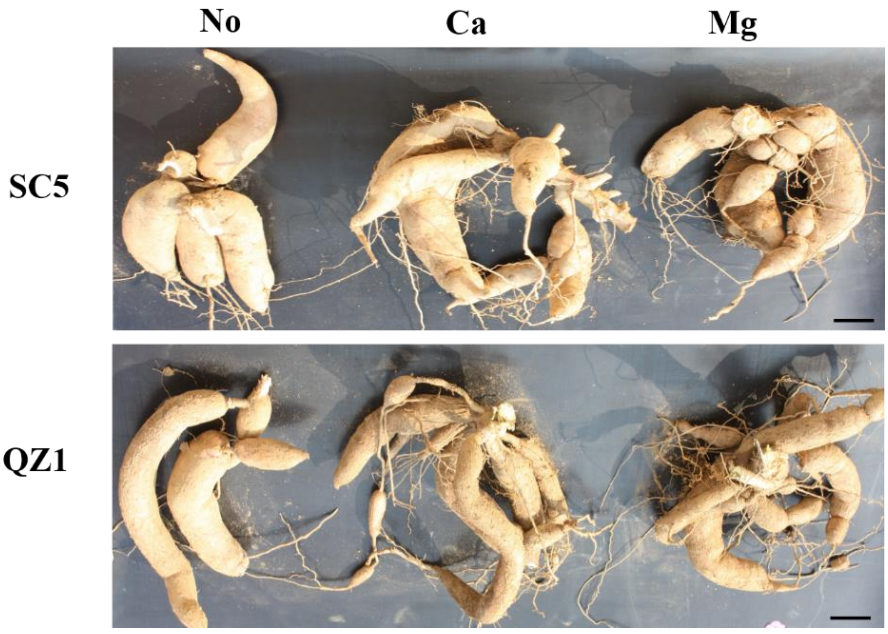

Fig. 12. Tubers aspect at different treatments at harvest eight months after planting.

No = control without any fertilizer; Ca= tubers obtained from plants receiving calcium fertilizer; Mg= tubers obtained from plants receiving magnesium fertilizer, bar= 10 cm.

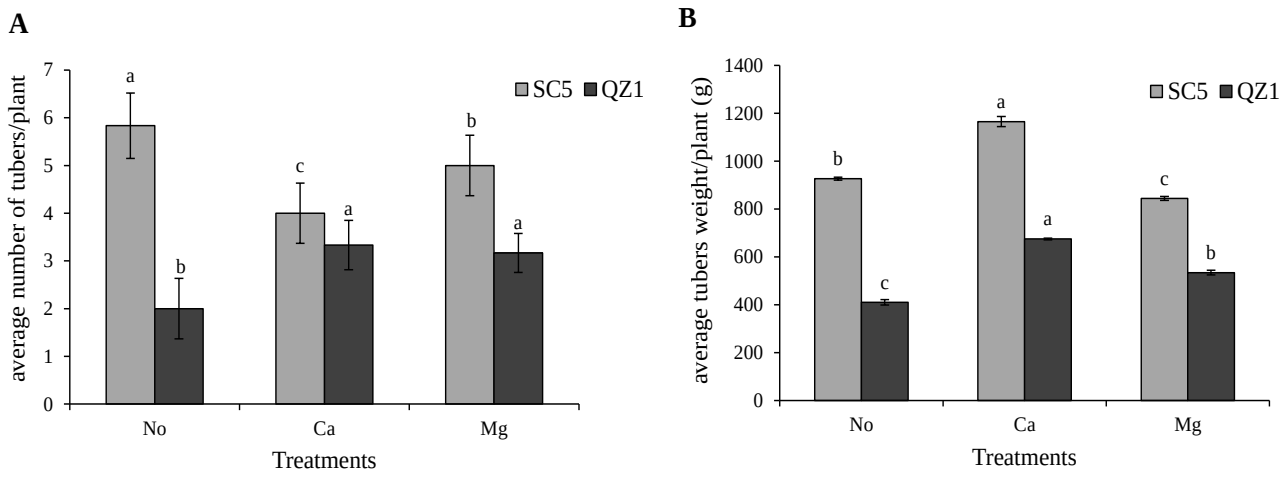

Fig. 13. Effect of Ca and Mg fertilization on the number of tubers (A) and weight of tubers (B) per plant.

III.1.2. Visual observation of change in storage root flesh during storage

III.1.2.1. PPD evaluation in the tubers from the field

At 0, 3, 6, 9, 15 and 30 days after harvest (DAH), roots were recorded to evaluate postharvest physiological deterioration (PPD) onset. Visual observation by transverse section of the roots showed differences in PPD onset and progression of between the two genotypes.

In SC5, tubers the first sign of PPD recognized as blue/black discoloration in parenchyma was observed at 6 DAH while in QZ1, it was at 15 DAH (Fig.14).

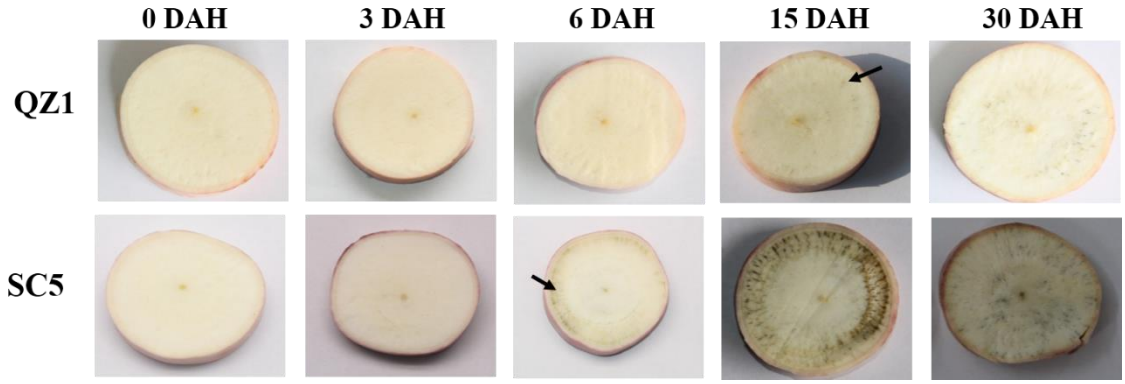

Fig. 14. Visual observation of PPD in slices during storage at 0, 3, 6, 9, 15 and 30DAH.
Black arrows show the onset of PPD during storage.

III.1.2.2. Effect of calcium and magnesium fertilization on PPD development on tubers from pots during storage

Morphological symptoms of PPD on storage roots were recorded and presented in Fig. 14. The first symptom of PPD was observed in the control of SC5 at 6 DAH while in magnesium-treated plants, discoloration related to PPD was observed at 30 DAH in storage roots. Delay on PPD onset was observed in SC5 in tubers from plants treated with Ca^{2+} fertilizer (Fig. 15A). In QZ1, the first sign of PPD was observed in the treatment with Mg^{2+} at 6 DAH followed by the treatment with Ca at 15 DAH (Fig. 15B).

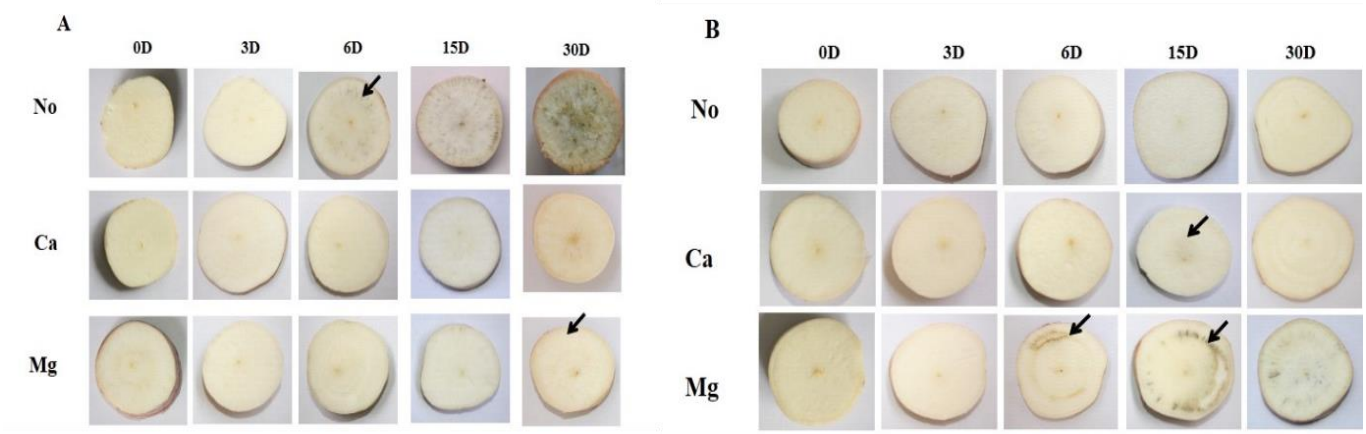

Fig. 15. Effect of calcium and magnesium fertilizers on the onset of PPD during storage at 0, 3, 6, 9, 15 and 30 DAH in SC5 (A) and QZ1 (B).

Like observed in the tubers from the field, the tubers obtained from the control in SC5 were the first affected by the development of PPD. PPD was delayed by the application either of calcium or magnesium fertilizer in SC5.

III.1.3. Histological changes in storage root flesh during storage

Tubers from the field were used to investigate the impact of PPD at histological level. Microscopic observation of cross sections of tubers showed a high number of starch granules in SC5 compared to QZ1 (Fig. 16. a1, b1). From 0 DAH to 15 DAH, a consistent decrease of the number of starch granules was observed in SC5 compared to QZ1 (Fig. 16. a3, b3). Staining with astrablue/safranin showed the presence of tyloses inside the xylem vessel in QZ1 at 0 and 15 DAH (Fig. 16. a4, a5). In SC5 no tyloses were observed at 0 DAH (Fig. 16. b4). However at 15 DAH a black discoloration was observed inside the xylem as an indicator of PPD development (Fig. 16. b5).

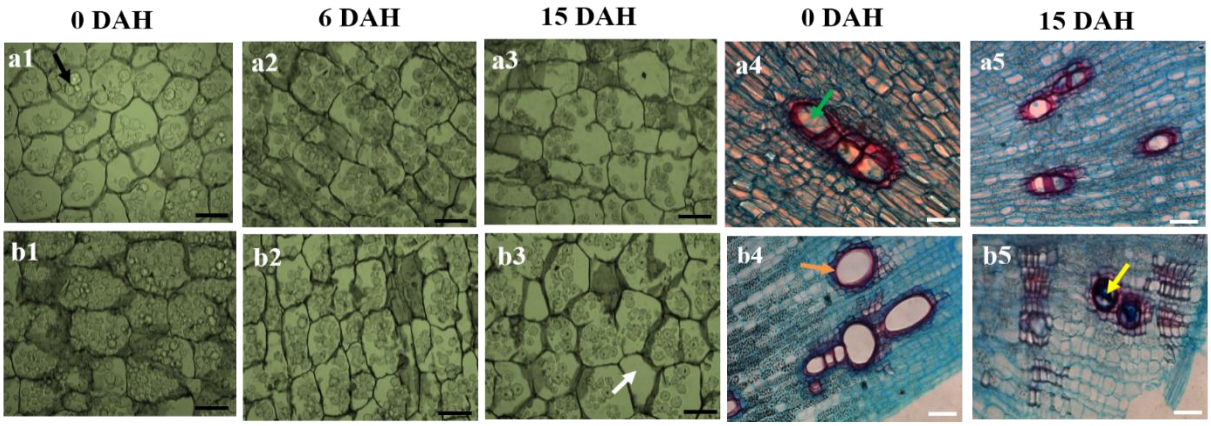

Fig. 16. Microscopic observation of parenchyma cells and xylem vessel at 0, 6 and 15 DAH.

Black: arrow starch granules (a1); white arrow: parenchyma cell (b3); orange arrow: xylem vessel (b4); green arrow: tyloses inside xylem; vessel (a4); and yellow arrow: black discoloration (b5). Black bar = 100 µm; white bar = 200 µm.

III.1.4. Change in starch content during PPD

III.1.4.1. Change in starch content during PPD in the tubers from the field

The change in the amount of starch during storage for the tubers from the field is presented in Fig. 17. At harvest, starch content was about 75.5 % in SC5 and 68.9 % in QZ1. This amount decreases significantly in SC5 from 0 DAH to 30 DAH. In QZ1, no significant

difference ($p<0.05$) was observed in starch content from 3 DAH to 15 DAH. A significant decrease ($p<0.05$) was observed at 30 DAH.

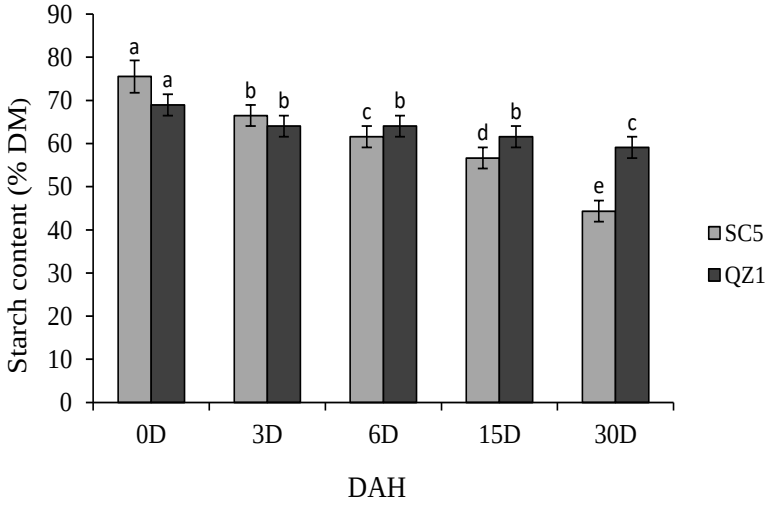

Fig. 17. Change in starch content in tubers from the field during storage.

III.1.4.2. Change in starch content during PPD after fertilization in the pots

The analysis of starch content during PPD after fertilization showed a decrease in starch content in both genotypes in response to soil fertilization from 0 to 30 DAH. In SC5 genotype, a significant decrease was observed in tubers from plants treated with Mg^{2+} fertilizer from 6 to 30 DAH (Table V). In QZ1, a slight decrease was observed in all the treatments from 0 to 15 DAH. Both genotypes showed considerable decrease of starch content at 30 DAH (Table V).

Table V. Interactive effect of calcium and magnesium fertilization on tuber starch content during storage.

		Starch content (%)				
		Day after harvest (DAH)				
Genotypes	Treatments	**0**	**3**	**6**	**15**	**30**
SC5	Control	68.97 a	66.5 a	54.19 d	51.72 c	46.8 c
	Calcium	66.5 b	61.58 c	59.11 c	59.11 b	51.72 a
	Magnesium	64.04 c	61.58 c	50.9 e	36.95 d	27.09 f
QZ1	Control	66.5 b	66.5 a	59.11 c	59.11 b	49.26 b
	Calcium	68.97 a	64.04 b	61.58 b	61.58 a	32.02 e
	Magnesium	66.5 b	66.5 a	64.04 a	61.58 a	41.87 d

Each value is the mean of three replicates. Values in same column followed by different letters are significantly different ($p < 0.05$) according to Tukey´s test.

III.1.5. Mineral content of the tubers at harvest in the field

Tuber mineral concentrations were in general highest in QZ1 compared to SC5 (Fig. 18). The amounts of 0.09 %, 0.11, 0.08 % and 0.57 % for Na, Ca, Mg and K respectively were obtained in SC5. The amounts of 0.15 %, 0.1, 0.1 % and 0.82 % for Na, Ca, Mg and K respectively were obtained in QZ1. High content of potassium was observed in both genotypes with QZ1 presenting the highest.

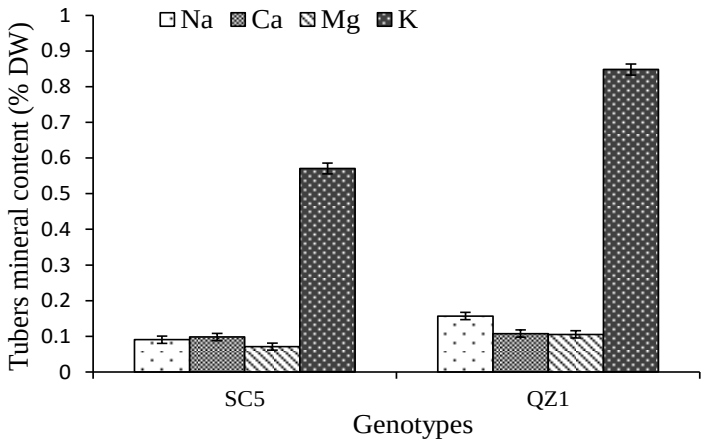

Fig. 18. Analysis of mineral content in QZ1 and SC5 after harvest in the field.

III.1.6. Mineral content of tubers after calcium and magnesium fertilization in the pots

The mineral profile of the tubers after fertilization is presented on table VI. Compared to control, the amount of Ca^{2+} and Mg^{2+} in cassava storage roots (CSRs) of QZ1 slightly increased with the application of calcium fertilizer; while in SC5, the content of Ca^{2+} slightly decreased. On the contrary, concentration of Ca^{2+} and Mg^{2+} in CSRs of SC5 slightly increased by the application of magnesium fertilizer. The K^{+} and Na^{+} contents were also affected by calcium and magnesium fertilization. A slight increase of K^{+} was observed in both genotypes treated with calcium and magnesium fertilizers compared to the control.While the content of Na^{+} decreased in QZ1, it increased in SC5 under magnesium fertilization.

Table VI. Interactive effect of calcium and magnesium fertilization on mineral content of cassava tubers.

Genotypes	Treatments	Na	Ca	Mg	K
SC5	Control	0.12 a	0.129 a	0.108 abc	1.35 ab
	Calcium	0.13 a	0.121 ab	0.112 ab	1.39 ab
	Magnesium	0.14 a	0.184 a	0.117 a	1.49 a
QZ1	Control	0.12 a	0.076 b	0.088 bc	1.17 b
	Calcium	0.13 a	0.118 b	0.088 bc	1.24 b
	Magnesium	0.03 a	0.139 ab	0.084 c	1.29 ab
LSD at 0.05		0.07	0.06	0.02	0.05

Each value is the mean of three replicates. Values in same column followed by different letters are significantly different ($p < 0.05$) according to Tukey´s test.

III.1.7. Proteomic results

III.1.7.1. Change in proteome profile during PPD development in cassava storage roots

Proteins extracted from cassava storage roots of QZ1 and SC5 during storage were separated by two dimension electrophoresis (2-DE). To ensure technical reproducibility of protein separation, three biological replicate of 2-D gels per sample were produced.The warps of the gels showing high variability (Appendix 1) compared to the control of each genotype are presented in Fig. 19 and Fig. 20. After isoelectric focalization (IEF) using 13 cm immobilised pH gradient (IPG) strips and SDS-PAGE, proteins were visualized with colloidal Coomassie Brilliant Blue and spot patterns were compared using image analysis software. 2-DE images of proteins extracted from 0 DAH time point in the storage roots of QZ1 and SC5 genotypes were used as control, respectively. A total of 120 proteins spots with greater than 2 fold change altered intensity in the pairwise comparison of 3DAH/0DAH, 6DAH/0DAH, 15DAH/0DAH and 30DAH/0DAH were detected in both genotypes, in which 106 proteins were identified using MALDI-TOF-MS/MS against cassava database (http://phytozome.jgi.doe.gov/pz/portal.html#!search) and classified at gene ontology (http://phytozome.jgi.doe.gov/pz/portal.html#!search) and confirmed by proteomexchange. These identified proteins were involved in eleven functional categories including carbohydrate/energy metabolism (23 spots, 21 %), chaperones (20 spots, 19 %), detoxifying/antioxidant (10 spots, 9 %), structure (2 spots, 2 %), inorganic ion transport and metabolism (1 spot, 1 %), proteins biosynthesis (13 spots, 12 %), amino acid metabolism (3

spots, 3 %), DNA and RNA metabolism (1 spot, 1 %), defense (23 spots, 22 %), signal transduction mechanism (5 spots, 5 %) and unknown (5 spots, 5 %). Of these, 63 differential proteins were from QZ1 and 75 proteins were identified from SC5 genotype. The most identified proteins expressed in both genotypes were associated to defense proteins (25 % and 23 %) for QZ1 and SC5 respectively. The second largest group represents proteins involved in carbohydrate and energy metabolism (19 % and 21 %) as well as chaperones (14 % and 21 %) for QZ1 and SC5 respectively (Fig. 21). The changes of the abundance of these proteins during storage are presented in Table VII and Table VIII for QZ1 and SC5 respectively.

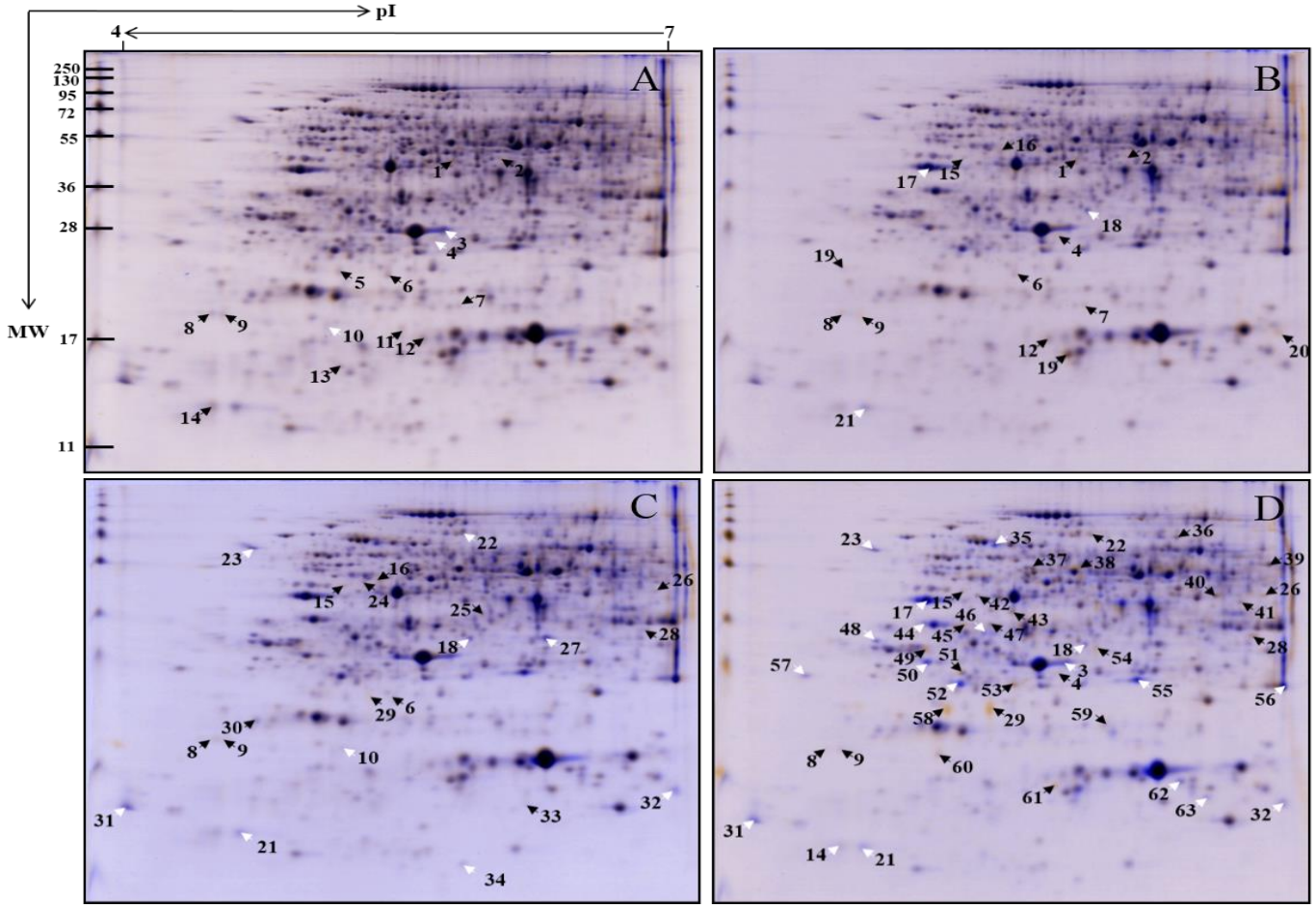

Fig. 19. Two dimension gel electrophoresis analysis of QZ1genotype at the time point from 0 DAH to 30 DAH.

Total of proteins (300 μg) were loaded on a 13 cm IPG strip with linear gradient (pH 4−7) and SDS-PAGE was performed on a 12 % gel. Proteins were stained with Coomassie blue G250 G-250. The wrapped 2-DE maps showed the pairwise comparison at the time-points of 3DAH/0DAH (A), 6DAH/0DAH (B), 15DAH/0DAH (C) and 30DAH/0DAH (D). The white and black arrows indicate proteins that showed detectable changes (>2.0-fold of the normalized volume) in abundance compared with those observed in the control of 0 h.White arrow presented a decreased match and black arrow indicated an increased match

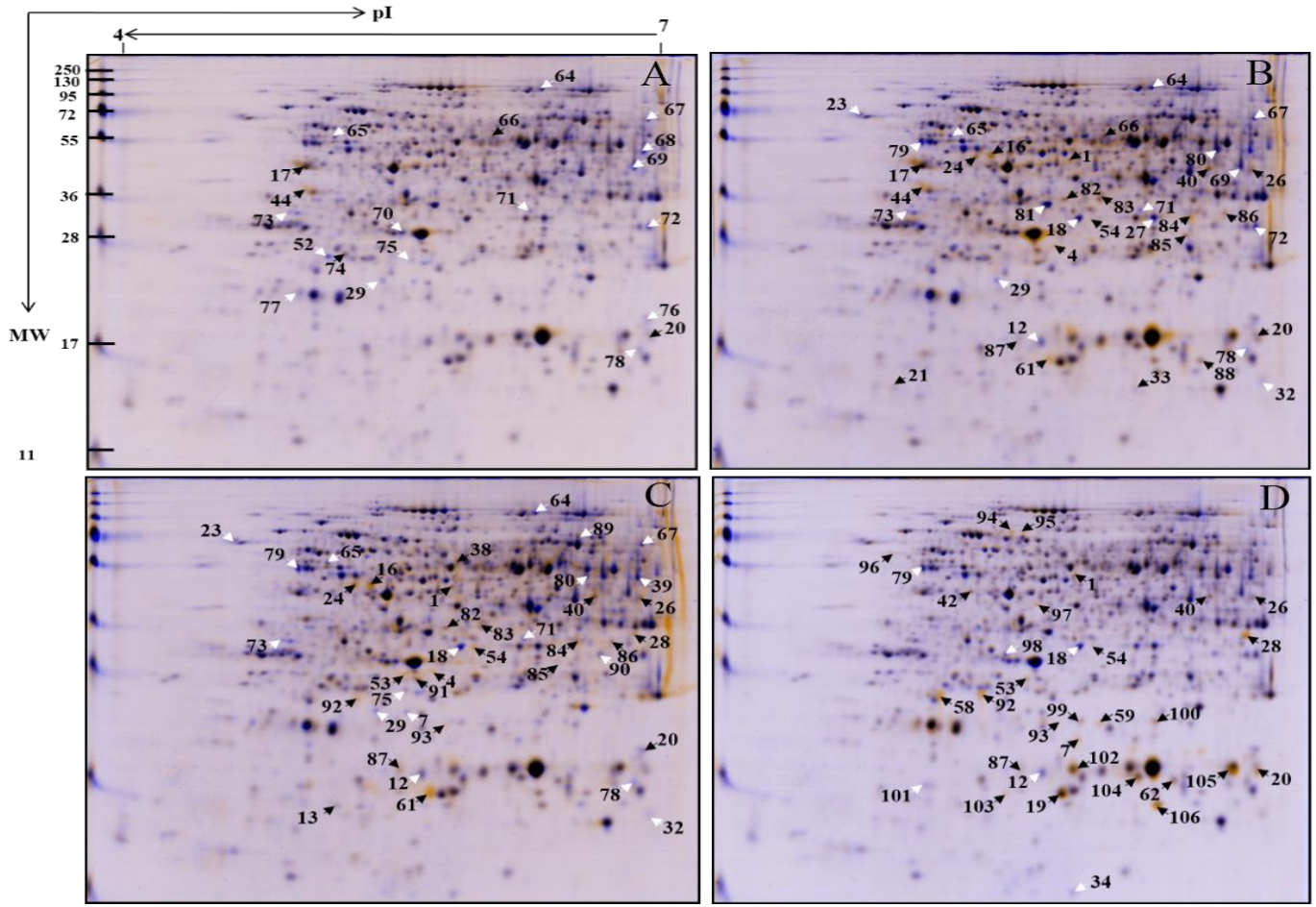

Fig. 20. Two dimension gel electrophoresis analysis of SC5 genotype at the time point from 0 DAH to 30 DAH.

Total of proteins (300 μg) were loaded on a 13 cm IPG strip with linear gradient (pH 4−7) and SDS-PAGE was performed on a 12 % gel. Proteins were stained with Coomassie blue G250 G-250. The wrapped 2-DE maps showed the pairwise comparison at the time-points of 3DAH/0DAH (A), 6DAH/0DAH (B), 15DAH/0DAH (C) and 30DAH/0DAH (D). The white and black arrows indicate proteins that showed detectable changes (>2.0-fold of the normalized volume) in abundance compared with those observed in the control of 0 h.White arrow presented a decreased match and black arrow indicated an increased match.

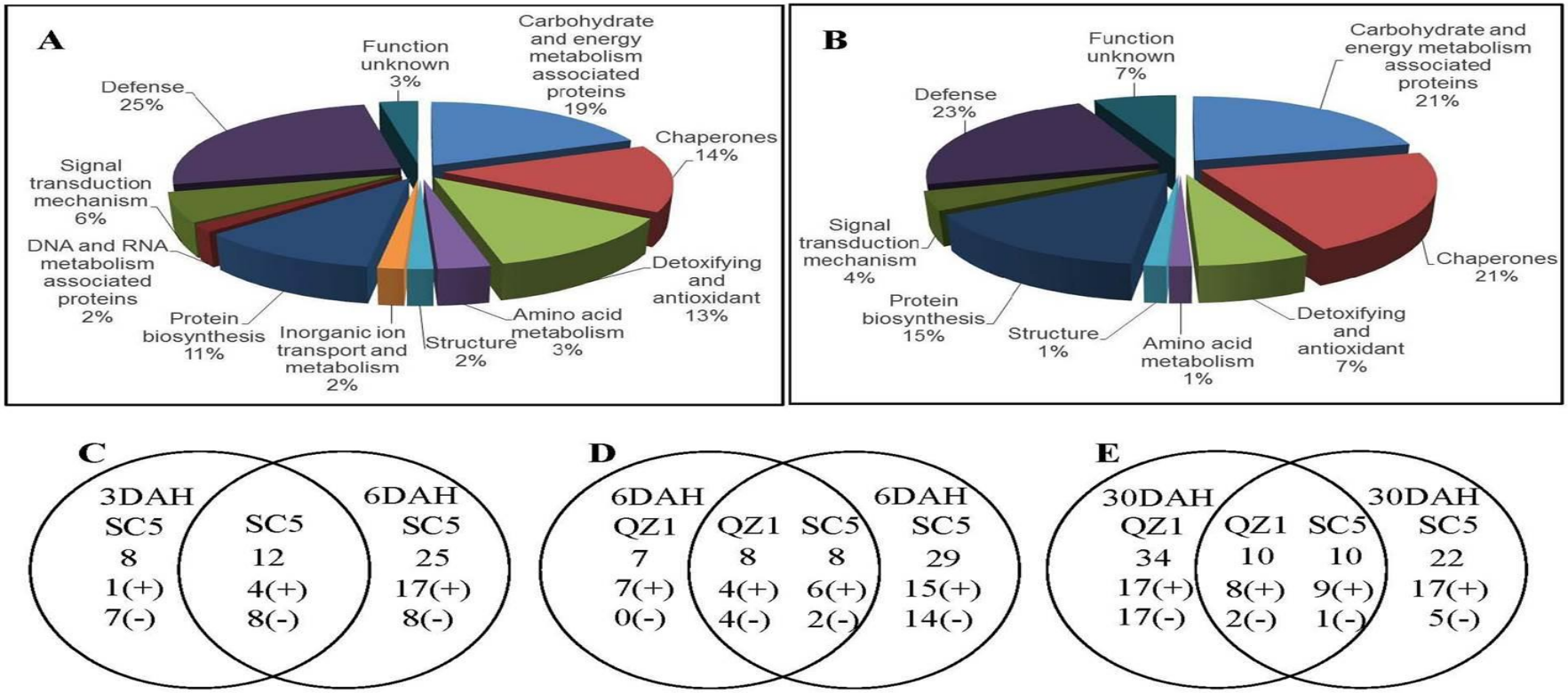

Fig. 21. Venn diagrams and functional categories of differential expressed proteins identified in cassava storage at the time points from 0 DAH to 30 DAH.

Unknown proteins included those whose functions had not been described. (A) and (B), functional categories of 63 and 75 differential proteins identified in QZ1 and SC5 genotypes roots respectively. Functional categorization of differential proteins from QZ1 (A) and SC5 (B) was performed according to the MIPS database (http://mips.gsf.de). C:the list of the SC5-specific at 3 DAH and the SC5-specific at 6 DAH and common inducted proteins appears near each circle; D:The list of the QZ1-specific at 6 DAH and the SC5-specific at 6 DAH and common inducted proteins appears near each circle; E:The list of the QZ1-specific and the SC5-specific during storage at 30 DAH and common inducted proteins appears near each circle. +:increased proteins; -:decreased protein.

Table VII. List of the identified proteins changing in abundance in QZ1 at different durations of storage.

Spot Number[a]	Identification	0DAH/3DAH	0DAH/6DAH	0DAH/15DAH	0DAH/30DAH	Accession no[b]	Proteins ID in string database
Carbohydrate and energy metabolism associated proteins (12)							
1	monodehydroascorbate reductase (NADH)	2.69 ± 0.08(+)	6.21 ± 0.09(+)	-	-	Manes.08G023200.1	MDAR1
4	Triose-phosphate isomerase / Triosephosphate mutase	∞ (-)	∞ (+)	-	∞ (+)	Manes.01G173500.1	TPI
7	Orotate phosphoribosyltransferase / Orotidylic acid phosphorylase	2.37 ± 0.03(+)	3.52 ± 0.04(+)	-	-	Manes.11G004200.1	AT3G54470
10	F-type H+-transporting ATPase subunit d	2.01 ± 0.20(-)	-	2.01 ± 0.20(-)	-	Manes.10G043000.1	ATPQ
28	Fructose-bisphosphate aldolase 3, chloroplastic	-	-	∞ (+)	∞ (+)	Manes.14G108900.1	PDE345
36	Malate dehydrogenase	-	-	-	2.83 ± 0.05(+)	Manes.16G109900.1	NADP-ME3
37	mitochondrial-processing peptidase subunit alpha	-	-	-	3.01 ± 0.25(+)	Manes.03G029000.1	AT1G51980
41	Alcohol dehydrogenase class-P	-	-	-	2.56 ± 0.05(+)	Manes.18G087400.1	ADH1
42	starch phosphorylase	-	-	-	2.20 ± 0.08(+)	Manes.02G052600.1	AT3G29320
46	2,3-dimethylmalate lyase / (2R,3S)-2,3-dimethylmalate pyruvate-lyase	-	-	-	∞ (-)	Manes.11G115000.1	AT2G43180
50	Ran-binding protein 1	-	-	-	∞ (-)	Manes.18G136500.1	RANBP1
60	F-type H+-transporting ATPase subunit d	-	-	-	2.41 ± 0.05(+)	Manes.10G043000.1	ATPQ
Chaperones (9)							
11	17.6 kDa class I heat shock protein 1	4.38 ± 0.02(+)	-	-	-	Manes.10G020600.1	HSP17.6A
13	25.3 kDa heat shock protein, chloroplastic	2.03 ± 0.04(+)	-	-	-	Manes.01G042200.1	AT5G51440
19	17.6 kDa class II heat shock protein	-	2.37 ± 0.21(+)	-	-	Manes.16G083600.1	HSP17.6II
20	17.6 kDa class I heat shock protein 1	-	∞ (+)	-	-	Manes.10G020600.1	HSP17.6A
30	25.3 kDa heat shock protein, chloroplastic	-	-	3.02 ± 0.05(+)	-	Manes.01G042200.1	AT5G51440
35	heat shock 70kDa protein 1/8	-	-	-	6.06 ± 0.12(-)	Manes.07G114200.1	HSC70-1
53	20 kDa chaperonin, chloroplastic	-	-	-	5.97 ± 0.10(+)	Manes.03G044000.1	CPN20

62	Small heat-shock protein HSP20 family	-	-	-	3.04 ± 0.09(-)	Manes.10G020100.1	AT2G29500
63	Small heat-shock protein HSP20 family	-	-	-	2.02 ± 0.05(-)	Manes.10G020100.1	AT2G29500
Detoxifying and antioxidant (8)							
3	L-ascorbate peroxidase 2, cytosolic	4.14 ± 0.06(-)	-	-	5.58 ± 0.07(-)	Manes.04G026800.1	APX2
5	Riboflavin synthase	2.47 ± 0.03(+)	-	-	-	Manes.13G146800.1	AT2G20690
14	Thioredoxin-like protein CXXS1	2.36 ± 0.06(+)	-	-	3.04 ± 0.23(-)	Manes.16G017200.1	CXXS1
17	Dehydrin	-	2.01 ± 0.21(-)	-	4.12 ± 0.21(-)	Manes.05G140400.1	COR47
21	Thioredoxin-like protein CXXS1	-	2.04 ± 0.05(-)	3.05 ± 0.14(-)	∞ (-)	Manes.16G017200.1	CXXS1
45	Dehydrin	-	-	-	∞ (+)	Manes.05G140400.1	COR47
56	Glutathione S-transferase	-	-	-	3.01 ± 0.14(-)	Manes.02G089200.1	GSTF9
58	Riboflavin synthase	-	-	-	∞ (+)	Manes.13G146800.1	AT2G20690
Amino acid metabolism (2)							
2	Fumarylacetoacetase / Fumarylacetoacetate hydrolase	∞ (+)	∞ (+)	-	-	Manes.09G070300.1	AT1G12050
47	Homocysteine S-methyltransferase 3	-	-	-	2.05 ± 0.09(+)	Manes.15G031300.1	HMT3
Structure (1)							
51	DREPP plasma membrane polypeptide	-	-	-	2.07 ± 0.11(+)	Manes.S071400.1	PCAP1
Inorganic ion transport and metabolism (1)							
31	calmodulin-5	-	-	2.08 ± 0.05(-)	7.08 ± 0.03(-)	Manes.01G002400.1	CAM7
Protein biosynthesis (7)							
12	translation initiation factor 5A	2.15 ± 0.05(+)	2.64 ± 0.04(+)	-	-	Manes.05G107000.1	ELF5A-1
16	Argininosuccinate synthase / Citrulline--aspartate ligase	-	7.07 ± 0.08(+)	8.48 ± 0.09(+)	-	Manes.03G085300.1	AT4G24830
22	Oligopeptidase A	-	-	2.03 ± 0.03(-)	-	Manes.13G103900.1	AT5G65620
25	protein disulfide-isomerase A6	-	-	2.26 ± 0.05(+)	-	Manes.05G002000.1	UNE5
39	Disulfide oxidoreductase	-	-	-	2.31 ± 0.13(+)	Manes.03G209100.1	mtLPD1
40	protein disulfide-isomerase A6	-	-	-	3.16 ± 0.09(+)	Manes.05G002000.1	UNE5
61	translation initiation factor 5A	-	-	-	∞ (+)	Manes.05G107000.1	ELF5A-1
DNA and RNA metabolism associated proteins (1)							
43	Nucleic acid-binding, OB-fold-like protein	-	-	-	2.50 ± 0.09(+)	Manes.02G024200.1	AT1G12800

Signal transduction mechanism (4)							
23	C2 domain	-	-	3.06 ± 0.06(-)	3.06 ± 0.06(-)	Manes.S104800.1	AT5G55530
26	Vesicle coat complex COPI, alpha subunit	-	-	∞ (+)	∞ (+)	Manes.12G101600.1	AT3G50590
48	14-3-3 protein	-	-	-	2.04 ± 0.09(-)	Manes.02G091000.1	At2g42590
49	14-3-3 protein	-	-	-	∞ (+)	Manes.15G093600.1	At2g42590
Defense (16)							
6	Ras-related protein Rab-11A	2.69 ± 0.05(+)	2.51 ± 0.04(+)	2.77 ± 0.04(+)	-	Manes.12G092100.1	RABA1f
8	Translationally controlled tumor protein	2.81 ± 0.19(+)	3.78 ± 0.04(+)	4.38 ± 0.04(+)	3.26 ± 0.09(+)	Manes.15G011800.1	TCTP
9	Translationally controlled tumor protein	5.66 ± 0.04(+)	7.10 ± 0.05(+)	6.77 ± 0.04(+)	3.56 ± 0.03(+)	Manes.15G011700.1	TCTP
15	suppressor of G2 allele of SKP1	-	∞ (+)	∞ (+)	∞ (+)	Manes.06G153900.1	SGT1B
18	thiamine thiazole synthase	-	2.04 ± 0.06(-)	2.79 ± 0.04(-)	3.08 ± 0.03(-)	Manes.15G075600.1	THI1
24	RING-H2 finger protein ATL61	-	-	3.28 ± 0.04(+)	-	Manes.07G090300.1	AT2G34990
27	thiamine thiazole synthase	-	-	2.02 ± 0.05(-)	-	Manes.15G075600.1	THI1
29	Ras-related protein Rab-11A	-	-	29.01± 0.03(+)	214 ± 0.13(+)	Manes.12G092100.1	RABA1f
34	RING-H2 finger protein ATL61	-	-	2.03 ± 0.02(-)	-	Manes.07G090300.1	AT2G34990
38	RING-H2 finger protein ATL61	-	-	-	∞ (+)	Manes.07G090300.1	AT2G34990
44	RING-H2 finger protein ATL61	-	-	-	5.09 ± 0.04(-)	Manes.07G090300.1	AT2G34990
52	thiamine thiazole synthase	-	-	-	5.05 ± 0.16(-)	Manes.15G075600.1	THI1
54	thiamine thiazole synthase	-	-	-	6.72 ± 0.06(+)	Manes.03G123800.1	THI1
55	Monothiol glutaredoxin-S16, chloroplastic	-	-	-	4.06 ± 0.15(-)	Manes.08G093600.1	CXIP2
57	thiamine thiazole synthase	-	-	-	3.05 ± 0.03(-)	Manes.15G075600.1	THI1
59	allene oxide cyclase	-	-	-	2.26 ± 0.04(+)	Manes.01G108000.1	AOC3
Function unknown (2)							
32	Universal stress protein family	-	-	2.06 ± 0.03(-)	5.02 ± 0.06(-)	Manes.08G082400.1	AT3G53990
33	Universal stress protein family	-	-	2.97 ± 0.02(+)	-	Manes.08G082400.1	AT3G53990

The spots showing differential expression (2.0-fold of the normalized volume) were counted after gel analysis and manual editing with Delta2D software. Each value represents the mean ± SD of triplicate. Protein spots whose abundance increased (+) or decreased (-) after matching with 0 DAH as a control were shown. a:The spot numbers corresponded to the 2-DE gel in Fig. 20; b:The accession numberin NCBI and cassava gene bank. Each value ismeans±SE.

Table VIII. List of identified proteins changing in abundance in SC5 at different durationsof storage.

Spot Number[a]	Identification	0DAH/3DAH	0DAH/6DAH	0DAH/15DAH	0DAH/30DAH	Accession no[b]	Proteins ID in string database
Carbohydrate and energy metabolism associated proteins (16)							
1	monodehydroascorbate reductase (NADH)	-	3.42 ± 0.12(+)	2.69 ± 0.12(+)	5.73 ± 0.09(+)	Manes.08G023200.1	MDAR1
4	Triose-phosphate isomerase / Triosephosphate mutase	-	∞ (+)	∞ (+)	-	Manes.01G173500.1	TPI
7	Orotate phosphoribosyltransferase / Orotidylic acid phosphorylase	-	-	∞ (-)	∞ (+)	Manes.11G004200.1	AT3G54470
28	Fructose-bisphosphate aldolase 3, chloroplastic	-	-	∞ (+)	11.04 ± 0.23(+)	Manes.14G108900.1	PDE345
42	starch phosphorylase	-	-	-	2.10 ± 0.14 (+)	Manes.02G052600.1	AT3G29320
64	ATP-dependent CLP protease	2.08 ± 0.11(-)	5.08 ± 0.19(-)	7.09 ± 0.08(-)	-	Manes.06G085600.1	CLPC1
68	Alcohol dehydrogenase class-P	4.05 ± 0.19(-)	-	-	-	Manes.18G087400.1	ADH1
69	Alcohol dehydrogenase class-P	2.09 ± 0.13(-)	5.10 ± 0.13(-)	-	-	Manes.18G087400.1	ADH1
71	Malate and lactate dehydrogenase	∞ (-)	∞ (-)	-	-	Manes.14G078500.1	MDH
74	Triosephosphate isomerase, chloroplastic	2.14 ± 0.12(+)	-	-	-	Manes.04G078300.1	TPI
82	Fructose-bisphosphate aldolase 3, chloroplastic	-	2.82 ± 0.13(+)	2.69 ± 0.04(+)	-	Manes.06G063500.1	FBA2
83	Fructose-bisphosphate aldolase 3, chloroplastic	-	18.02± 0.13(+)	-	-	Manes.06G063500.1	FBA2
85	nitrogen metabolic regulation protein NMR	-	2.65 ± 0.07(+)	-	-	Manes.09G092700.1	AT2G37660
89	ATP-dependent CLP protease	-	-	2.09 ± 0.09(-)	-	Manes.06G085600.1	CLPC1
90	Nitrogen metabolic regulation protein	-	-	2.05 ± 0.05(-)	-	Manes.09G092700.1	AT2G37660
98	Lactoylglutathione lyase glyoxalase I	-	-	-	2.06 ± 0.11(-)	Manes.16G112000.1	AT1G67280
Chaperones (16)							
13	25.3 kDa heat shock protein, chloroplastic	-	-	2.07 ± 0.04(+)	-	Manes.01G042200.1	AT5G51440
19	17.6 kDa class II heat shock protein	-	-	-	2.23 ± 0.35(+)	Manes.16G083600.1	HSP17.6II
20	17.6 kDa class I heat shock protein 1	2.20 ± 0.08(+)	3.12 ± 0.10(+)	2.18 ± 0.07(+)	5.76 ± 0.15(+)	Manes.10G020600.1	HSP17.6A

53	20 kDa chaperonin, chloroplastic	-	-	∞ (+)	4.05 ± 0.02(+)	Manes.03G044000.1	CPN20
62	Small heat-shock protein HSP20 family	-	-	-	2.01 ± 0.13(+)	Manes.10G020100.1	AT2G29500
77	25.3 kDa heat shock protein, chloroplastic	2.26 ± 0.05(-)	-	-	-	Manes.01G042200.1	AT5G51440
78	17.6 kDa class I heat shock protein 1	∞ (-)	∞ (-)	2.19 ± 0.12(-)	-	Manes.10G020600.1	HSP17.6A
87	17.6 kDa class I heat shock protein 1	-	2.31 ± 0.03(+)	6.64± 0.04(+)	6.82 ± 0.05(+)	Manes.10G020600.1	HSP17.6A
88	Small heat-shock protein HSP20 family	-	2.04 ± 0.09(+)	-	-	Manes.10G020100.1	AT2G29500
94	heat shock 70 kDa protein 5	-	-	-	∞ (+)	Manes.11G067600.1	Hsp70b
97	chaperonin 60 subunit beta 1, chloroplastic	-	-	-	∞ (+)	Manes.03G086700.1	CPN60B
100	26.5 kDa heat shock protein, mitochondrial	-	-	-	∞ (+)	Manes.04G113600.1	AT1G07400
103	17.6 kDa class II heat shock protein	-	-	-	∞ (+)	Manes.16G083600.1	HSP17.6II
104	17.6 kDa class I heat shock protein 1	-	-	-	2.05 ± 0.10(+)	Manes.02G124600.1	HSP17.6A
105	17.6 kDa class I heat shock protein 1	-	-	-	2.56 ± 0.10(+)	Manes.10G020600.1	HSP17.6A
106	Small heat-shock protein HSP20 family	-	-	-	5.67 ± 0.24(+)	Manes.15G154400.1	AT1G53540
Detoxifying and antioxidant (5)							
17	Dehydrin	2.86 ±0.14(+)	7.75 ± 0.16(+)	-	-	Manes.05G140400.1	COR47
21	Thioredoxin-like protein CXXS1	-	2.06 ± 0.08(+)	-	-	Manes.16G017200.1	CXXS1
58	Riboflavin synthase	-	-	-	2.36 ± 0.22(+)	Manes.13G146800.1	AT2G20690
84	L-ascorbate peroxidase 2, cytosolic	-	4.22 ± 0.07(+)	2.63 ± 0.05(+)	-	Manes.04G026800.1	APX2
91	L-ascorbate peroxidase 2, cytosolic	-	-	3.82 ± 0.18(+)	-	Manes.04G026800.1	APX2
Amino acid metabolism (1)							
81	proline iminopeptidase	-	3.15 ± 0.11(-)	-	-	Manes.07G049300.1	PIP
Structure (1)							
70	DREPP plasma membrane polypeptide	∞ (-)	-	-	-	Manes.S071400.1	PCAP1
Protein biosynthesis (11)							
12	translation initiation factor 5A	-	3.07 ± 0.04(-)	2.01± 0.05(-)	2.07 ± 0.04(-)	Manes.05G107000.1	ELF5A-1
16	Argininosuccinate synthase / Citrulline--aspartate ligase	-	10.05 ± 0.25(+)	13.05 ± 0.25(+)	-	Manes.03G085300.1	AT4G24830

39	Disulfide oxidoreductase	-	-	5.05 ± 0.08(-)	-	Manes.03G209100.1	mtLPD1
40	protein disulfide-isomerase A6	-	3.99 ± 0.22(+)	5.24 ± 0.18(+)	2.69 ± 0.10(+)	Manes.05G002000.1	UNE5
61	translation initiation factor 5A	-	∞ (+)	∞ (+)	-	Manes.05G107000.1	ELF5A-1
65	26S proteasome regulatory subunit T5	∞ (-)	-	∞ (-)	-	Manes.08G117200.1	RPT5A
73	20S proteasome subunit alpha 6	∞ (-)	∞ (-)	∞ (-)	-	Manes.05G157400.1	PAF1
75	20S proteasome subunit beta 1	∞ (-)	-	∞ (-)	-	Manes.03G001100.1	PBA1
79	protein disulfide-isomerase A6	-	2.19 ± 0.11(-)	4.01 ± 0.17(-)	3.01 ± 0.17(-)	Manes.05G002000.1	UNE5
80	elongation factor 1-gamma	-	3.15 ± 0.11(-)	-	-	Manes.10G116000.1	AT1G57720
95	peptidyl-prolyl cis-trans isomerase FKBP62	-	-	-	∞ (+)	Manes.12G050700.1	ROF1
Signal transduction mechanism (3)							
23	C2 domain	-	4.08 ± 0.08(-)	3.08 ± 0.04(-)	-	Manes.S104800.1	AT5G55530
26	Vesicle coat complex COPI, alpha subunit	-	3.99 ± 0.12(+)	4.21 ± 0.14(+)	2.35 ± 0.07(+)	Manes.12G101600.1	AT3G50590
92	2',3'-cyclic-nucleotide 3'-phosphodiesterase	-	-	16.01 ± 0.09(+)	40.5 ± 0.05(+)	Manes.17G061800.1	AT4G18930
Defense (17)							
18	thiamine thiazole synthase	-	3.01 ± 0.11(-)	6.06 ± 0.15(-)	3.15 ± 0.08(-)	Manes.15G075600.1	THI1
24	RING-H2 finger protein ATL61	-	5.16 ± 0.16(+)	8.50 ± 0.22(+)	-	Manes.07G090300.1	AT2G34990
27	thiamine thiazole synthase	-	4.01 ± 0.08(-)	-	-	Manes.15G075600.1	THI1
29	Ras-related protein Rab-11A	6.05 ± 0.23(-)	∞ (-)	∞ (-)	-	Manes.12G092100.1	RABA1f
34	RING-H2 finger protein ATL61	-	-	-	9.48 ± 0.01(-)	Manes.07G090300.1	AT2G34990
38	RING-H2 finger protein ATL61	-	-	2. 60 ± 0.16(+)	-	Manes.07G090300.1	AT2G34990
44	RING-H2 finger protein ATL61	9.74 ± 0.08(+)	18.02± 0.13(+)	-	-	Manes.07G090300.1	AT2G34990
52	thiamine thiazole synthase	∞ (-)	-	-	-	Manes.15G075600.1	THI1
54	thiamine thiazole synthase	-	∞ (+)	∞ (+)	∞ (+)	Manes.03G123800.1	THI1
59	allene oxide cyclase	-	-	-	∞ (+)	Manes.01G108000.1	AOC3
66	RING-H2 finger protein ATL61	2.89 ± 0.13(+)	2.12 ± 0.12(+)	-	-	Manes.07G090300.1	AT2G34990
67	stress-induced-phosphoprotein 1	4.07 ± 0.08(-)	3.50 ± 0.06(-)	6.09 ± 0.08(-)	-	Manes.01G075100.1	Hop3
72	thiamine thiazole synthase	5.05 ± 0.11(-)	5.07 ± 0.11(-)	-	-	Manes.15G075600.1	THI1
86	thiamine thiazole	-	2.65 ± 0.07(+)	-	-	Manes.15G075600.1	THI1
93	allene oxide cyclase	-	-	∞ (+)	-	Manes.01G108000.1	AOC3
96	RING-H2 finger protein ATL61	-	-	-	8.28 ± 0.06(+)	Manes.07G090300.1	AT2G34990

99	RING-H2 finger protein ATL61	-	-	-	2.48 ± 0.07(+)	Manes.07G090300.1	AT2G34990
Function unknown (5)							
32	Universal stress protein family	-	2.03 ± 0.03(-)	3.02 ± 0.03(-)	-	Manes.08G082400.1	AT3G53990
33	Universal stress protein family	-	5.07 ± 0.03(+)	-	-	Manes.08G082400.1	AT3G53990
76	Universal stress protein family	2.05 ± 0.03(-)	-	-	-	Manes.08G082400.1	AT3G53990
101	Universal stress protein family	-	-	-	6.01 ± 0.03(-)	Manes.03G204200.1	AT3G17020
102	Universal stress protein family	-	-	-	2.68 ± 0.26(+)	Manes.03G204200.1	AT3G17020

The spots showing differential expression (2.0-fold of the normalized volume) were counted after gel analysis and manual editing with Delta2D software. Each value represents the mean ± SD of triplicate. Protein spots whose abundance increased (+) or decreased (-) after matching with 0 DAH as a control were shown. a:The spot numbers corresponded to the 2-DE gel in Fig. 21; b:The accession numberin NCBI and cassava gene bank. Each value ismeans±SE.

III.1.7.2. Comparative analysis of proteomes from QZ1 and SC5 flesh during storage

Proteins have been previously used to investigate the protein expression pattern in cassava storage during PPD (Owiti et *al.*, 2011; Vandershuren et *al.*, 2014). However, few studies have been conducted to investigate a comparative analysis about protein profiles between susceptible and tolerant cassava genotypes to PPD. The following are the differential expressed protein functions recorded in SC5 and QZ1 storage roots during storage.

III.1.7.2.1. Carbohydrate and energy metabolism

Storage roots showed a high regulation of carbohydrate and energy metabolism associated proteins. The most regulation of these proteins was observed in SC5 between 3DAH and 15DAH while in QZ1, it was at 30 DAH. There were 6 common proteins in both genotypes including monodehydroascorbate reductase (NADH) (spot 1), triose-phosphate isomerase (spot 4), orotate phosphoribosyltransferase (spot 7), fructose-bisphosphate aldolase 3 up-regulated (spot 28 in QZ1, and spots 28, 82, 83 in SC5), starch phosphorylase (spot 42) and alcohol dehydrogenase class-P (spot 41 up-regulated in QZ1, and spots 68, 69 down-regulated in SC5). The abundance of fructose-bisphosphate aldolase 3 (spot 28 in QZ1, and spots 28, 82, 83 in SC5) and alcohol dehydrogenase class-P (spot 41) in QZ1 increases more than that in SC5 (spot 28, spots 68 and 69) at 0DAH/30DAH, while the expression of starch phosphorylase (spot 42) increased in both genotypes.

III.1.7.2.2. Chaperones

Heat-shock proteins (Hsps) function as molecular chaperones that assist protein folding or assembly and prevent irreversible protein aggregation to maintain cellular homeostasis under both optimal and adverse developmental conditions. Based on their approximate molecular weights, 5 major families of Hsps are recognized: Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsp (sHsp) families. The study showed that PPD process mostly involved the expression of sHsp family. These proteins were generally up-regulated in both genotypes during storage. There were 5 common proteins in both genotypes including 17.6 kDa class I Hsp (spots 11, 20 in QZ1, and spots 20, 78, 87, 104, 105 in SC5), 17.6 kDa class Hsp (spot 19 in QZ1, and spots 19, 103 in SC5), 25.3 kDa Hsp (spots 13, 30 in QZ1, and spots 13, 77 in SC5), 20 kDa chaperonin (spot 53), and sHsp20 family (spots 62, 63 in QZ1, and spots 62, 88, 106 in SC5. In SC5, the up-regulation of 17.6 kDa small heat shock protein class II (spot 19) and class I (spots 20, 87, 104, 105 and 106) was especially observed

following the duration of storage. In parallel, the decrease of abundance of Hsp70 (spot 35) and Hsp20 (spot 63) was especially observed in QZ1.

III.1.7.2.3. Detoxifying and antioxidant associated proteins

There were 4 common proteins in both genotypes including L-ascorbate peroxidase 2 (spot 3 in QZ1, and spots 84, 91 in SC5), riboflavin synthase (spots 5, 58 in QZ1, and spot 58 in SC5), thioredoxin-like protein CXXS1 (spots 14, 21 in QZ1, and spot 21 in SC5) and dehydrin (spots 17, 45 in QZ1, and spot 17 in SC5). All the identified proteins in this group were up-regulated in SC5. These included dehydrin (spot 17), thioredoxin-like protein (spot 21) and Two isoforms of the L-ascorbate peroxidase 2 (spots 84 and 91). A part from dehydrin for which the up-regulation started at 3DAH, the up-regulation of other proteins of this group was observed between 6DAH and 15DAH. In QZ1, two proteins, riboflavin synthase (spot 5) and thioredoxin-like protein (spot 14) were observed. In addition, the down-regulation L-ascorbate peroxidase (spot 3) and glutathione-S-transferase (spot 53) were notified.

III.1.7.2.4. Proteins related to defense

Defense proteins were the second most represented proteins identified in both genotypes. A total of 10 proteins were identified as common in both genotypes. The increase of abundance of translationally controlled tumor protein (spots 8 and 9) and Ras-related proteins (spots 6, 29) were especially observed in QZ1 following the duration of storage. In parallel, the increase of abundance of RING-H2 finger ATL61 (spots 44, 66) was observed in SC5 at 3DAH and 6DAH. The down-regulation of thiamine thiazole (spots 18, 27) was observed in both genotypes.

III.1.7.2.5. Proteins related to structure

The DREPP plasma membrane polypeptide (spot 51 in QZ1, and spot 70 in SC5) was common protein in both genotypes, in which it was up-regulated in QZ1 at 0DAH/30DAH, and down-regulated in SC5 at 0DAH/3DAH.

III.1.7.2.6. Signal transduction proteins

The decrease of calmodulin (CaM) abundance (spot 31), the most important calcium sensor protein, was observed only in QZ1 at 0DAH/15DAH and 0DAH/30DAH, while the up-regulation of vesicle coat complex COPI (spot 26) and the down-regulation of C2-domain (spot 23) were observed in both genotypes.

III.1.7.2.7. Protein biosynthesis

There were four common proteins in both genotypes, including Translation initiation factor 5A (spots 12, 61), and argininosuccinate synthase/citrulline aspartate ligase (spot 16), protein disulfide-isomerase A6 (spots 25, 40 in QZ1, and spots 40, 79), and disulfide oxidoreductase (spot 39), in which argininosuccinate synthase/citrulline aspartate ligase is up-regulated in both genotypes at 0DAH/6DAH and 0DAH/15DAH. The up-regulation of disulfide oxidoreductase in QZ1 at 0DAH/3DAH while the down-regulation in SC5 at 0DAH/15DAH.

III.1.7.2.8. DNA and RNA metabolism proteins

The increase of nucleic acid-binding (spots 43) abundance was only observed in QZ1 at 0DAH/30DAH.

Differential expressed proteins not associated to an existing biological function were also recorded. A total of two and five proteins recorded in this group were differentially expressed in QZ1 and SC5 respectively. These proteins will require further study in the future. Since they were differentially expressed in the two genotypes, most of them can give much information for the understanding of PPD.

III.1.7.3. Protein-protein interaction and plant metabolism networks

Proteins control all biological systems in a cell, and while many proteins perform their functions independently, the vast majority of proteins interact with others for proper biological activity. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database provides a critical assessment and integration of protein–protein interactions, including direct (physical) as well as indirect (functional) associations. A network was used to show the interactions of the identified proteins in each genotype and revealed the potential information at the protein level (Fig. 22A and B). The model plant *Arabidopsis thaliana* database was used as model (Szklarczyk et *al.,* 2015). The protein-protein interaction **(**PPI) performed revealed a complexity network in SC5 compared to QZ1.

The PPI networks in QZ1 was constructed with 33 nodes and 87 edges (including 24 expected edges) by choosing the top 61 protein pairs (PPI enrichment p-value: < 1.0e-16) (Fig. 21A), and in SC5 35 nodes and 126 edges (including 26 expected edges) from the top 75 protein pairs (PPI enrichment p-value: <1.0e-16) (Fig. 22B). In QZ1, AT3G54470 (spot 7,

orotate phosphoribosyltransferase/orotidylic acid phosphorylase), Hsp70-1 (spot 35, heat shock 70 kDa protein 1/8), TPI (spot 4, triosephosphate isomerase/triosephosphate mutase) and CPN20 (spot 53, 20 kDa chaperonin) were viewed as hub proteins with a degrees of 18, 16, 11 and 10, respectively. In SC5, AT3G54470 (spot 7, orotate phosphoribosyltransferase/ orotidylic acid phosphorylase), Hsp70b (spot 94, heat shock 70 kDa protein 5), CPN60B (spot 97, Chaperonin 60 subunit beta 1), Hop3 (spot 67, stress-induced-phosphoprotein 1), AT2G29500 (spot 62, small heat-shock protein Hsp20 family) were hub proteins each with a degree of 22, 17, 16, 12, and 11, respectively. In QZ1, the 4 hub proteins have the common functions which could be in response to inorganic substance, stress, metal ion, cadmium ion, and stimulus. In addition, Hsp70-1 and CPN20 could respond to temperature stimulus; AT3G54470 has the response to oxidative stress; and TPI has the functions with osmotic stress and oxidative stress (Appendix 3A). In SC5, there are the common functions including response to stress and stimulus in the 5 hub proteins. Moreover, Hop3 and AT2G29500 have the functions with response to temperature stimulus, inorganic substance and oxidative stress; AT3G54470 has the functions of inorganic substance, cadmium ion, and small molecule metabolic process; Hsp70b has the response to temperature stimulus, CPN60B has the functions of temperature stimulus and cold, and CPN20 has functions with the response to temperature stimulus, inorganic substance, cadmium ion, and cold (Appendix 3B). Proteins related with carbohydrate and energy metabolism, chaperones, defence system, including detoxifying and antioxidant activities were identified as the most expressed proteins and they constituted the hub nodes in the PPI networks.

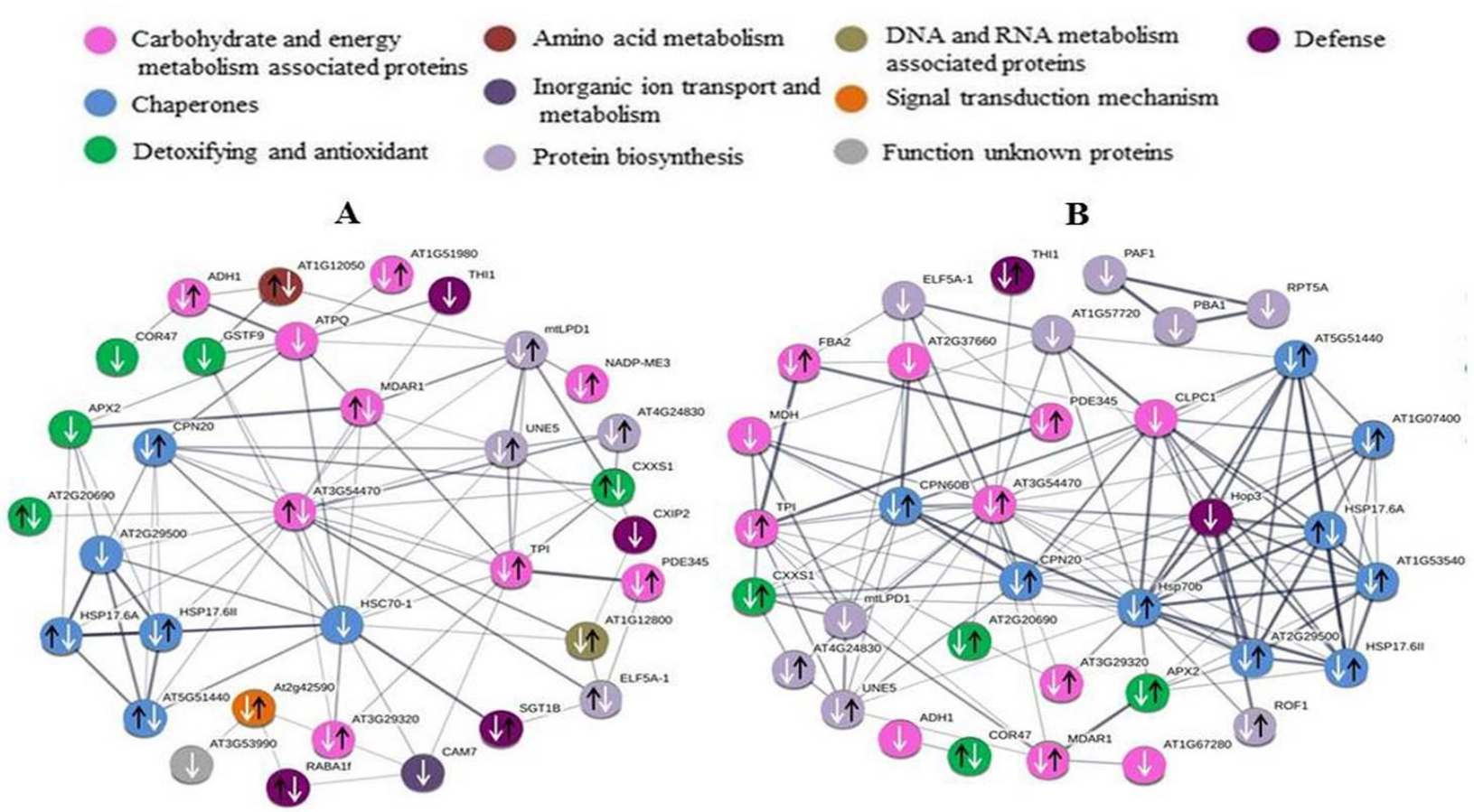

Fig. 22. Protein-protein interaction of differential expressed proteins in QZ1 (A) and SC5 (B) by string online software according to the database of *A. thaliana* PPI. White arrows indicate down-regulated proteins, and black arrows indicated up-regulated proteins. The different colour circle indicates the proteins with different biological functions. The network nodes are proteins, whereas the edges represent the predicted or known functional association.

III.1.8. Changes in hydrogen peroxide (H_2O_2) and ethylene during PPD in the tubers from the field

III.1.8.1. Changes in hydrogen peroxide (H_2O_2) content during storage

The result of the quantification of hydrogen peroxide (H_2O_2) is summarized in Fig. 23. A quantitative difference in H_2O_2 content was observed during the storage with SC5 presenting a high accumulation of H_2O_2 compared to QZ1. Two peaks of the accumulation were observed in SC5 at 6 and 30DAH; the first one corresponding to the onset of PPD as observed in (Fig. 14). In QZ1, the highest accumulation of H_2O_2 was observed at 15DAH.

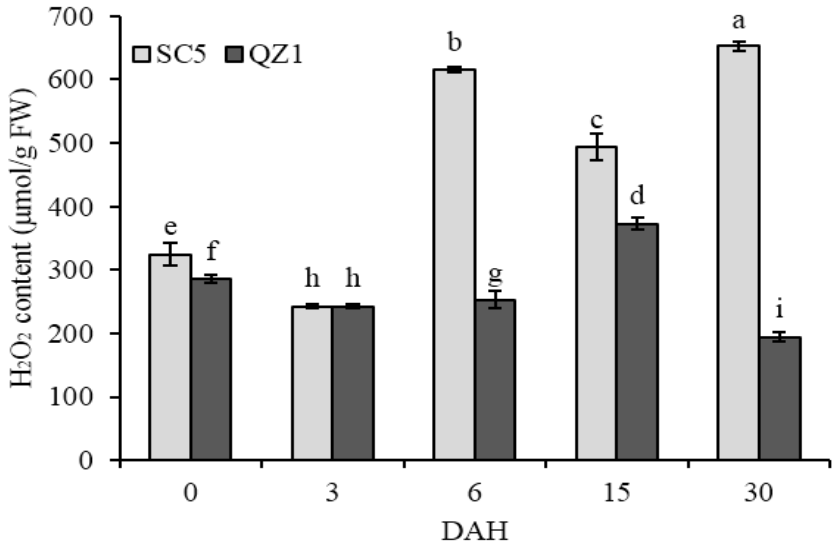

Fig. 23. Changes in total H_2O_2 content during PPD.

III.1.8.2. Changes in ethylene content during storage

The concentration of ethylene was higher in QZ1 compared to SC5 at harvest. Ethylene concentration decreases in QZ1 at 3DAH and 6DAH (Fig. 24). Thereafter, the amount gradually increases until 30DAH where the maximum accumulation was observed. In SC5, the amount of ethylene gradually increases from 0DAH to 15DAH followed by a high accumulation at 30DAH (Fig. 24).

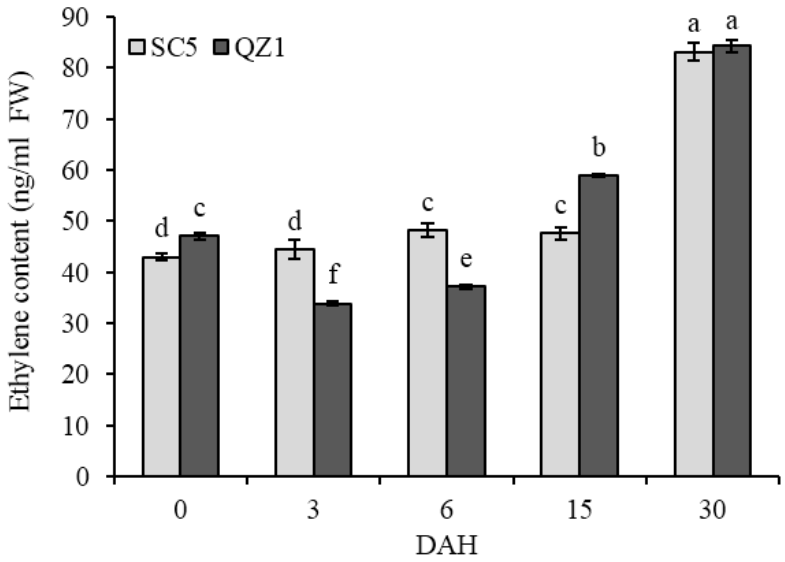

Fig. 24. Changes in total ethylene content during PPD.

III.1.8.3. Changes in superoxide dismutase (SOD) and peroxidase (POD) activities during PPD

The activity of peroxidase (POD) gradually decreased in QZ1 with no significant difference between the time 0-3DAH and 6-15DAH. A slight increase was observed in SC5 at 3DAH and 15DAH (Fig. 25A). The superoxide dismutase (SOD) assay kit used in the assay was not able to readily distinguish between the activities of any of the three forms of SODs but rather gave the total activity of all the isoforms (Fig. 25B). In SC5, the highest activity of SOD was observed at 6 DAH. Thereafter, the activity decreases until 30DAH. In QZ1, no significant difference was observed from 0 to 3DAH. At 6DAH, the slight increase of the activity was followed by a decrease at 15DAH and the activity remained stable until 30 DAH.

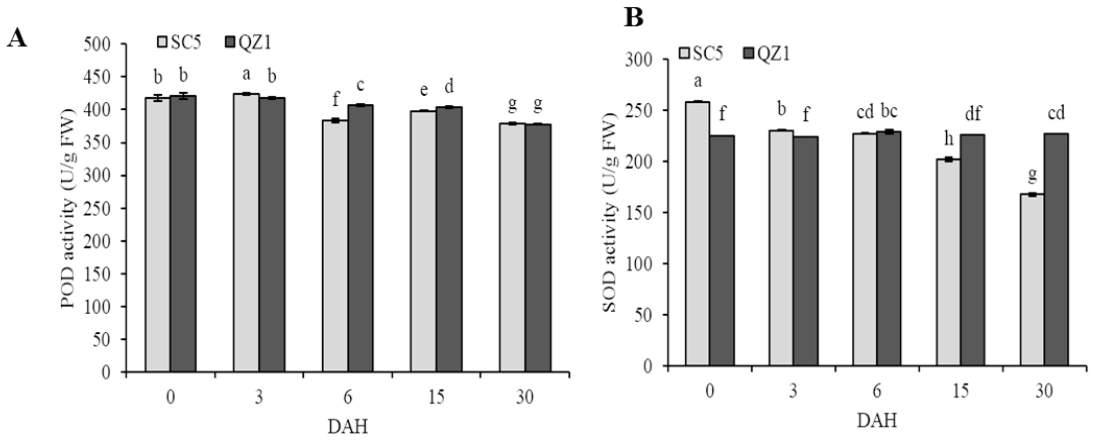

Fig. 25. Changes in total POD (A) and SOD (B) activities during PPD.

III.1.8.4. Changes in catalase (CAT) and ascorbate peroxidase (APX) activities during PPD

Except at 0 and 3DAH where the activity of catalase (CAT) was stable in SC5, the activity of CAT gradually increases over time (Fig. 26A). In QZ1, the activity increases from 0 to 3DAH followed by a slight decrease at 6 DAH and the activity continues to increase till 30DAH. It is important to highlight the high increase of CAT activity from 0 to 30DAH in both genotypes. In SC5 the activity increased by 8-fold while in QZ1 it increased by 10-fold. No significant difference was observed in the activity of ascorbate peroxidase (APX) in QZ1 following the duration of storage (Fig. 26B). In SC5, no significant difference was observed from 0 to 15DAH. The lowest activity was observed at 30DAH.

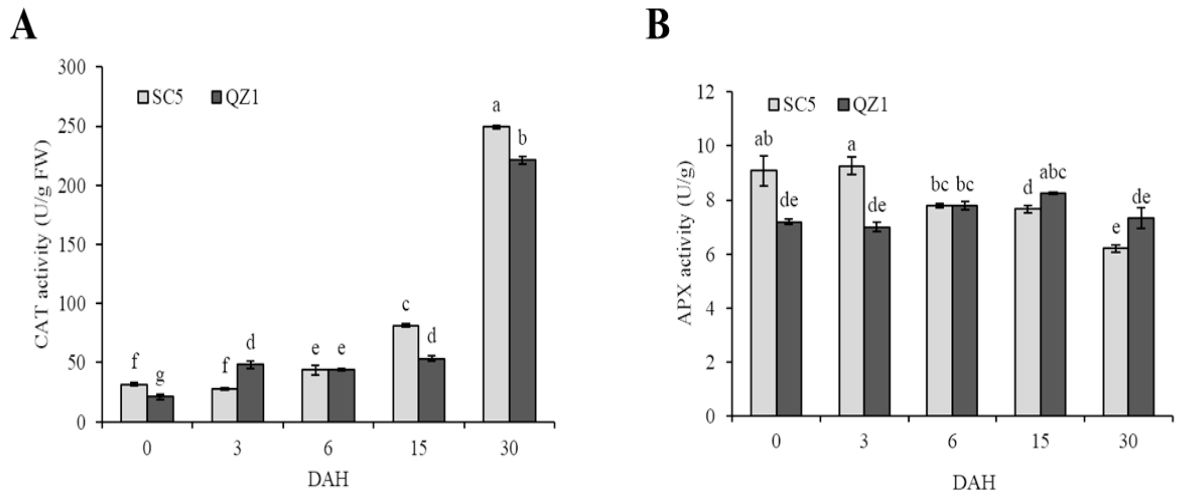

Fig. 26. Changes in catalase (A) and peroxidase (B) activities during PPD.

III.1.9. Effect of fertilizers on H_2O_2 and antioxidants during PPD in the tubers from the pots

The effects of fertilization on antioxidant activities and H_2O_2 for SC5 and QZ1 are presented in Table X and Table XI. High accumulation of H_2O_2 was observed at harvest in both genotypes under the treatment with Ca and Mg compared to the control. However, a remarkable increase in H_2O_2 was observed in the control and the treatment with Mg from 0 to 3DAH. In SC5, the activity of SOD was higher in the treated samples than in the control from 0 to 3DAH. Then, the SOD activity decreased in these treatments at 6DAH but increased in the control. In QZ1, SOD activity was high in the control and the treatment with Mg from 0 to 3DAH, becoming slightly the same at 6DAH in all the treatments and thereafter increased in the treatment with Ca and Mg at 15 and 30 DAH respectively. A high activity of POD was observed at harvest in the treatment with Mg in SC5 as in the treatment with Ca in QZ1. A consistent decrease was observed in the treatment with Mg at 6DAH in SC5 while in the control of QZ1 the decrease occurred at 30DAH.

At harvest, the high activity of CAT was observed in SC5 and QZ1 in the treatments with Mg and Ca respectively. In QZ1, the activity of CAT gradually increased from 0 to 30 DAH, being stimulated mainly by the treatment with Mg. In SC5, a high activity of APX was observed in the control and in the treatment with Ca. Thereafter, the APX activity decreased in both treatments while it increased in the treatment with Mg at 3DAH. The opposite situation was observed at 6DAH where the activity increased in tubers from Mg-treated plants and decreased in the control and tubers from Ca-treated plants. At 30DAH, the activity

decreased in all the treatments. In QZ1 at 0DAH, the APX activity was higher in the treatments compared to the control. At 15DAH, the activity increased especially in those treated with Mg.

Table IX. Interactive effect of calcium and magnesium fertilization on peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities of tubers during storage.

		POD (U/g)					SOD (g/mol)				
		Days after harvest									
		0	**3**	**6**	**15**	**30**	**0**	**3**	**6**	**15**	**30**
SC5	**Control**	408.1d	428.4a	405.2c	404.8a	261.9e	234.7a	221.5c	225.9a	216.9b	241.6c
	Calcium	388.8e	423.7a	403.7c	386.0c	334.4c	228.5b	228.6b	210.5b	213.1b	248.9b
	Magnesium	434.4b	407.8c	206.5d	394.1b	294.4d	216.9c	228.5b	178.3c	214.7b	222.4d
QZ1	**Control**	423.7c	396.7d	418.4b	408.5a	235.6f	225.2b	234.6a	213.4b	215.9b	214.3e
	Calcium	566.7a	384.4e	428.9a	376.3d	357.8b	217.3c	189.5d	213.1b	233.0a	207.0f
	Magnesium	431.5b	417.8b	427.3a	388.5bc	375.0a	217.8c	234.3a	213.2b	204.3c	254.5a
L.S.D at 0.05				5.82					4.3		
		CAT (U/g)					**APX** (U/g)				
		0	**3**	**6**	**15**	**30**	**0**	**3**	**6**	**15**	**30**
SC5	**Control**	26.8 c	23.9 c	34.2 a	28.4 e	63.2 e	8.6 c	6.7 d	8.3 d	5.0 f	5.35 d
	Calcium	24.5 a	21.6 d	43.9 d	28.5 d	81.9 a	9.6 b	5.4 f	7.8 e	8.2 c	6.7 b
	Magnesium	25.9 d	29.1 d	15.7 b	57.2 a	106.9 b	6.9 e	8.5 b	4.6 f	7.2 e	6.1 c
QZ1	**Control**	18.2 b	22.4 c	79.2 d	40.4 f	68.2 f	6.1 f	7.7 c	10.5 c	7.4 d	4.6 f
	Calcium	37.1 b	16.8 c	35.2 c	46.7 f	179.9 d	8.2 d	6.2 e	10.7 b	8.7 b	5.1 e
	Magnesium	12.1 b	57.2 b	62.9 c	101.4 b	141.3 c	9.8 a	9.9 a	12.3 a	11.1 a	7.2 a
L.S.D at 0.05				3.3					0.09		

Each value is the mean of three replicates. Values in same column followed by different letters are significantly different ($p < 0.05$) according Tukey´s test.

Table X. Interactive effect of calcium and magnesium fertilization on hydrogen peroxide content of tubers during storage.

Hydrogen peroxide (µmol/g of Fresh weight)						
		Day after harvest (DAH)				
Genotypes	Treatments	**0**	**3**	**6**	**15**	**30**
SC5	Control	245.8 c	326.6 a	383.0 a	223.3 d	246.1 b
	Calcium	255.4 b	313.8 b	306.0 b	288.6 b	255.6 f
	Magnesium	169.5 e	276.4 c	190.4 d	345.1 a	216.3 c
QZ1	Control	263.6 b	334.5 a	304.9 b	274.6 c	147.7 a
	Calcium	275.0 a	225.2 d	272.6 c	268.5 c	158.8 e
	Magnesium	215.2 d	306.0 b	305.1 b	353.5 a	185.1 d
LSD at 0.05		9.2				

Each value is the mean of three replicates. Values in same column followed by different letters are significantly different ($p < 0.05$) according Tukey´s test.

III.1.10. Western Blot

An additional analysis of some differential expressed proteins identified during PPD investigation (APX, FeSOD, Cu/ZnSOD and Hsp70) were detected by western blot and the result was analysed by Image J sofware. The results present a decrease of the expression of APX (Fig. 27A) at 15 and 30DAH in QZ1. In SC5 the expression was very low at 30DAH. The second band of Cu/ZnSOD (Fig. 27C) was markedly expressed in SC5 at 15 and 30DAH. The expression of Hsp70 (Fig. 27D) was considerably lower at 30 DAH in SC5.

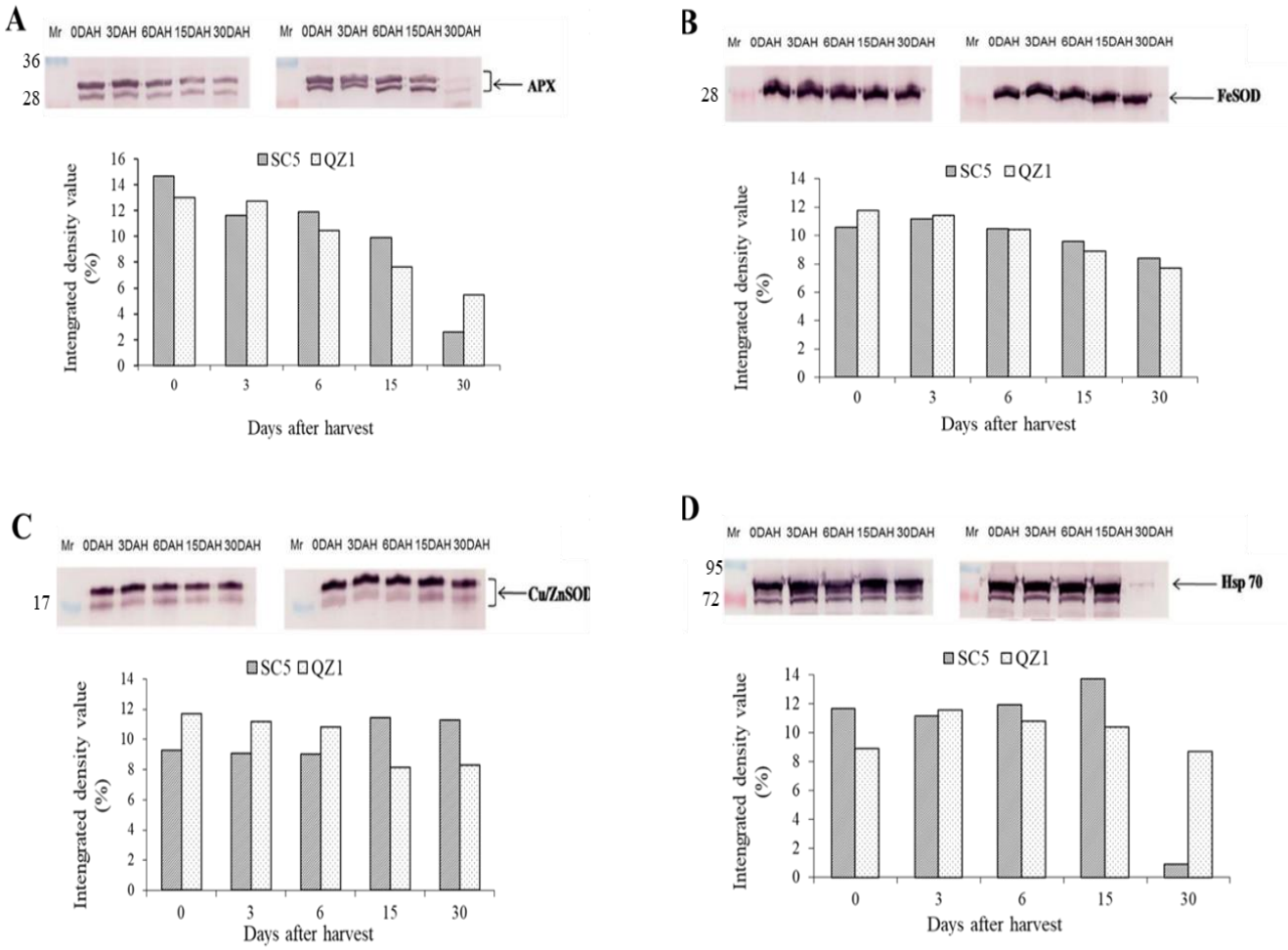

Fig. 27. Western blotting of APX (A), FeSOD (B), Cu/ZnSOD (C) and Hsp 70 (D).

The expression of APX, FeSOD, Cu/ZnSOD, Hsp 70, actin and SPS were detected by western blotting using anti-APX AS08368, anti- Cu/ZnSOD AS06170 and anti-Hsp70 AS08348 from Agrisera® respectively.

III.1.11. PCR results

III.1.11.1. Analysis of gene expression in the tubers from the field

The highest expression level of calmodulin gene was observed in SC5 the day of harvest (Fig. 28A). At 3DAH, the expression level of this gene decreased in both genotypes with SC5 still presenting the high value. A significant difference was observed at 6 and 15 DAH where the expression level was high in QZ1 compared to SC5. Aspartic proteinase expression increased in QZ1 from 0 DAH to 6DAH and thereafter slowly decreased until 30 DAH. In SC5, the expression decreased from 0DAH to 6DAH and a slight increase was observed at 15DAH and 30DAH. The highest expression level was observed in QZ1 at 6 DAH while the lowest expression level was observed in SC5 at the same time (Fig. 28B).The expression level of Ras-related Nuclear protein (RanGTPase) was high in QZ1 compared to

SC5 at all the time points of storage. In SC5, no significant difference was observed from 0 to 3DAH. However, the expression decreased at 6DAH followed by a consistent increase at 15 DAH and 30DAH (Fig. 28C). High in QZ1 compared to SC5 at harvest, the expression of NADH ubiquinone-oxidoreductase, decreased in both genotypes at 3DAH followed by an increase until 15DAH (Fig. 28D).

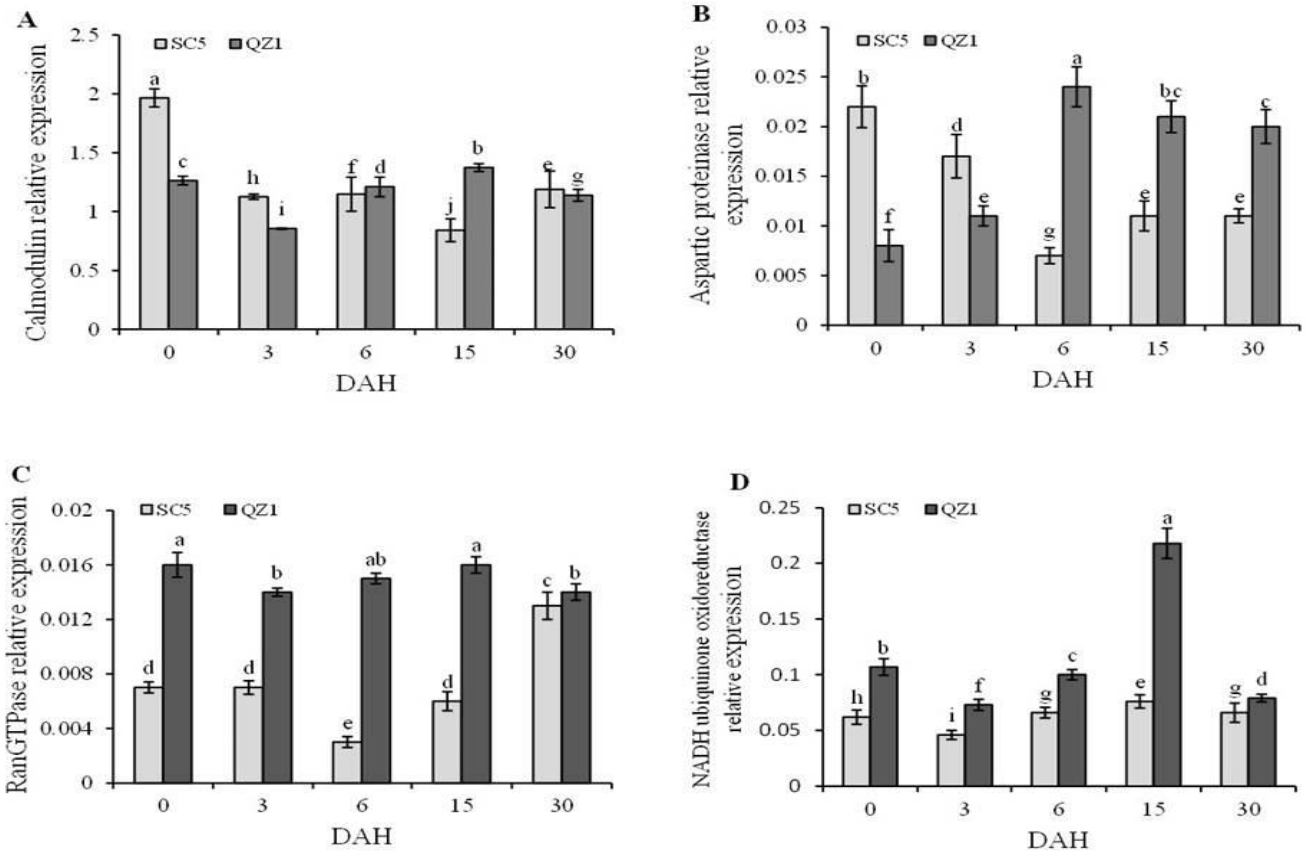

Fig. 28. Relative expression levels of some genes of tubers from the field during storage from 0 DAH to 30 DAH.

A- Calmodulin; B- RanGTPase; C- NADH ubiquinone oxidoreductase. Data are presented as means ± SD of three independent RNA samples. Values labeled by different letters are significantly different accordingto Duncan's multiple comparison tests at $p < 0.05$.

III.1.11.2. Effect of fertilizer on gene expression in the tubers from the pots

The expression level of calmodulin (CaM) was high in the Ca-treated plants at 0DAH and slightly decreased in SC5 at 3DAH and increased up till 15DAH. In the control treatment, the expression level was low until 6DAH. A high expression level was observed in the treatment with Mg at 3 and 15DAH (Fig. 29A). In QZ1, the expression level of CaM was high in the control and Ca-treated plants at harvest. The expression level later decreased in the control but increased for the treatment with Ca. Apart from the day of harvest, the expression

level of CaM remained high in Mg-treated plants during the period of storage (Fig. 29A).The highest expression level of aspartic proteinase was recorded by the treatment with Ca at 0 DAH (Fig. 29B and 30B). Then, the expression level decreased in all the treatments at 3DAH. Thereafter the expression increased highly in the treatment with Ca at 6 and 15DAH. At 30DAH the treatment with Ca presented the lowest expression. The expression level of NADH ubiquinone oxidoreductase decreased in all the treatments at 3DAH (Fig. 29C and 30C). The highest expression level was recorded by the treatment with Mg at 6 and 15 DAH. In Ca treated plants, the expression gradually increased in storage roots from 3 to 6 DAH and thereafter decreased until 30DAH. In the control, the high expression level at the day of harvest decreased at 3DAH and remained stable until 30DAH. In RanGTPase, the highest expression level was recorded by the treatment with Mg at the day of harvest (Fig. 29D and 30D), a slight decrease was observed in this treatment and the expression increased again at 6 and 30DAH. In the treatment with Ca, the expression level of RanGTPase gradually inceased from 0 to 30DAH.

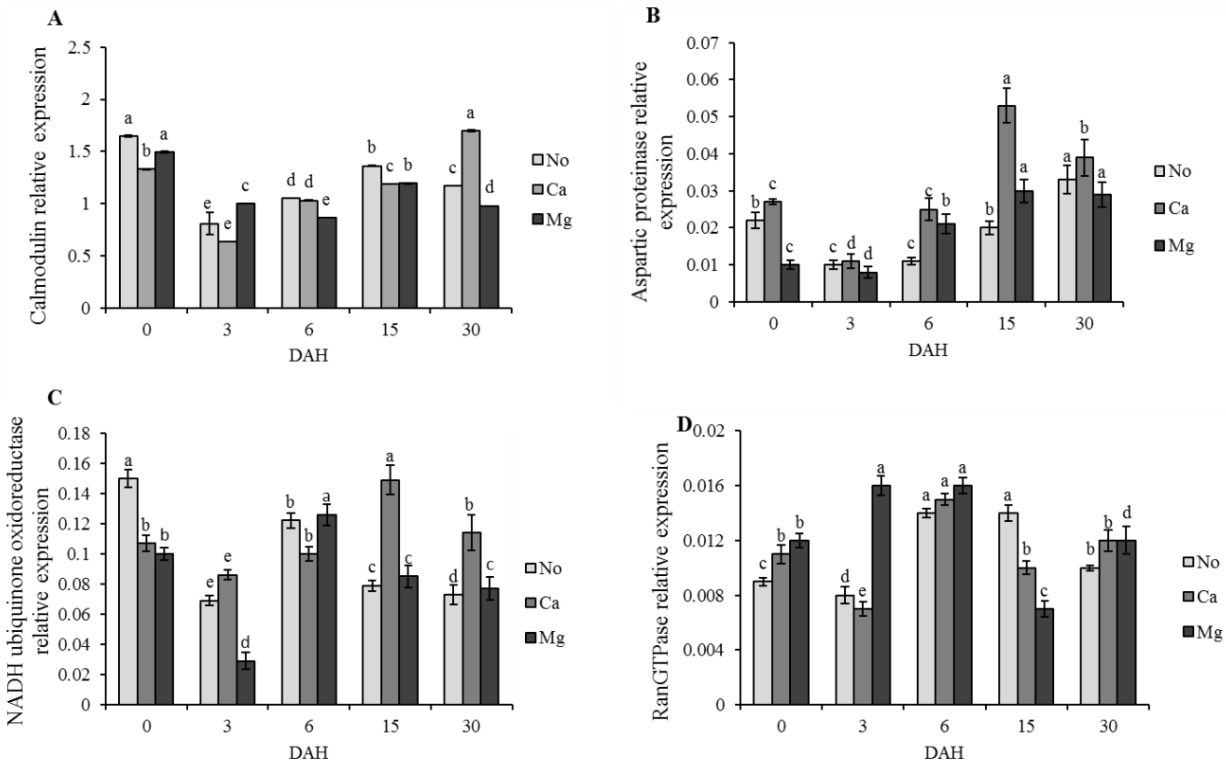

Fig. 29. Effect of fertilization with Ca and Mg on relative expression levels of some genes during storage of SC5 cassava storage roots from 0 DAH to 30DAH.

A: Calmodulin; B:Aspartic proteinase; C:NADH ubiquinone oxidoreductase; D: RanGTPase. Data are presented as means ± SD of three independent RNA samples. Values labeled by different letters are significantly different according toDuncan's multiple comparison tests at $p < 0.05$ at each time point.

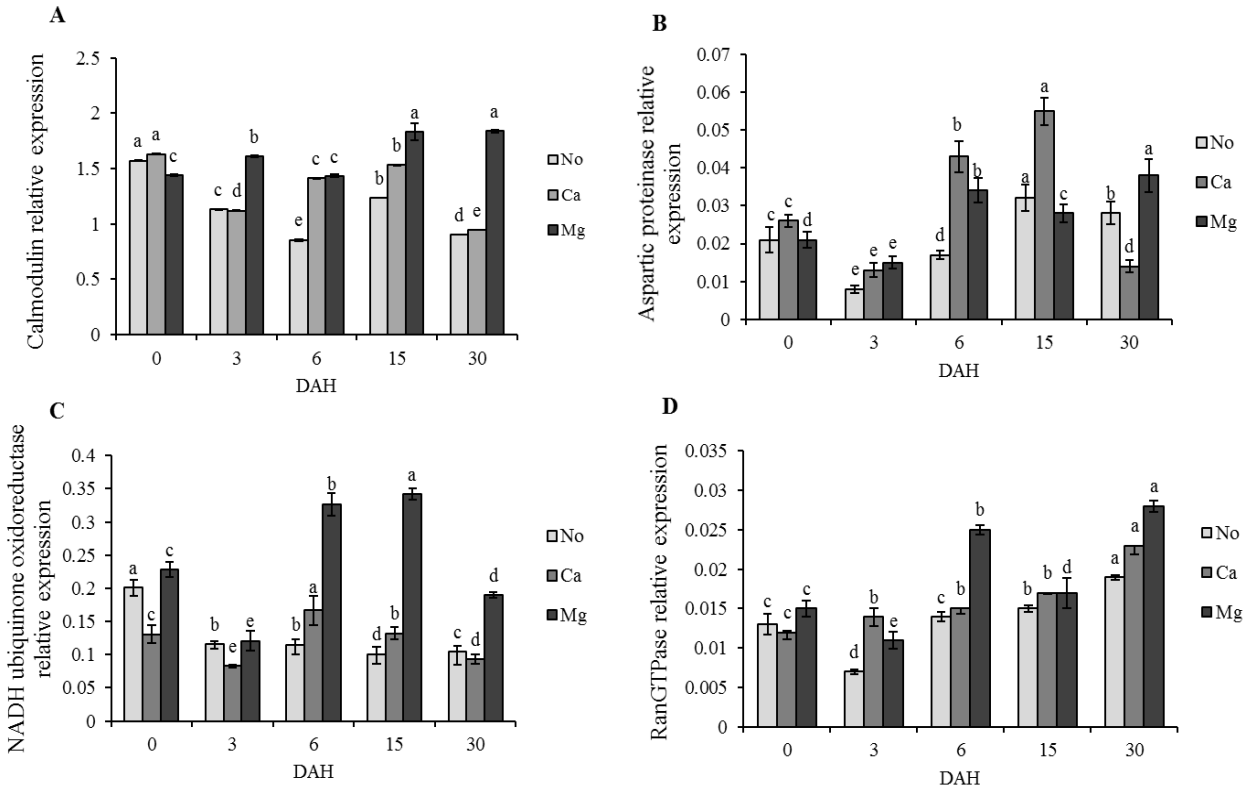

Fig. 30. Effect of fertilization with Ca and Mg on relative expression levels of some genes during storage of QZ1 cassava storage roots from 0 DAH to 30DAH.

A- Calmodulin; B- Aspartic proteinase; C- NADH ubiquinone oxidoreductase; D- RanGTPase; Data are presented as means ± SD of three independent RNA samples. Values labeled by different letters are significantly different according toDuncan's multiple comparison tests at $p< 0.05$ at each time point.

III.2. DISCUSSION

III.2.1. Postharervest physiological deterioration (PPD) onset in cassava storage roots

Tubers from the field were harvested at 10 months after planting. It is considered that tubers from 10 months planting gave the highest values for most physical and chemical characters of tuber roots as well as total yield of fresh tuber roots, starch and dry matter with reduced carbohydrates/nitrogen ratio and total fiber percentage of tuber roots (Hasan, 2012). In order to mimic the common conditions of roots storage, entire roots were selected for the experiment as described by Salcedo et *al.* (2010). The morphological observations of cassava storage roots during storage showed the blue-black discoloration as a visual sign of PPD (Buschmann et *al.*, 2000; Beeching et *al.*, 2004; Owiti et *al.*, 2011). Delay was observed in discoloration development according to the genotype with SC5 presenting the most susceptibility. In fact, difference in PPD susceptibility according to genotype has been previously reported by Morante et *al* (2010).

The effect of fertilization on PPD development is poorly studied in cassava. No sign of PPD was observed in the control of QZ1 up to 30DAH confirming the tolerance of this genotype to PPD. Soil fertilization with calcium and magnesium delayed the onset of PPD symptoms up to 10 days in SC5, a PPD-susceptible cassava genotype. This is consistent with the report of Aghofack-Nguemezi & Tatchago (2010) who also observed the prolongation of the shelf-life of the red-ripe tomato as well as the delay of ripening of mature green tomato in plants fertilized with fertilizers containing calcium and magnesium. In fact, it is well established that Ca ion can delay ripening and senescence, by stabilizing cell membrane and increasing the rigidification of monolayers (Aghofack-Nguemezi & Tatchago, 2010). In addition, the severity and incidence of the rot of potato tuber was minimized by the application of calcium fertilizer during potato growth (Conway et *al.*, 1992; Mantsebo et *al.*, 2014). Increasing plant Ca^{2+} content has been shown to enhance resistance of plant tissue to bacterial phytopathogens (Arvin et *al.*, 2005). The Mg ion affects electrostatic cross linking between membrane components to a lesser extent than Ca ion (Leshem, 1991; Aghofack-Nguemezi & Tatchago, 2010).

III.2.2. Effect of fertilizer on tuber weight and size

Fertilization with calcium reduced tuber number and increased tuber weight in SC5. This result is similar to that reported by Ogzen & Palta (2004) where an improvement in tuber grade and the decrease in tuber numbers were observed in potato affected by soil Ca^{2+} application. On the contrary, supplemental Ca induced an enhancement of yield in strawberry cultivars grown at hight NaCl salinity (Kaya et *al.,* 2002). An increase in Ca^{2+} concentration in the soil may suppress tuberization signalling by increasing gibberellic acid (Ozgen & Palta, 2004). There has been evidence for the critical role of gibberellic acid in tuberization (Koda & Okazawa, 1983; Xu et *al.,* 1998). Gibberellic acid in plants can also be modulated by a Ca^{2+}-calmodulin pathway (Bush et *al.,* 1993; Gilroy & Jones, 1993). It is indicated that Ca^{2+} concentration influences the tuberization signal by changing the biochemical processes such as altering the hormonal balance at the stolon tip (Iqbal et *al.,* 2011).

III.2.3. Effect of fertilizer on tuber mineral contents

Results showed that the application of Ca^{2+} enhances Ca^{2+} and Mg^{2+} contents in QZ1. This is in line with another study on potato, of which the concentration of Ca^{2+} was significantly increased by supplemental calcium application (Palta, 2010). It has been suggested that the application of Ca^{2+} or Mg^{2+} in the soil may increase the uptake of these minerals differentially in each genotype. This was seen where the application of Ca^{2+} or Mg^{2+} fertilizers either on the soil or on the aerial part of tomato plants led to an increase in Ca^{2+} concentration in the fruits (Aghofack-Nguemezi & Tatchago, 2010). Fertilization contributes to the direct availability of calcium in the soil, which suggests that tubers can absorb calcium directly from the soil solution (Schroeder et *al.,* 2001). In SC5, the application of Mg^{2+} induced an increase of Ca^{2+} and Mg^{2+} contents in the tubers. An increase in the level of Ca^{2+}in banana (*Musa acuminata* colla) fruits after treatment by dipping in $MgSO_4$ solution was previously observed by Aghofack-Nguemezi & Dassie (2007). However, further studies are needed in order to understand how Mg^{2+} fertilizer triggers an increase in Ca^{2+} content in cassava tubers. The K content increased in the tubers of both genotypes of the plants fertilized. Fertilization either with Ca^{2+} or Mg^{2+} had a positive effect on K uptake in both genotypes. Poljak et *al.* (2007) showed that Ca^{2+} fertilizers increase K, and phosphorus concentrations in the plant. The specific effect of K on the antioxidant level by the activation of protein synthesis, osmoregulation, energy transfer and stress resistance was mentioned by

other authors (Devi et *al*, 2012; Marschner, 2012). This may explain the improvement of antioxidant level and consequently the the delay of the onset of PPD in SC5 (susceptible genotype) after fertilization.

III.2.4. Proteome analysis reveals candidate proteins involved in PPD tolerance during storage

The differential regulated proteins during PPD in cassava storage roots were investigated in this work using 2D-PAGE. Differences in protein profiles of two genotypes different by their susceptibility to PPD were compared in the tubers from the field. Many proteins appeared specific to some time points and proteins expressed at different time points showed changes in abundance. A situation in which multiple spots corresponded to one unique protein was observed in this study. There were also several proteins that appeared to be specially expressed in each genotype following the duration of storage. This suggests that qualitative and quantitative changes in protein profiles may contribute to PPD development in cassava storage roots. As expected, energy and carbohydrate metabolism were affected by wound induced PPD during tuber storage. The large number of this group of proteins suggests the dynamic influence of PPD on starch and energy as observed in histology via the decrease of starch granules and decrease of starch amount during storage. PPD has been shown to induce starch degradation thereby increasing sugar content (Sanchez et *al.*, 2013). In another tuberous plant like sweet potato, it is suggested that during storage, the degradation of starch can produce energy to maintain the lowest level of metabolism which can be used during tuber germination (Jiang et *al.*, 2012). Since cassava tuberous roots have no reproductive function, the fate of the sugars resulting from starch degradation during a process remains unclear. In the processes of glycolytic synthesis of ATP, triose phosphate isomerase catalyzed the isomerization of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate. The decrease of abundance of triose phosphate isomerase was observed at early stage of conservation in QZ1. This decrease could lead to an inhibition of glycolysis and the tricarboxylic acid cycle and, consequently, arrest mitochondrial metabolism under oxidative stress conditions, leading to the prevention of the deleterious production of reactive oxygen species (Ito et *al.*, 2003).

Heat shock proteins play an important role in cells both under normal conditions and when stressed (Wangxia et *al.*, 2004). Even if the precise functional role of heat shock proteins remains unclear, it is suggested that their accumulation can act to decrease the

intracellular level of reactive oxygen species (Heckathorn et *al.,* 2002). Furthermore, Hsp70 was up-regulated in SC5 at 30 DAH, indicating its fundamental role in response to PPD development.

Stress induced by wounding can repress the synthesis of proteins. Some isoforms of translation initiation factors were up-regulated during storage in QZ1 while down-regulated in SC5. This may reveal the complexity of expression patterns for initiation following PPD development. In some previous studies, a high accumulation of translation factors has been reported to induce cellular reorganization leading to programmed cell death (PCD) during stress (Rollins et *al.,* 2013). In fact, QZ1 may display PCD process in order to control the spread of the discoloration during storage, hence reducing the development of PPD. Translation factors display chaperone activity, and it has been suggested that high temperature-induced accumulation of translation factors is important for high temperature stress tolerance in plants; cultivars expressing a higher translation factor under high temperature stress were more tolerant to high temperature stress (Bita & Gerats, 2013). Two 14-3-3 isoforms (spots 48 and 49) were detected in QZ1, indicating that the 14-3-3 proteins were phosphorylated during storage to face PPD development and reduce its impact. The 14-3-3 group of proteins is ubiquitous and multifunctional regulators in many cellular signaling pathways. They interact with a number of signaling molecules, such as calcium-dependent protein kinase (CDPK), and mitogen activated protein kinase (MAPK) (Rosenquist et *al.,* 2001; Comparot et *al.,* 2003; Swatek et *al.,* 2014). Importantly, 14-3-3 proteins can also act as positive regulators of plasma membrane (PM) H^+-ATPase by interacting with the C terminus, which is essential for the control of ion transport and cytoplasmic pH. They can play roles in stress response at multiple levels including regulating target proteins with functions including signaling, transcription activation and defense (Rosenquist et *al.,* 2001; Comparot et *al.,* 2003).

Calmodulin (CaM) was found down regulated in QZ1 from 15DAH to 30DAH while the expression level was high at 15DAH. The Ca^{2+}/calmodulin pathways have been implicated in mediating stress responses and tolerance in plants. In rice, salt inducible and developmentally regulated plasma membrane polypeptides (DREPP PM) containing a Glu-rich site at the C terminus is proposed to be responsible for calcium binding, and association with the Ca^{2+} signal transduction pathway under salt stress (Yuasa et *al.,* 2000; Cheng et *al.,* 2009). Proteomic study revealed the increase of abundance of Ras-related protein in QZ1

following the duration of storage. Plant Ras-related group proteins have been shown to be mobilized by hormones, pathogen elicitors and abiotic stresses (Gu et *al.,* 2004; Nibau et *al.,* 2006). Ras-related protein can also interact with multiple effectors, affecting cellular and biochemical systems that regulate reactive oxygen species production, proteolysis, and gene expression (Nibau et *al.,* 2006).

III.2.5. PPD development and changes in ROS activities in cassava storage roots

Previous reports indicated that PPD was associated with ROS production (Uarrota et *al.*, 2015; Reilly et *al.,* 2004; Zidenga et *al.,* 2012 and Xu et *al.,* 2013). PPD onset was mostly regulated by the balance between ROS and changes in the activities of antioxidant enzymes (Ma et *al.,* 2016). H_2O_2 is moderately reactive. It has a relatively long half-life and high permeability across membranes (Uarrota et *al.,* 2014). In the present study, H_2O_2 content increased apparently to the highest value in the storage roots of SC5 and QZ1 at 6 DAH and 15 DAH respectively (Fig. 23). SOD, APX and CAT activities increased to the highest values in PPD-tolerant genotype QZ1 at 3DAH; however, CAT activity in PPD-susceptible genotype SC5 reached the higher values at the time-point of 15 DAH (Fig. 24A). SOD has been reported to work in collaboration with CAT which acts in tandem to remove H_2O_2 (Sohal et *al.,* 1996; Isamah et *al.,* 2003). It seems to show that the high activities of SOD and CAT antioxidants in QZ1 may be used to remove the increased H_2O_2. It means SOD in combination with CAT would be the first line of defense against PPD for the PPD-tolerant cassava variety, and could be used as a signaling to detect PPD. This is consistent with those observed by Qin et *al* (2017). Then, the first line of defense against PPD in SC5 was weak and resulted in the production of PPD phenomenon at 6 DAH. POD was likely to participate with PPD onset because its activity was increased during storage roots in response to PPD, whilst high tolerant cultivars exhibited lower levels of POD activity during the post-harvest period (Uarrota et *al.,* 2015). Xu et *al.* (2014) reported that APX was used as simultaneously activated antioxidant to participate in the defense mechanisms via cyclic ROS scavenging. APX increased in the PPD-susceptible cultivars SAN and IAC during storage for 3 days, producing PPD phenomenon. In the PPD-tolerant BRA cultivar PPD was found at 5 days (Uarrota et *al.,* 2016), suggesting that APX may participate in the construction of the second line of defense in order to maintain the low levels of ROS produced from PPD. The second line of defense against PPD is the presence of endogenous antioxidant chemicals, such as other antioxidant enzymes (Xu et *al.,* 2014; Uarrota et *al.,* 2016; Apel & Hirt, 2004; Van

Doorn & Ketsa, 2014). In the protein-protein interaction (PPI) networks of both genotypes, APXs were the connections between detoxifying and antioxidant, carbohydrate and energy metabolism associated proteins and chaperones confirming the association of APX to PPD process as observed by Reilly et *al.* (2007). A high activity of ascorbate peroxidase enzyme in SC5 was correlated to the up-regulation of many isoforms of this protein at 3DAH and 6DAH especially in SC5. In fact, enzymes of the ascorbate-glutathione cycle contribute to the control of ROS primarily due to the activity of ascorbate peroxidase which converts H_2O_2 to H_2O and molecular oxygen via oxidation of ascorbic acid to monohydrogenoascorbate (Birgit et *al.*, 2010).

The results showed a change in ethylene content especially in QZ1. The gaseous hormone ethylene is an important signalling component for many abiotic and biotic stresses (O'Donnell et *al.*, 1996; Wi et *al.*, 2010). H_2O_2 accumulation and simultaneous increase of ethylene production were observed in camptothecin (De jong et *al.*, 2000) and cadmium-induced cell death (Yakimova et *al.*, 2006). A significant positive correlation observed between CAT and ethylene suggests a cross-talk between ROS and this hormone in response to PPD. Xu et *al.* (2013) have demonstrated that transgenic cassava storage roots overexpressing CAT and SOD can limit the accumulation of ROS independently from the ascorbate pool. The detection of multiple isoelectric species for heat shock proteins and ascorbate peroxidase and the difference in their expression levels suggest a possible post-translational modification during PPD.

III.2.6. Fertilization with Ca and Mg impact on ROS activities and PPD onset

Fertilization with calcium and magnesium had a positive effect on the production of antioxidants in both genotypes. Siddiqui et *al.* (2012) demonstrated that the application of calcium improved the activities of antioxidant enzymes and solutes. This may contribute to a better reaction against oxidative stress and subsequently results in the delay of the onset of PPD. An increase in antioxidant activity was observed in tomato fruits growing under high proportion of Mg (Fanasca et *al.*, 2006). It is also believed that calcium has an influence on the production of heat shock proteins that help the plant tolerate the stress of prolonged heat (Chang et *al.*, 2006). Furthermore, the addition of Ca^{2+} to the pollen tube tip increases ROS accumulation (Potocký et *al.*, 2007, 2012; Wilkins et *al.*, 2011). On the contrary, a decrease of antioxidant enzymes was observed in wheat after foliar application of calcium (Dolatabadian

et *al.,* 2013) suggesting the implication of Ca in the protection of plants against oxidative stress

III.2.6. Candidate genes involved in PPD tolerance

In response to stress, expression of many genes gets altered. In the present study, gene relative expression levels of calmodulin (CaM), aspartic proteinase, RanGTPase and NADH ubiquinone-oxidoreductase linked with calcium signalling, ROS and programmed cell death (PCD) were evaluated by qRT-PCR. The detection of CaM at protein level coupled to the change of its gene expression level during conservation of tubers can be attributed to the increase of Ca^{2+} flux (Beneloujaephajri et *al.,* 2013; Sagi & Fluhr, 2006). Thus, the increase in Ca^{2+} flux sensed by CaM is suggested as a possible trigger of ROS during wound induced PPD in CSR. In SC5, Ca^{2+}/CaM may increase H_2O_2 generation through Ca^{2+}/CaM dependent NAD kinase which affects the concentration of available NADPH (Harding et *al.,* 1997). High expression level of MeCaM coupled to APX was recently reported by Qin et *al.* (2017) as a line of defence in cassava PPD-susceptible genotype. The increase of expression level of NADH ubiquinone oxidoreductase was correlated to the change of CaM expression level in QZ1, suggesting a possible crosstalk between Ca^{2+}-CaM and NADH during the oxidative burst causing PPD on cassava storage roots. Calmodulin gene expression increased in both genotypes under Ca Fertilization. It was suggested that external calcium might increase soluble Ca^{2+} and the expression of the CaM gene to stimulate an intracellular Ca^{2+}/calmodulin signaling system. This has been seen in the increase in the level of calmodulin expression in apple fruits cultivated under hydroponic conditions' supplemented by $CaCl_2$ (Jing-Wen et *al.,* 2011). Ca/Mg appears to be a key player in the modulation of enzymes and gene expression during PPD in cassava storage roots. The role of calmodulin in signal transduction of the activation of numerous target proteins has been observed in many plant stress responses (Bouché et *al.,* 2005). Our results confirmed the concept that the mRNA level is not well correlated with the protein level (Gygi et *al.,* 1999).

There is increasing evidence demonstrating the implication of small GTase in mediating numerous processes such as pollen growth as well as abiotic stress. This protein has been found to be essential for nuclear translocation, nuclear assembly, mRNA processing, and cell cycle control (Brandizzi et *al.,* 2004). Decreased ATP levels induced by oxidative stress were correlated to the decrease in Ran–GTP levels and disordered Ran distribution

(Yasuda et *al.*, 2006). In *Oryza sativum*, the overexpression of OSRAN1 in transgenic plants enhanced resistance to cold stress by maintaining cell division (Xu & Cai, 2014). Our results indicate a consistent up-regulation of RanGTPase during postharvest physiological deterioration in QZ1. The up-regulation of this protein may be correlated to the tolerance of this genotype to stress induced by wounding in cassava storage roots. Calmodulin gene expression increased in both genotypes under calcium fertilization.

II.2.7. Crosstalk among Ca^{2+} signaling, ROS and PCD integration network in cassava to fine-tune PPD syndrome

Based on the data described above, we proposed a mechanism of PPD development in cassava storage roots. All impact factors associated with PPD were used to generate a biological interaction network using Pathway Studio. This network includes cell processes, functional class proteins, small molecules and osmotic stress treatments. Mechanical damage caused by wounds induces oxidative burst and stimulates Ca^{2+} influx. This flux is sensed by Ca^{2+} binding proteins such as CaM which has several Ca^{2+}-dependent *in vitro* activities. It is involved in regulating various cellular and biochemical processes, such as PPD. Ca^{2+} signaling is essential for activating the NO and ROS production induced by mechanical damage. Therefore, Ca^{2+}-CaM complex is a key center to regulate ROS homeostasis. Other key points are ROS and apoptosis. ROS are responsible for mediating cellular defense responses in cassava. The production of ROS, mediated through NADPH oxidase, increases under stress conditions such as wound damage, causing oxidative burst and impairment of normal metabolism. ROS are also key elements in cassava PCD which is essential for microbial infection. In the model, a negative control on cell death-dependent ROS accumulation, promoted by salycilic acid and ethylene is limited by jasmonic acid (Fig. 31). CAT and SOD can interfere with hormones such as ethylene to scavenge the flux of ROS produced (Fig. 31), restore the equilibrium and PPD can be delayed. The susceptibility to PPD may be associated to high ROS activities especially H_2O_2, CAT as well as high amounts of ethylene production. The lack/low activation of CaM which relates to Ca^{2+} flux may exacerbate the phenomenon since the increasing production of ROS may interfere with mitochondria and stimulate the production of MAPK. MAPK can act with heat shock proteins and other cell death pathways inducing cell wall degradation and subsequently PPD. In QZ1, high expression levels of aspartic proteinase at early days after harvest and high expression levels of RanGTPase following the duration of tuber storage coupled to high expression levels

of structural protein related, may contribute to the delay in the onset of PPD. In fact, the different flux of Ca^{2+} as well as the change in ROS activities increases the expression levels of genes such as calmodulin, and the novo proteins synthesis or the up/down regulation of the existing proteins. As a summary, we consider PPD in cassava as a complex process in which the crosstalk among Ca^{2+} signaling, ROS and PCD are integrated to fine-tune PPD syndrome.

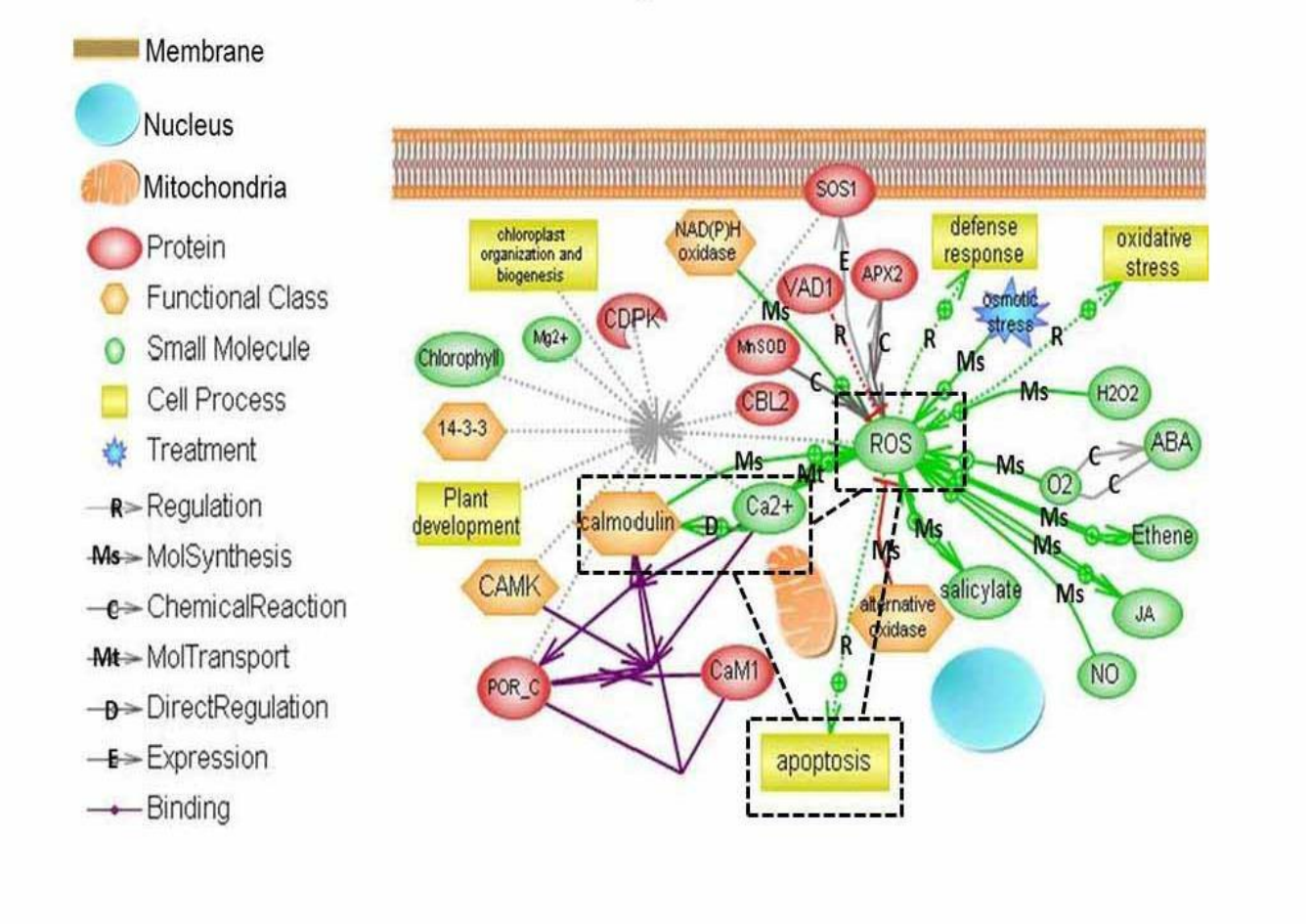

Fig. 31. Putative crosstalk among Ca^{2+}, ROS and apoptosis in cassava storage roots during PPD.

The network was generated with Pathway Studio software default. Regulation is marked as an arrow with R, MolSynthesis as an arrow with Ms, Chemical Reaction as an arrow with C, MolTransport as an arrow with Mt, Direct Regulation as an arrow with D, Expression as an arrow with E and Binding as an arrow without any marks

CONCLUSION AND PERSPECTIVES

The short shelf life of cassava storage roots is an important constraint that limits potential and commercial vulgarization of cassava especially in developing countries that rely on cassava for food. Postharvest physiological deterioration (PPD) is an inherent constraint since wounding and mechanical damage cannot be successfully prevented during the process of harvesting cassava storage roots. PPD is a complex phenomenon involving different metabolic pathways in which reactive oxygen species (ROS) are undoubtedly implicated and make connections with other pathways. The first objective of this thesis was to access the interaction between Ca^{2+}-calmodulin, ROS, proteome and programmed cell death to fine tune the PPD process. Secondly an approach to delay PPD onset was investigated using calcium and magnesium fertilizers.

The results showed a qualitative and quantitative change in proteome profiles of the two genotypes following different storage durations. Proteins related to carbohydrate and energy metabolism associated proteins, defense, chaperones and detoxifying/antioxidant were the most identified in both genotypes. In SC5, the susceptible genotype, the up-regulation of proteins related to antioxidants such as L-ascorbate peroxidase, many heat shock proteins, dehydrin, arginosuccinate and ring-H2 finger protein were observed while in QZ1 the tolerant genotype, the down regulation of calmodulin and the up-regulation of fumarylacetoacetase as well as proteins related to programmed cell death (PCD) such as Ras-related protein and translational controlled tumor protein were observed. This suggests that the tolerance to PPD may be due to the activation of PCD process which is lacking or low in the susceptible genotype.The results also support the implication of calmodulin in PPD processes since the protein-protein interaction showed a connection between this protein and Hsp70, Ras-related proteins and carbohydrate and energy metabolism associated proteins in QZ1 the PPD tolerant genotype. The high expression levels of RanGTPase and NADH ubiquinone oxidoreductase observed in the tolerant genotype suggest further investigations about how they interact with ROS and phytohormones or may be other proteins of programmed cell death like 14-3-3 regarding the complex signalling networks involved in PPD process. The high amounts of H_2O_2 and ethylene in SC5 support the susceptibility of this genotype compared to QZ1. Ethylene may act alone as an up-stream signal or simultaneously with catalase in response to the accumulation of hydrogen peroxide.

Ca and Mg appear to be involved in the modulation of enzymatic activities during PPD in cassava storage roots. Furthermore, these results elucidate the implication of Ca^{2+}

signaling in the PPD process and its connection with ROS, since an increase of antioxidant activities was observed in the tubers from plants treated with Ca fertilizer.

Antioxidants are undoubtedly important in the physiological responses of cassava storage roots to wounding that incurres at harvest. However, other potential contributors such as Ca^{2+}-calmodulin signalling and PCD might provide opportunities for developing alternative strategies for modulating PPD. This information lead to a better understanding of the protein function and metabolic pathways involved in PPD as well as the identification of particular proteins that can be applied as potential PPD-molecular markers in cassava. Cassava tolerant genotype to PPD (QZ1) showed high expression level of Ras-related protein, translationally controlled tumor protein and 14-3-3 protein. Transgenic approaches by overexpression of calmodulin and Ras-related protein would give valuable information about PPD tolerance mechanisms in cassava. This has been already initiated with calmodulin but due to the time-consuming transformation procedure and the long life cycle of cassava, transgenic plants have to be analyzed in prospective experiments. However these markers can be used in future research through screening to discriminate PPD tolerant and susceptible genotypes. The increase of Ca^{2+} content under calcium fertilization followed by a delay of PPD onset for 10 days in SC5 (the susceptible genotype) has provided an important clue for PPD control by manipulating soil Ca and mineral contents. In this case, investigation on different calcium concentrations with different calcium sources as well as the direct application of calcium on tuber during storage will be usefull for small holder farmers whose cassava is their main income generation source. This work also offers to food technology, the opportunity to build objective indicators for monitoring which will be useful in the implementation of strategies to control cassava postharvest physiological deterioration.

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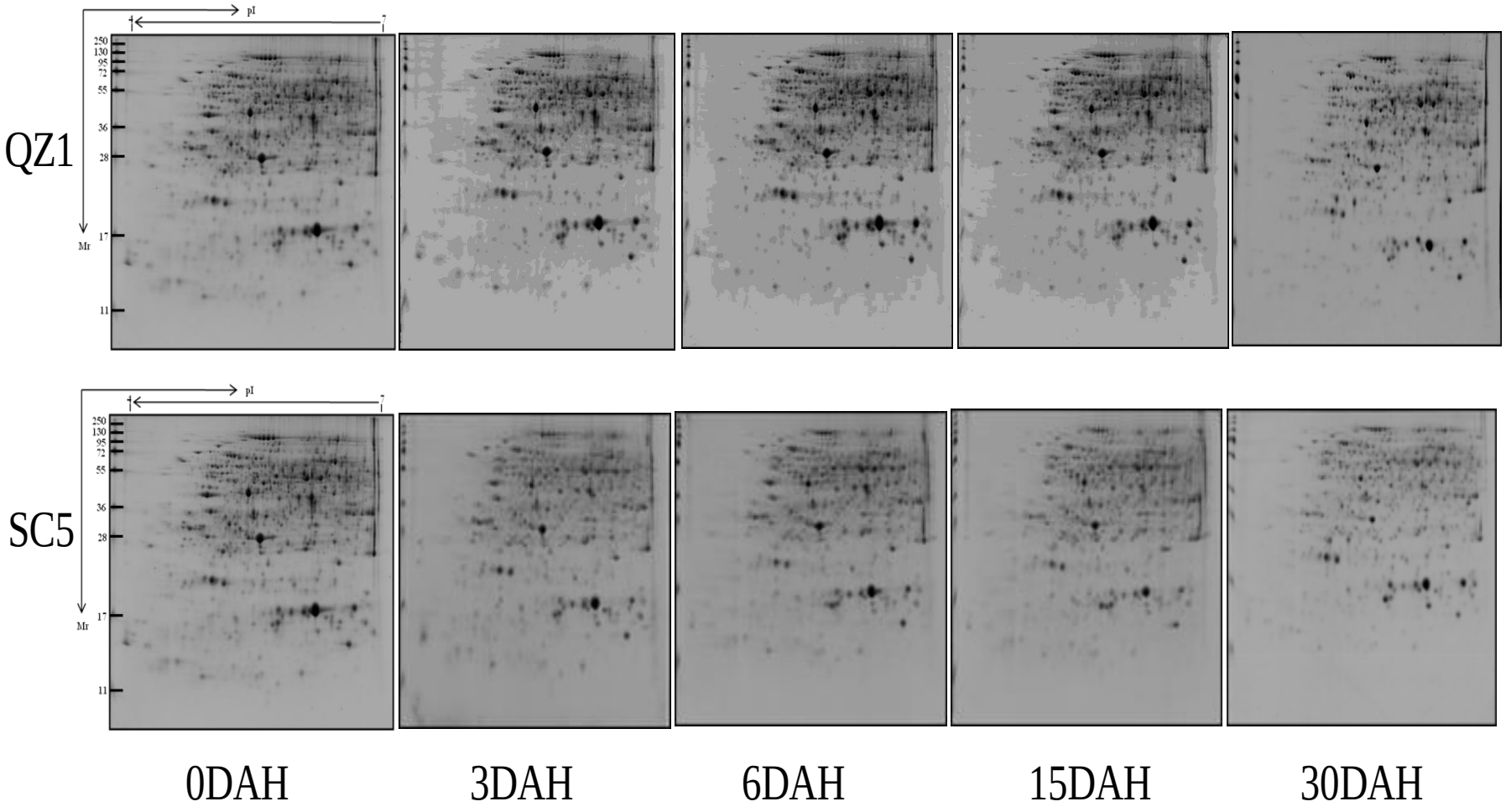
APPENDIX

Appendix 1. 2-DE image profile of QZ1 and SC5 flesh at 0, 3, 6, 15 and 30 DAH.



Appendix 2. List of the total identified proteins changing in abundance during storage

Spot Number[a]	Identification	Accession no[b]	Theoretical pI/Mw(kDa)	Coverage (%)	# of unique peptides	Peptide query	Protein ID in string database
Carbohydrate and energy metabolism associated proteins （23）							
1	Monodehydroascorbate reductase (NADH)	Manes.08G023200.1	5.43/47.32	6.91	3	384,93,7	MDAR1
4	Triose-phosphate isomerase/Triosephosphate mutase	Manes.01G173500.1	5.80/27.73	20.00	4	345;142;424;335;425;423	TPI
7	Orotate phosphoribosyltransferase/ Orotidylic acid phosphorylase	Manes.11G004200.1	6.87/51.65	2.11	1	108	AT3G54470
10	F-type H+-transporting ATPase subunit d	Manes.10G043000.1	4.82/19.66	30.36	6	59,190,98,95,43,9	ATPQ
28	Fructose-bisphosphate aldolase 3, chloroplastic	Manes.14G108900.1	8.64/43.05	5.58	2	67,321,97	PDE345
36	Malate dehydrogenase	Manes.16G109900.1	6.37/65.25	9.98	5	286,90,309,228,2,52,98,88,89	NADP-ME3
37	Mitochondrial-processing peptidase subunit alpha	Manes.03G029000.1	6.15/55.11	10.04	5	271,123,104,272,194,102,122,269,103	AT1G51980
41	Alcohol dehydrogenase class-P	Manes.18G087400.1	6.44/39.24	14.89	6	90;210;75;71;102;91;103;180;72;76	ADH1
42	Starch phosphorylase	Manes.02G052600.1	6.21/10.81	12.91	12	155;122;322;79;88;144;82;110;161;3;134;253;160;115;313;109;137;145;321;132	AT3G29320
46	2,3-dimethylmalate lyase/(2R,3S)-2,3-dimethylmalate pyruvate-lyase	Manes.11G115000.1	7.25/33.57	6.95	2	56;144;15	AT2G43180
50	Ran-binding protein 1	Manes.18G136500.1	4.71/24.15	38.25	7	6;458;81;112;327;4;393;141;224;325	RANBP1
60	F-type H+-transporting ATPase subunit d	Manes.10G043000.1	4.82/19.66	30.36	6	59,190,98,95,43,9	ATPQ
64	ATP-dependent CLP protease	Manes.06G085600.1	5.93/101.05	7.91	7	182;259;250;98;180;296;144;13;300;195	CLPC1
68	Alcohol dehydrogenase class-P	Manes.18G087400.1	6.44/39.24	14.89	6	90;210;75;71;102;91;103;180;72;76	ADH1
69	Alcohol dehydrogenase class-P	Manes.18G087400.1	6.44/39.24	14.89	6	90;210;75;71;102;91;103;180;72;76	ADH1
71	Malate and lactate dehydrogenase	Manes.14G078500.1	8.73/43.53	10.92	4	170;28;118;262;168;172;255;175;117;29;31;171	MDH
74	Triosephosphate isomerase, chloroplastic	Manes.04G078300.1	7.39/34.64	16.61	4	371;330;420;331;281;353;99;376;383;96;100;301;300;149;334;304;95;283;97;279;299;377;98;256;101;423	TPI

82	Fructose-bisphosphate aldolase 3, chloroplastic	Manes.06G063500.1	8.97/42.91	3.30	1	106,72,295,284	FBA2
83	Fructose-bisphosphate aldolase 3, chloroplastic	Manes.06G063500.1	8.97/42.91	3.30	1	272 ;283 ;68	FBA2
85	Nitrogen metabolic regulation protein NMR	Manes.09G092700.1	5.49/27.51	18.04	5	365;217;213;115;14;215;116;366;24;214;216	AT2G37660
89	ATP-dependent CLP protease	Manes.06G085600.1	5.93/101.05	7.91	7	182;259;250;98;180;296;144;13;300;195	CLPC1
90	Nitrogen metabolic regulation protein	Manes.09G092700.1	5.49/27.51	6.67	2	137 ;136 ;5	AT2G37660
98	Lactoylglutathione lyase glyoxalase I	Manes.16G112000.1	7.90/40.28	16.39	7	286;165;36;7;167;8;163;116;162;292;186;178;164;19	AT1G67280
Chaperones (20)							
11	17.6 kDa class I heat shock protein 1	Manes.10G020600.1	9.02/18.75	12.73	2	342;71;80;17;2;336;79;77;18;72;338;16;74;339;3;75;305;14;15;109;335;73;340;19;76	HSP17.6A
13	25.3 kDa class I heat shock protein, chloroplastic	Manes.01G042200.1	8.20/26.26	5.13	1	257	AT5G51440
19	17.6 kDaclass II heat shock protein	Manes.16G083600.1	5.85/17.67	19.23	4	33;32;26;30;264;27;252;28;250;61;268;254;49;31;35;53;267;266;263;262;48;269;29;50;251;261;36;64;51;47;52;60;34;265;66;260	HSP17.6II
20	17.6 kDa class I heat shock protein 1	Manes.10G020600.1	9.02/18.75	8.48	1	74;71;3;323;322;20;22;324;4;18;352;24;73;351	HSP17.6A
30	25.3 kDa heat shock protein, chloroplastic	Manes.01G042200.1	8.20/26.26	38.89	12	241;139;7;17;336;18;144;141;147;339;108;135;145;224;148;140;223;150;152;142;330;208;341;143;434;106;219;6;149;221;138;146;137;337;216;335;340;242	AT5G51440
35	Heat shock 70 kDa protein 1/8	Manes.07G114200.1	4.92/71.46	7.72	4	312;194;248;192;279	HSC70-1
53	20 kDa chaperonin, chloroplastic	Manes.03G044000.1	8.73/26.70	7.42	2	179,30	CPN20
62	Small heat-shock protein HSP20 family	Manes.10G020100.1	6.56/17.96	31.01	8	127;276;71;455;102;80;26;331;453;324;99;244;366;72;329;27;74;272;463;259;458;424;451;447;243;103;462;31;330;266;78;505;77;106;325;133;29;1	AT2G29500

						23;261;97;81;499;459;101;260;76;365;274;370;129;327;506;136;30;100;25;28;120;75;134;250;268;135;254;24;104;271;130;131;328;245;267;262;121;79;22;269;23;105;257;251;326;253;448;98;132;256;270;242	
63	Small heat-shock protein Hsp 20 family	Manes.10G020100.1	6.56/17.96	20.89		345;90;399;346;91	AT2G29500
77	25.3 kDaheat shock protein, chloroplastic	Manes.01G042200.1	8.20/26.26	38.89	12	67;470;198;102;227;358;353;99;351;100;110;120;108;465;115;112;109;178;177;175;10;343;113;104;11;467;174;352;96;29;355;3;97;9;111;199;12;472;356;98;101;195	AT5G51440
78	17.6 kDa class I heat shock protein 1	Manes.10G020600.1	9.02/18.75	8.48	1	308 ;319 ;90 ;89 ;2	HSP17.6A
87	17.6 kDa class I heat shock protein 1	Manes.10G020600.1	9.02/18.75	12.73	2	342;71;80;17;2;336;79;77;18;72;338;16;74;339;3;75;305;14;15;109;335;73;340;19;76	HSP17.6A
88	Small heat-shock protein HSP20 family	Manes.10G020100.1	6.56/17.96	31.01	8	127;276;71;455;102;80;26;331;453;324;99;244;366;72;329;27;74;272;463;259;458;424;451;447;243;103;462;31;330;266;78;505;77;106;325;133;29;123;261;97;81;499;459;101;260;76;365;274;370;129;327;506;136;30;100;25;28;120;75;134;250;268;135;254;24;104;271;130;131;328;245;267;262;121;79;22;269;23;105;257;251;326;253;448;98;132;256;270;242	AT2G29500
94	Heat shock 70 kDa protein 5	Manes.11G067600.1	5.06/72.16	5.50	3	197,251,209,278,242	Hsp70b
97	Chaperonin 60 subunit beta 1, chloroplastic	Manes.03G086700.1	5.54/64.92	2.14	1	182	CPN60B
100	26.5 kDaheat shock protein, mitochondrial	Manes.04G113600.1	8.64/26.09	19.38	6	131;234;198;129;200;136;133;297;84;197;233;190;85;134;194;199;299;191;215;232;196	AT1G07400
103	17.6 kDa class II heat shock protein	Manes.16G083600.1	5.85/17.67	15.38	3	114;111;108;12;18;13;110;113	HSP17.6II
104	17.6 kDa class I heat shock protein 1	Manes.02G124600.1	6.29/17.87	10.19	2	90;188;407;119;180;116;179;162;186	HSP17.6A

						;110;182;310;95;458;400;305;115;316;184;112;92;109;254;178;89;10;187;113;11;155;181;314;91;93;154;461;13;157;96;185;183;97;94;111;114;12;153;408;317;312;56;117	
105	17.6 kDa class I heat shock protein 1	Manes.10G020600.1	9.02/18.75	20.61	4	127;7;188;129;2;125;44;246;128;190;488;194;134;484;192;400;421;191;395;49;414;490;124;131;189;412;53;239;394;491;48;413;46;487;399;133;422;126;6;123;50;486;3;332;51;9;47;8;52;482;4;483;45;43;493;485;242;5	HSP17.6A
106	Small heat-shock protein Hsp20 family	Manes.15G154400.1	6.53/15.65	33.09	4	32;342;346;314;26;394;22;344;399;30;23;29;25;27;147;28;310;396;357;24;31;311	AT1G53540
Detoxifying and antioxidant (10)							
3	L-ascorbata peroxidase 2, cytosolic	Manes.04G026800.1	5.19/27.77	34.8	9	104;189;309;314;188;22;413;419;257;6;197;326;199;417;202;454;408;92;312;196;260;311;5	APX2
5	Riboflavin synthase	Manes.13G146800.1	8.08/30.34	30.47	7	27;840;210;239;208;413;687;265;414;264	AT2G20690
14	Thioredoxin-like protein CXXS1	Manes.16G017200.1	4.46/14.12	22.40	3	109,93,108,76	CXXS1
17	Dehydrin	Manes.05G140400.1	5.16/24.67	65.14	15	71;102;520;737;376;844;522;587;27;517;20;109;633;634;584;849;287;857;106;742;378;29;65;203;199;429;861;642;432;73;101;284;371;519;67;204;289;585;727;739;418;590;202;69;24;178;285;588;516;104;646;282;420;639;154;23;426;369;176;629;372;643;21;63;524;523;288;521;586;72;179;526;433;637;647;108;740;430;103;201;379;414;415;423;862;632;107;867;419;290;422;592;64;631;854;417;731;66;866;370;70;593;68;638;645;635;41	COR47

						6;641;100;25;640;738;286;28;283;156;589;308;421;177;373;155;281;22;644;583;105;630;518;425;591;832;736	
21	Thioredoxin-like protein CXXS1	Manes.16G017200.1	4.46/14.12	29.6	6	204;21;206;139;26;331;327;180;18;144;141;329;25;334;333;182;20;24;140;181;330;328;142;207;267;208;143;22;205;212;214;23;325;210;326;138;183;160;211;137;209;19	CXXS1
45	Dehydrin	Manes.05G140400.1	5.16/24.67	20.18	5	240;123;120;234;118;239;236;122;367;4;351;117	COR47
56	Glutathione S-transferase	Manes.02G089200.1	6.73/24.37	18.10	7	274;127;21;165;102;188;129;193;17;416;162;16;222;128;190;161;418;194;134;192;220;218;313;135;14;20;163;191;224;223;130;189;314;107;419;219;13;133;221;417;12;15;98;12;216;132;164;196;19;195	GSTF9
58	Riboflavin synthase	Manes.13G146800.1	8.08/30.34	30.47	7	27;840;210;239;208;413;687;265;414;264	AT2G20690
84	L-ascorbata peroxidase 2, cytosolic	Manes.04G026800.1	5.19/27.77	34.80	9	104;189;309;314;188;22;413;419;257;6;197;326;199;417;202;454;408;92;312;196;260;311;5	APX2
91	L-ascorbata peroxidase 2, cytosolic	Manes.04G026800.1	5.19/27.77	9.60	2	182,258	APX2
Structure (2)							
51	DREPP plasma membrane polypeptide	Manes.S071400.1	4.62/22.50	31.70	7	371;124;373;71;70;366;16;29;27;228;368;230;15;231;367;34;229;232;148	PCAP1
70	DREPP plasma membrane polypeptide	Manes.S071400.1	4.62/22.50	31.70	7	371;124;373;71;70;366;16;29;27;228;368;230;15;231;367;34;229;232;148	PCAP1
Inorganic ion transport and metabolism (1)							
31	Calmodulin-5	Manes.01G002400.1	3.88/16.89	36.24	6	238;74;234;118;114;68;299;115;17;119;294;116;72;73;235	CAM7
Protein biosynthesis (13)							
12	Translation initiation factor 5A	Manes.05G107000.1	5.87/17.71	7.55	1	159;35;221;342;16	ELF5A-1
16	Argininosuccinate synthase / Citrulline--	Manes.03G085300.1	8.18/54.99	12.17	7	25;120;26;119;116;89;132;140;133;8	AT4G24830

	aspartate ligase					2	
22	Oligopeptidase A	Manes.13G103900.1	6.30/88.23	8.62	7	243;166;154;94;30;295;107	AT5G65620
25	Protein disulfide-isomerase A6	Manes.05G002000.1	5.40/40.29	10.58	4	67,303,126,20	UNE5
39	Disulfide oxidoreductase	Manes.03G209100.1	7.40/54.24	16.67	8	6,165,97,108,153,109,318,169,311,31	mtLPD1
40	Protein disulfide-isomerase A6	Manes.05G002000.1	5.40/40.29	10.58	4	67,303,126,20	UNE5
61	Translation initiation factor 5A	Manes.05G107000.1	5.87/17.71	7.55	1	159;35;221;342;16	ELF5A-1
65	26S proteasome regulatory subunit T5	Manes.08G117200.1	4.73/47.70	4.96	3	398;155;204;206;118;262;193;407;121;119;200;375;154;338;13;378;334;261;120;310;61;199;226;258;312;337;56;335;151;404	RPT5A
73	20S proteasome subunit alpha 6	Manes.05G157400.1	4.72/29.96	5.49	2	71,10	PAF1
75	20S proteasome subunit beta 1	Manes.03G001100.1	5.61/24.99	11.69	2	265 ;261 ;263	PBA1
79	Protein disulfide-isomerase A6	Manes.05G002000.1	5.40/40.29	10.58	4	67,303,126,20	UNE5
80	Elongation factor 1-gamma	Manes.10G116000.1	6.55/48.42	11.58	5	128;6;239;264;263;140	AT1G57720
95	Peptidyl-prolyl cis-trans isomerase FKBP62	Manes.12G050700.1	5.00/62.99	11.23	6	123;6;190;102;188;258;222	ROF1
Amino acid metabolism (3)							
2	Fumarylacetoacetase / Fumarylacetoacetate hydrolase	Manes.09G070300.1	6.35/46.36	4.04	2	243,10,242	AT1G12050
47	Homocysteine S-methyl transferase 3	Manes.15G031300.1	4.89/37.64	17.84	5	240,238,237,149,276,261,210,266,236,208,277,211,209,212	HMT3
81	Proline iminopeptidase	Manes.07G049300.1	6.33/44.34	7.40	4	114;115;41;121;42;112;172;43;171;113	PIP
DNA and RNA metabolism associated proteins (1)							
43	Nucleic acid-binding, OB-fold-like protein	Manes.02G024200.1	6.05/83.01	0.81	1	3	AT1G12800
Signal transduction mechanismst (5)							
23	C2 domain	Manes.S104800.1	4.45/45.84	3.32	1	65,601	AT5G55530
26	Vesicle coat complex COPI, alpha subunit	Manes.12G101600.1	10.77/23.03	4.66	1	91	AT3G50590
48	14-3-3 protein	Manes.02G091000.1	4.43/29.32	14.73	6	63;21;7;324;18;179;162;264;16;246;240;272;182;61;20;163;10;11;142;266;263;65;123;39;64;138;12;41;15;137;321;66;19;62;247;67;70;241;139;68;322;2;17;136;141;40;134;323;236;14;13	At2g42590

						5;69;178;140;271;181;267;122;262;121;269;42;158;235;159;3;36;9;160;176;8;265;37;164;43;242	
49	14-3-3 protein	Manes.15G093600.1	4.57/29.71	10.38	3	123,53,28,226,258,151,29,54	At2g42590
92	2',3'-cyclic-nucleotide 3'-phosphodiesterase	Manes.17G061800.1	5.09/21.87	7.69	2	6 ;114	AT4G18930
Defense (23)							
6	Ras-related protein Rab-11A	Manes.12G092100.1	5.38/24.21	13.89	3	84;226;223;113	RABA1f
8	Translationally controlled tumor protein	Manes.15G011800.1	4.20/19.08	17.26	3	128;127;296;145;3;297;126	TCTP
9	Translationally controlled tumor protein	Manes.15G011700.1	4.29/19.17	22.62	4	104;280;106;70;125;105	TCTP
15	Suppressor of G2 allele of SKP1	Manes.06G153900.1	4.74/41.34	14.21	6	159;149;74;155;283;75;17;1;18;106;16	SGT1B
18	Thiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	41.86	7	371;365;306;370;358;359;631;3;369;248;303;374;305;451;361;308;619;256;372;343	THI1
24	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	131	AT2G34990
27	Thiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	18.14	3	158,149,165	THI1
29	Ras-related protein Rab-11A	Manes.12G092100.1	5.38/24.21	13.89	3	84;226;223;113	RABA1f
34	RING-H2 finger protein ATL61	Manes.07G090300.1	6.66/25.92	3.51	1	92,91	AT2G34990
38	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	131	AT2G34990
44	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	93	AT2G34990
52	Tthiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	6.05	1	257	THI1
54	Thiamine thiazole synthase	Manes.03G123800.1	6.14/33.22	17.68	4	283;282;198;230;281;200;177;229;187	THI1
55	Monothiol glutaredoxin-S16, chloroplastic	Manes.08G093600.1	9.23/33.66	14.80	4	159,158,283,185,186,235	CXIP2
57	Thiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	17.67	3	426;310;462;449;420	THI1
59	Allene oxide cyclase	Manes.01G108000.1	9.20/28.05	40.48	11	519;127;17;520;521;506;522;255;16;647;650;310;20;313;414;646;626;511;257;508;513;515;39;185;36;509;561;651;41;649;568;312;256;37;43;507;311	AOC3
66	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	131	AT2G34990
67	Stress-induced-phosphoprotein 1	Manes.01G075100.1	6.46/65.37	15.10	8	274;67;225;239;102;68;281;180;179;	Hop3

						301;300;299;137;112;4;151;5;223	
72	Thiamine thiazole synthase	Manes.15G075600.1	6.51/37.63	10.20	3	165,242,223,222	THI1
86	Thiamine thiazole synthase	Manes.15G075600.1	6.51/37.63	7.32	2	197,268	THI1
93	Allene oxide cyclase	Manes.01G108000.1	9.20/28.05	29.37	7	391;384;389;227;17;18;383;228;381;268;501;392;395;49;10;224;11;53;387;385;502;267;480;269;481;46;13;388;479;50;390;51;9;12;226;47;52;482;483;265;393;45;478;54	AOC3
96	RING-H2 finger protein ATL61	Manes.07G090300.1	6.66/25.92	3.51	1	126	AT2G34990
99	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	96	AT2G34990
Function unknown (5)							
32	Universal stress protein family	Manes.08G082400.1	6.54/18.04	27.33	5	131;130;102;129;107;212;99;18;106;16;23;133;105;100;84;85;108;109;216;132;101;113	AT3G53990
33	Universal stress protein family	Manes.08G082400.1	6.54/18.04	27.33	5	131;130;102;129;107;212;99;18;106;16;23;133;105;100;84;85;108;109;216;132;101;113	AT3G53990
76	Universal stress protein family	Manes.08G082400.1	6.54/18.04	10.56	2	140 ;100	AT3G53990
101	Universal stress protein family	Manes.03G204200.1	8.33/24.27	9.30	2	81,126	AT3G17020
102	Universal stress protein family	Manes.03G204200.1	8.33/24.27	9.30	2	81,126	AT3G17020

The spots showing differential expression (2.0-fold of the normalized volume) were counted after gel analysis and manual editing with Delta2D software. Each value represents the mean ± SD of triplicate. Protein spots whose abundance increased (+) or decreased (-) after matching with 0 DAH as a control were shown. a,The spot numbers corresponded to the 2-DE gel in Fig. 20 et Fig. 21; b,The accession numberin NCBI and cassava gene bank. Each values were means±SE.

Appendix 3. List of the common identified proteins in QZ1 and SC5.

Spot Number[a]	Identification	Accession no[b]	Theoretical pI/Mw(kDa)	Coverage (%)	# of unique peptides	Protein ID in string database
Carbohydrate and energy metabolism associated proteins (5)						
1	monodehydroascorbate reductase (NADH)	Manes.08G023200.1	5.43/47.32	6.91	3	MDAR1
4	Triose-phosphate isomerase / Triosephosphate mutase	Manes.01G173500.1	5.80/27.73	20.00	4	TPI
7	Orotate phosphoribosyltransferase / Orotidylic acid phosphorylase	Manes.11G004200.1	6.87/51.65	2.11	1	AT3G54470
28	Fructose-bisphosphate aldolase 3, chloroplastic	Manes.14G108900.1	8.64/43.05	5.58	2	PDE345
42	starch phosphorylase	Manes.02G052600.1	6.21/10.81	12.91	12	AT3G29320
Chaperones (5)						
13	25.3 kDa class I heatshockprotein, chloroplastic	Manes.01G042200.1	8.20/26.26	5.13	1	AT5G51440
19	17.6 kDa class II heatshockprotein	Manes.16G083600.1	5.85/17.67	19.23	4	HSP17.6II
20	17.6 kDa class I heatshockprotein 1	Manes.10G020600.1	9.02/18.75	8.48	1	HSP17.6A
53	20 kDa chaperonin, chloroplastic	Manes.03G044000.1	8.73/26.70	7.42	2	CPN20
62	Small heat-shock protein HSP20 family	Manes.10G020100.1	6.56/17.96	31.01	8	AT2G29500
Detoxifying and antioxidant (3)						
17	Dehydrin	Manes.05G140400.1	5.16/24.67	65.14	15	COR47
21	Thioredoxin-like protein CXXS1	Manes.16G017200.1	4.46/14.12	29.6	6	CXXS1
58	Riboflavin synthase	Manes.13G146800.1	8.08/30.34	30.47	7	AT2G20690
Protein biosynthesis (5)						
12	translation initiation factor 5A	Manes.05G107000.1	5.87/17.71	7.55	1	ELF5A-1
16	Argininosuccinate synthase / Citrulline--aspartate ligase	Manes.03G085300.1	8.18/54.99	12.17	7	AT4G24830
39	Disulfide oxidoreductase	Manes.03G209100.1	7.40/54.24	16.67	8	mtLPD1
40	protein disulfide-isomerase A6	Manes.05G002000.1	5.40/40.29	10.58	4	UNE5
61	translation initiation factor 5A	Manes.05G107000.1	5.87/17.71	7.55	1	ELF5A-1
Signal transduction mechanisms (2)						
23	C2 domain	Manes.S104800.1	4.45/45.84	3.32	1	AT5G55530
26	Vesicle coat complex COPI, alpha subunit	Manes.12G101600.1	10.77/23.03	4.66	1	AT3G50590
Defense (12)						
18	thiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	41.86	7	THI1
24	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	AT2G34990
27	thiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	18.14	3	THI1
29	Ras-related protein Rab-11A	Manes.12G092100.1	5.38/24.21	13.89	3	RABA1f

34	RING-H2 finger protein ATL61	Manes.07G090300.1	6.66/25.92	3.51	1	AT2G34990
38	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	AT2G34990
44	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	AT2G34990
52	thiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	6.05	1	THI1
54	thiamine thiazole synthase	Manes.03G123800.1	6.14/33.22	17.68	4	THI1
55	Monothiol glutaredoxin-S16, chloroplastic	Manes.08G093600.1	9.23/33.66	14.80	4	CXIP2
57	thiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	17.67	3	THI1
59	allene oxide cyclase	Manes.01G108000.1	9.20/28.05	40.48	11	AOC3
Function unknown (2)						
32	Universal stress protein family	Manes.08G082400.1	6.54/18.04	27.33	5	AT3G53990
33	Universal stress protein family	Manes.08G082400.1	6.54/18.04	27.33	5	AT3G53990

Appendix 4. List of the unique identified proteins in SC5 and QZ1.

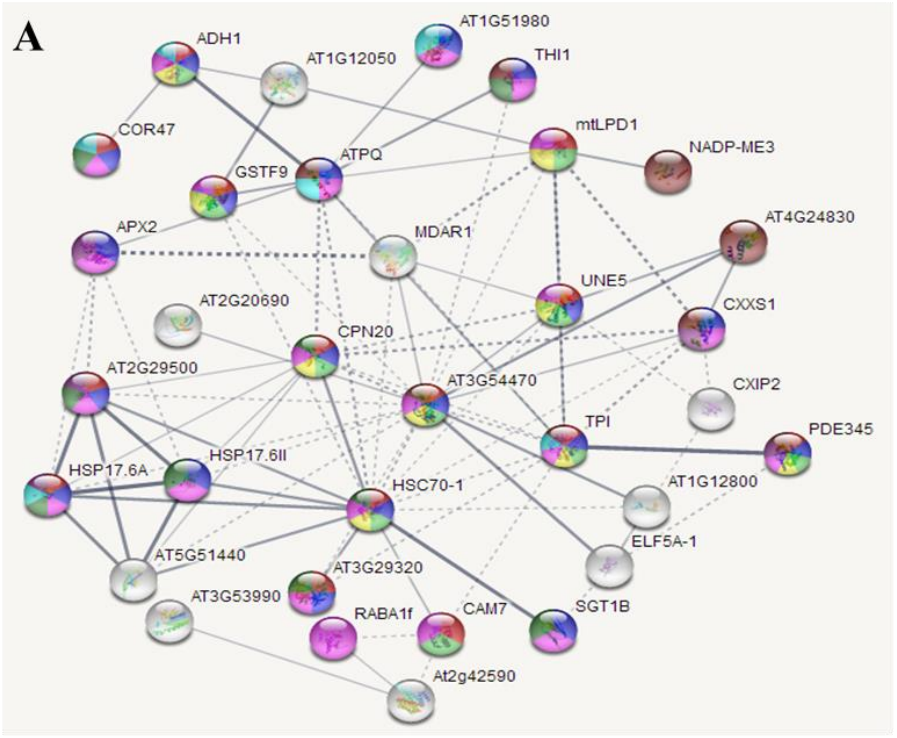
Spot Number[a]	Identification	Accession no[b]	Theoretical pI/Mw(kDa)	Coverage (%)	# of unique peptides	Protein ID in string database
QZ1						
Carbohydrate and energy metabolism associated proteins						
10	F-type H+-transporting ATPase subunit d	Manes.10G043000.1	4.82/19.66	30.36	6	ATPQ
36	Malate dehydrogenase	Manes.16G109900.1	6.37/65.25	9.98	5	NADP-ME3
37	mitochondrial-processing peptidase subunit alpha	Manes.03G029000.1	6.15/55.11	10.04	5	AT1G51980
41	Alcohol dehydrogenase class-P	Manes.18G087400.1	6.44/39.24	14.89	6	ADH1
46	2,3-dimethylmalate lyase / (2R,3S)-2,3-dimethylmalate pyruvate-lyase	Manes.11G115000.1	7.25/33.57	6.95	2	AT2G43180
50	Ran-binding protein 1	Manes.18G136500.1	4.71/24.15	38.25	7	RANBP1
60	F-type H+-transporting ATPase subunit d	Manes.10G043000.1	4.82/19.66	30.36	6	ATPQ
Chaperones						
11	17.6 kDa class I heatshockprotein 1	Manes.10G020600.1	9.02/18.75	12.73	2	HSP17.6A
30	25.3 kDa heat shock protein, chloroplastic	Manes.01G042200.1	8.20/26.26	38.89	12	AT5G51440
35	heat shock 70 kDa protein 1/8	Manes.07G114200.1	4.92/71.46	7.72	4	HSC70-1
63	Small heat-shock protein Hsp20 family	Manes.10G020100.1	6.56/17.96	20.89		AT2G29500
Detoxifying and antioxidant						
3	L-ascorbate peroxidase 2, cytosolic	Manes.04G026800.1	5.19/27.77	34.8	9	APX2
5	Riboflavin synthase	Manes.13G146800.1	8.08/30.34	30.47	7	AT2G20690
14	Thioredoxin-like protein CXXS1	Manes.16G017200.1	4.46/14.12	22.40	3	CXXS1
45	Dehydrin	Manes.05G140400.1	5.16/24.67	20.18	5	COR47
56	glutathione S-transferase	Manes.02G089200.1	6.73/24.37	18.10	7	GSTF9
Structure						
51	DREPP plasma membrane polypeptide	Manes.S071400.1	4.62/22.50	31.70	7	PCAP1
Inorganic ion transport and metabolism						
31	calmodulin-5	Manes.01G002400.1	3.88/16.89	36.24	6	CAM7
Protein biosynthesis						

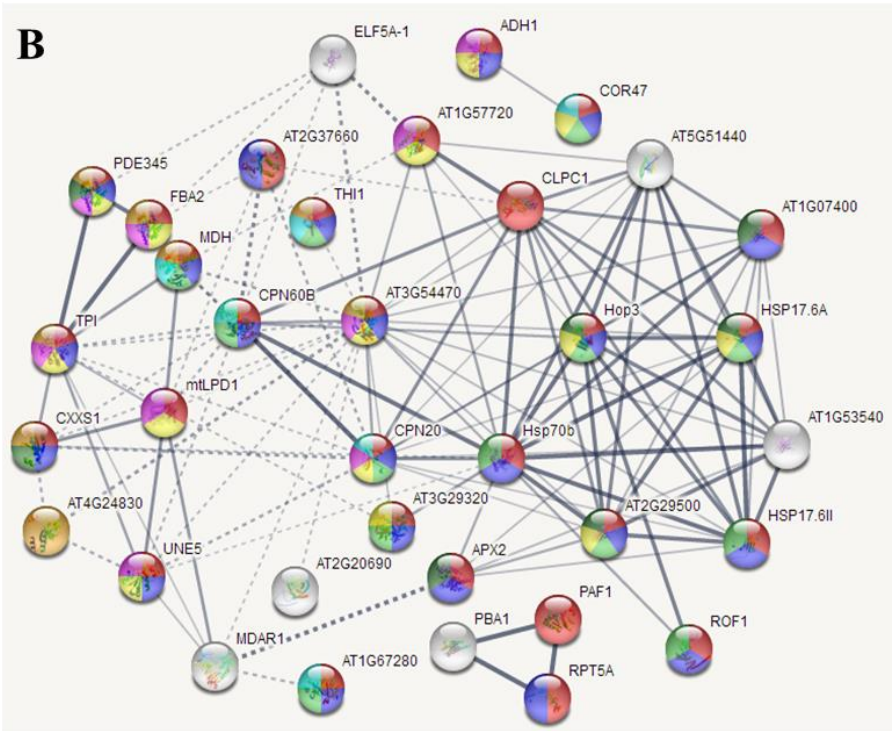
22	Oligopeptidase A	Manes.13G103900.1	6.30/88.23	8.62	7	AT5G65620
25	protein disulfide-isomerase A6	Manes.05G002000.1	5.40/40.29	10.58	4	UNE5
65	26S proteasome regulatory subunit T5	Manes.08G117200.1	4.73/47.70	4.96	3	RPT5A
73	20S proteasome subunit alpha 6	Manes.05G157400.1	4.72/29.96	5.49	2	PAF1
75	20S proteasome subunit beta 1	Manes.03G001100.1	5.61/24.99	11.69	2	PBA1
79	protein disulfide-isomerase A6	Manes.05G002000.1	5.40/40.29	10.58	4	UNE5
80	elongation factor 1-gamma	Manes.10G116000.1	6.55/48.42	11.58	5	AT1G57720
95	peptidyl-prolyl cis-trans isomerase FKBP62	Manes.12G050700.1	5.00/62.99	11.23	6	ROF1
Amino acid metabolism						
2	Fumarylacetoacetase / Fumarylacetoacetate hydrolase	Manes.09G070300.1	6.35/46.36	4.04	2	AT1G12050
47	homocysteine S-methyl transferase 3	Manes.15G031300.1	4.89/37.64	17.84	5	HMT3
81	proline iminopeptidase	Manes.07G049300.1	6.33/44.34	7.40	4	PIP
DNA and RNA metabolism associated proteins						
43	Nucleic acid-binding, OB-fold-like protein	Manes.02G024200.1	6.05/83.01	0.81	1	AT1G12800
Signal transduction mechanisms						
48	14-3-3 protein	Manes.02G091000.1	4.43/29.32	14.73	6	At2g42590
49	14-3-3 protein	Manes.15G093600.1	4.57/29.71	10.38	3	At2g42590
Defense						
6	Ras-related protein Rab-11A	Manes.12G092100.1	5.38/24.21	13.89	3	RABA1f
8	Translationally controlled tumor protein	Manes.15G011800.1	4.20/19.08	17.26	3	TCTP
9	Translationally controlled tumor protein	Manes.15G011700.1	4.29/19.17	22.62	4	TCTP
15	suppressor of G2 allele of SKP1	Manes.06G153900.1	4.74/41.34	14.21	6	SGT1B
55	Monothiol glutaredoxin-S16, chloroplastic	Manes.08G093600.1	9.23/33.66	14.80	4	CXIP2
57	thiamine thiazole synthase	Manes.15G075600.1	5.30/22.97	17.67	3	THI1
SC5						
Carbohydrate and energy metabolism associated proteins						
64	ATP-dependent CLP protease	Manes.06G085600.1	5.93/101.05	7.91	7	CLPC1
68	Alcohol dehydrogenase class-P	Manes.18G087400.1	6.44/39.24	14.89	6	ADH1
69	Alcohol dehydrogenase class-P	Manes.18G087400.1	6.44/39.24	14.89	6	ADH1

71	Malate and lactatedehydrogenase	Manes.14G078500.1	8.73/43.53	10.92	4	MDH
74	Triosephosphate isomerase,chloroplastic	Manes.04G078300.1	7.39/34.64	16.61	4	TPI
82	Fructose-bisphosphate aldolase 3, chloroplastic	Manes.06G063500.1	8.97/42.91	3.30	1	FBA2
83	Fructose-bisphosphate aldolase 3, chloroplastic	Manes.06G063500.1	8.97/42.91	3.30	1	FBA2
64	ATP-dependent CLP protease	Manes.06G085600.1	5.93/101.05	7.91	7	CLPC1
68	Alcohol dehydrogenase class-P	Manes.18G087400.1	6.44/39.24	14.89	6	ADH1
69	Alcohol dehydrogenase class-P	Manes.18G087400.1	6.44/39.24	14.89	6	ADH1
71	Malate and lactatedehydrogenase	Manes.14G078500.1	8.73/43.53	10.92	4	MDH
74	Triosephosphate isomerase,chloroplastic	Manes.04G078300.1	7.39/34.64	16.61	4	TPI
82	Fructose-bisphosphate aldolase 3, chloroplastic	Manes.06G063500.1	8.97/42.91	3.30	1	FBA2
83	Fructose-bisphosphate aldolase 3, chloroplastic	Manes.06G063500.1	8.97/42.91	3.30	1	FBA2
85	Nitrogen metabolic regulation protein NMR	Manes.09G092700.1	5.49/27.51	18.04	5	AT2G37660
89	ATP-dependent CLP protease	Manes.06G085600.1	5.93/101.05	7.91	7	CLPC1
90	Nitrogen metabolic regulation protein	Manes.09G092700.1	5.49/27.51	6.67	2	AT2G37660
Chaperones						
77	25.3 kDa heat shock protein, chloroplastic	Manes.01G042200.1	8.20/26.26	38.89	12	AT5G51440
78	17.6 kDa class I heatshockprotein 1	Manes.10G020600.1	9.02/18.75	8.48	1	HSP17.6A
87	17.6 kDa class I heatshockprotein 1	Manes.10G020600.1	9.02/18.75	12.73	2	HSP17.6A
88	Small heat-shock protein HSP20 family	Manes.10G020100.1	6.56/17.96	31.01	8	AT2G29500
94	Heat shock 70 kDaprotein 5	Manes.11G067600.1	5.06/72.16	5.50	3	Hsp70b
97	Chaperonin 60 subunit beta 1, chloroplastic	Manes.03G086700.1	5.54/64.92	2.14	1	CPN60B
100	26.5 kDa heatshockprotein, mitochondrial	Manes.04G113600.1	8.64/26.09	19.38	6	AT1G07400
103	17.6 kDa class II heatshockprotein	Manes.16G083600.1	5.85/17.67	15.38	3	HSP17.6II
104	17.6 kDa class I heatshockprotein 1	Manes.02G124600.1	6.29/17.87	10.19	2	HSP17.6A
105	17.6 kDa class I heatshockprotein 1	Manes.10G020600.1	9.02/18.75	20.61	4	HSP17.6A
106	Small heat-shock protein HSP20 family	Manes.15G154400.1	6.53/15.65	33.09	4	AT1G53540
Detoxifying and antioxidant						
84	L-ascorbate peroxidase 2, cytosolic	Manes.04G026800.1	5.19/27.77	34.80	9	APX2
91	L-ascorbate peroxidase 2, cytosolic	Manes.04G026800.1	5.19/27.77	9.60	2	APX2
Structure (2)						

70	DREPP plasma membrane polypeptide	Manes.S071400.1	4.62/22.50	31.70	7	PCAP1
Protein biosynthesis						
65	26S proteasome regulatory subunit T5	Manes.08G117200.1	4.73/47.70	4.96	3	RPT5A
73	20S proteasome subunit alpha 6	Manes.05G157400.1	4.72/29.96	5.49	2	PAF1
75	20S proteasome subunit beta 1	Manes.03G001100.1	5.61/24.99	11.69	2	PBA1
79	protein disulfide-isomerase A6	Manes.05G002000.1	5.40/40.29	10.58	4	UNE5
80	elongation factor 1-gamma	Manes.10G116000.1	6.55/48.42	11.58	5	AT1G57720
95	peptidyl-prolyl cis-trans isomerase FKBP62	Manes.12G050700.1	5.00/62.99	11.23	6	ROF1
73	20S proteasome subunit alpha 6	Manes.05G157400.1	4.72/29.96	5.49	2	PAF1
75	20S proteasome subunit beta 1	Manes.03G001100.1	5.61/24.99	11.69	2	PBA1
Amino acid metabolism						
81	proline iminopeptidase	Manes.07G049300.1	6.33/44.34	7.40	4	PIP
Signal transduction mechanisms						
92	2',3'-cyclic-nucleotide 3'-phosphodiesterase	Manes.17G061800.1	5.09/21.87	7.69	2	AT4G18930
Defense						
66	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	AT2G34990
67	stress-induced-phosphoprotein 1	Manes.01G075100.1	6.46/65.37	15.10	8	Hop3
72	thiamine thiazole synthase	Manes.15G075600.1	6.51/37.63	10.20	3	THI1
86	thiamine thiazole synthase	Manes.15G075600.1	6.51/37.63	7.32	2	THI1
93	allene oxide cyclase	Manes.01G108000.1	9.20/28.05	29.37	7	AOC3
96	RING-H2 finger protein ATL61	Manes.07G090300.1	6.66/25.92	3.51	1	AT2G34990
99	RING-H2 finger protein ATL61	Manes.07G090300.1	7.26/11.40	8.00	1	AT2G34990
Function unknown						
76	Universal stress protein family	Manes.08G082400.1	6.54/18.04	10.56	2	AT3G53990
101	Universal stress protein family	Manes.03G204200.1	8.33/24.27	9.30	2	AT3G17020
102	Universal stress protein family	Manes.03G204200.1	8.33/24.27	9.30	2	AT3G17020

Appendix 5. PPI network with Kmeans clustering and the most biological process involved in QZ1 (A) and SC5 (B).





Biological Process (GO)		
pathway description	*count in gene set*	*false discovery rate*
response to inorganic substance	14	4.58e-11
response to stress	19	3.44e-09
response to metal ion	10	1.16e-08
response to cadmium ion	9	1.93e-08
response to stimulus	22	2.25e-08
response to temperature stimulus	9	8.56e-07
response to osmotic stress	6	0.00392
response to oxidative stress	5	0.0172
small molecule metabolic process	8	0.0274

Biological Process (GO)			
pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0050896	response to stimulus	28	7.7e-14
GO:0006950	response to stress	23	2.21e-13
GO:0009266	response to temperature stimulus	13	3.97e-12
GO:0010035	response to inorganic substance	14	3.1e-11
GO:0046686	response to cadmium ion	9	1.98e-08
GO:0006979	response to oxidative stress	7	0.000142
GO:0009409	response to cold	6	0.000624
GO:0044281	small molecule metabolic process	8	0.023

Appendix 6. Table of correlation

Index	H_2O_2	POD	SOD	CAT	APX	ETH	CaM	ASP	NAD	RAS
H_2O_2	1.000									
POD	-0.756*	1.000								
SOD	-0.733*	0.533	1.000							
CAT	0.483	-0.774*	-0.660*	1.000						
APX	-0.488	0.328	0.648*	-0.472	1.000					
ETH	0.474	-0.752*	-0.519	0.900**	-0.328	1.000				
CaM	0.000	-0.172	0.198	0.117	0.242	0.385	1.000			
ASP	0.221	-0.102	-0.182	-0.284	-0.037	-0.115	0.296	1.000		
NAD	0.219	-0.451	-0.274	0.522	-0.246	0.295	-0.528	-0.285	1.000	
RAS	-0.411	0.129	0.344	-0.133	0.533	-0.084	-0.235	-0.377	0.094	1.000

Appendix 7. Proteins quantification by Bradford

1. Standard

BSA (µl)	0	2.5	5	7.5	10	12.5
Quantity (µg)	0	5	10	15	20	25
Loading buffer (SB) µl	5	5	5	5	5	5
Distilled water (µl)	145	142.5	140	137.5	135	132.5
Bradford reagent (µl)	150	150	150	150	150	150
Total volume (µl)	300	300	300	300	300	300

2. Samples

	Sample 1	Sample 2	Sample 3	Sample 4
Loading Vol (µl)	5	5	5	5
Distilled water (µl)	145	145	145	145
Bradford reagent (µl)	150	150	150	150
Total volume (µl)	300	300	300	300

Appendix 8: SDS-PAGE for small gels

1. Composition of 12% of SDS-PAGE Resolving gel for small gels

Solution components (12 %)	Component volume (ml) per gel mold volume of						
	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
ddH_2O	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% acrylamide	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH. 8.8)	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10 % Sodium dodecylsulfate (SDS)	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% Ammonium persulfate (APS)	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.006	0.008	0.01	0.012	0.016	0.02

2. Composition of 5 % of SDS-PAGE Stacking gel for small gels

Solution components (12 %)	Component volume (ml) per gel mold volume of						
	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
ddH_2O	1.41	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH. 6.8)	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10 % Sodium dodecylsulfate (SDS)	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% Ammonium persulfate (APS)	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Appendix 7. Composition of 12% of Acrylamide gel for 2D electrophoresis: 4 gels (150 ml)

30 % acrylamide	6.5 ml
40 % bisacrylamide	52 ml
1 M Tris HCl (pH. 8.8)	56.3 ml
H_2O (distilled water)	32.25 ml
10 % Sodium dodecylsulfate (SDS)	1.5 ml
10% Ammonium persulfate (APS)	1.5 ml
TEMED	60 µl

Appendix 3.Overview of cassava growing in the field (A), the storage conditions of cassava storage roots after harvest (B and C), and the experimental design in the pots (D).

PUBLICATIONS

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Djabou M.A.S., Yuling Q., Boudjeko T., Figueiredo P.G., An F., An F., Luiz J.C.B. Carvalho, Omokolo N.D., Li K., Niemenak N., Chen S., 2018. Effects of calcium and magnesium fertilization on antioxidant activities during cassava postharvest physiological deterioration. *Crop Sci.* 58:1–8. doi: 10.2135/cropsci2017.09.0526.

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REVIEW

Cassava postharvest physiological deterioration: a complex phenomenon involving calcium signaling, reactive oxygen species and programmed cell death

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Abstract Postharvest physiological deterioration (PPD) of cassava (*Manihot esculenta*) storage roots is a complex physiological and biochemical process which involve many regulatory networks linked with specific proteins modulation and signaling transduction pathways. However, it is poorly understood regarding biological regulation, and the interactions among protein groups and signals to determine PPD syndrome in cassava storage roots. This review sheds some light on the possible molecular mechanisms involved in reactive oxygen species (ROS), calcium signaling transduction, and programmed cell death (PCD) in cassava PPD syndrome. A model for predicting crosstalk among calcium signaling, ROS and PCD is suggested to fine-tune PPD syndrome. This would clues to cassava molecular breeding to alleviate the PPD effects on the shelf-life.

Keywords *Manihot esculenta* · Postharvest physiological deterioration · Calcium signaling · ROS · Programmed cell death · Crosstalk

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Introduction

Cassava (*Manihot esculenta* Crantz) is a vegetative propagated shrub belonging to the Euphorbiaceae family. In the tropics, where it is a major staple food crop, cassava is the 4th most important source of calories (Bradbury 1988). Resilience to drought and disease and tolerance to low-soil fertility enable it to grow well under a wide range of climatic conditions, where few crops could survive without costly external inputs. Despite these agronomic advantages, cassava storage roots (CSR) are far more perishable after harvest compared to other storage root and tuber crops, such as sweet potato, true yam, corn and potato. Therefore, cassava is range as a sensitive species of postharvest deterioration (An et al. 2012). The rapid deterioration of CSR significantly shortens its shelf-life for fresh consumption and impacts transportation and potential for income generation (Westby 2002; Iyer et al. 2010). This phenomenon is known as postharvest physiological deterioration (PPD). Estimated losses reach up to 8, 10, and 29% in Asia, Latin America and Caribbean, and Africa, respectively (FAO 2000). Root damage during harvest alters the equilibrium of natural physiological process of the exposed cells and subsequently their oxidative burst. Since PPD is a complex biological phenomenon, it is expected to involve early events (Buschmann et al. 2000a) as the observed dark strips of vessels due to oxidation of cell components (Apostol et al. 1989; Reilly et al. 2004) as tissue wounding reaction (Beeching et al. 2002). Later on, deterioration of cell allows microorganism growth.

This review summarized the current knowledge on oxidative events, participation of calcium signaling events and programmed cell death in association with PPD syndrome. An exploratory model was proposed for prediction of crosstalk among calcium (Ca^{2+}) signaling, reactive

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oxygen species (ROS) production and scavenge, and apoptosis. The model could be used to predict PPD syndrome.

Morphological and histological changes of storage root due to exposure of root cells to ambient air

Cassava storage roots are far more perishable than other staple food crops. Subsistence and commercial utilization of cassava are affected by its short shelf-life due to a rapid postharvest physiological deterioration process (Westby et al. 2002). The duration of cassava shelf-life depends on the cultivars, harvest practices and handing, and storage conditions. However, PPD commonly occurs within 72 h after harvest and renders the root unpalatable (Buschmann et al. 2000b; Iyer et al. 2010). Formation and growth of cassava storage root are resulted from the swelling of primary roots due to the secondary growth which forms three tissue layers. The first, second and third tissue layers are composed of phellogen and phelloderm, cambium and phloem, and secondary vessels and store parenchyma cells, respectively (De Souza et al. 2006). PPD syndrome is first observed in the third tissue layer (the edible part of CSR) by visualization of dark colors changes in a cross section of a storage root (Fig. 1). This is associated with formation of the so-called dark strip of xylem vessels. Microscopic tissue sectioning observation (Fig. 2) shows that the dark strip is associated with the formation of tylose occlusions inside the secondary vessels and may be the oxidized candidate cell structure since the xylem is a dead cell. PPD considered as primary deterioration, is initiated by mechanical damage which occurs during harvesting. This is known as PPD. The visible signs are black blue to black discoloration or vascular streaking which begins at the broken or cut surfaces and subsequently spreads to the adjacent storage parenchyma and the stored starch undergoes structural changes. This primary deterioration is characterized by physiological and biochemical changes, and does not involve microorganisms (Noon and Booth 1977). Therefore, it is a biological active process and distinct from the secondary deterioration caused by microbial infection leading to softening of the root tissue (Sánchez et al. 2013; García et al. 2013). The later events of PPD syndrome involve the formation of callus from the exposed cells as healing process proceeds in addition to cell death. Difference in susceptibility to PPD amount cassava varieties has been reported (Aristizábal and Sánchez 2007; Morante et al. 2010a, b; Salcedo et al. 2010). In addition, an inversely correlation between light yellow parenchyma color of roots associated to high amount of carotenoid content and delaying of PPD was reported by Chavez et al. (2007). In fact, it is suggested that cassava wound repair can occur if the root remains attached to the plant (Plumbey and Rickard 1991; Reilly et al. 2004). Then, the problem takes place once the root is detached. Although the wound response is present, the healing process and the subsequent down-regulation of the signals are insufficient or too low (Salcedo and Siritunga 2011). It is thought that at some point during evolution cassava roots lost their efficiency in wound repair (Reilly et al. 2004). In addition, extent of PPD damage and speed of symptom development in roots was also associated to the genotypic as well as the environmental conditions increasing the complexity of the phenomenon (Reilly et al. 2004).

In the level of histology, the increase of oxidative stress caused by wounding may induce a metachromatic reaction observed by staining with toluidine blue indicating the presence of acidic polysaccharides in the cell wall and around starch granules (Uarrota et al. 2014). Acidic polysaccharides may act as reducing PPD stress. In addition, the degradation of starch granules during PPD evolution was clearly observed after staining with periodic

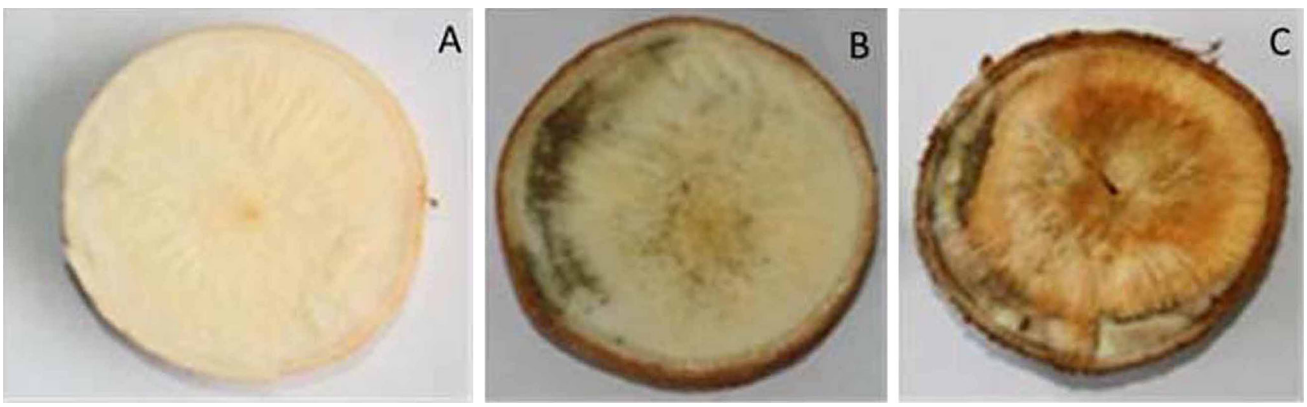

Fig. 1 Cross sections of cassava freshly harvested storage roots exposed to air for zero (**a**), three (**b**), and ten days (**c**). Blue–black/brown discolorations recognized as a visual sign of PPD are clearly observed at 3 days. The discolorations continued until ten days following by the softening of the roots given place to development of microorganisms (secondary deterioration)

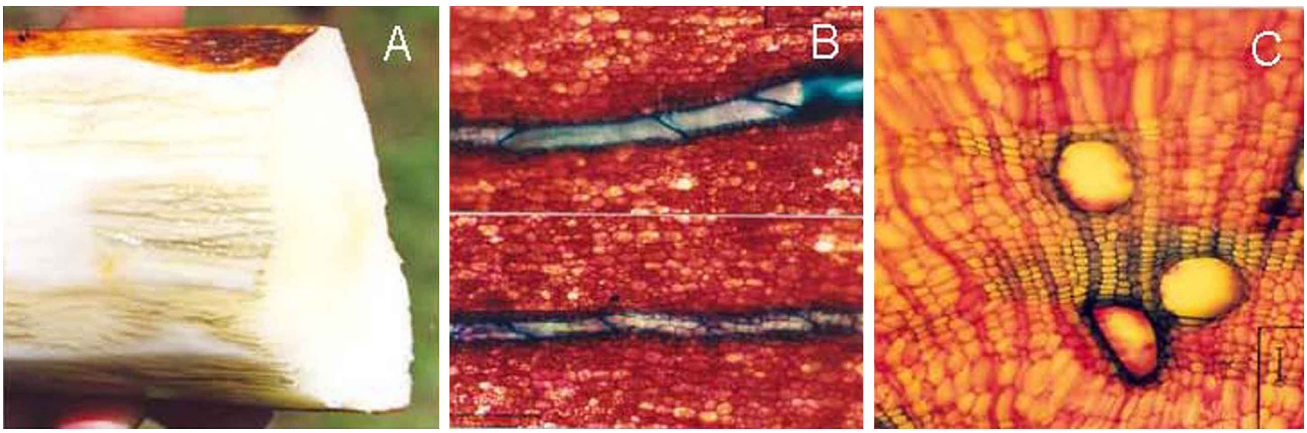

Fig. 2 Microscopic observation of cassava storage roots undergoing PPD. **a** Dark strip formation in vessels of tissue system III resulting from oxidative process. **b** Close longitudinal cutting showing detailed vessels fully observable of tylose occlusion. **c** Close-up of tylose occlusion formation inside secondary vessels was observed

acid Schiff (Uarrota and Maraschin 2015). Previously, Canto et al. (2013) did not observe alterations in the primary xylem after storage during PPD, suggesting that PPD process happens mainly in the peripheric region of the root without affecting the vascular cambium (primary xylem).

Biochemical features of PPD syndrome

Several studies have been performed to investigate the biochemical feature and molecular events related to PPD syndrome (Reilly et al. 2004, 2007; Owiti et al. 2011). Changes in response to cell damages after CSR harvest included accumulation of fluorescent compounds and secondary metabolites (Buschmann et al. 2000a, b), decrease of starch content in the profit of sugar (Sánchez et al. 2013); the main soluble sugars found by high performance light chromatography were raffinose, sucrose, fructose, and glucose (Uarrota et al. 2014); increase in cell respiration and enzymatic activities including regulation of ROS synthetase (Xu et al. 2013; Zidenga et al. 2012) as well as phenylammonia-lyase. PPD has been found to be correlated with the content of β-carotene since Morante et al. (2010a, b) observed a less susceptibility to PPD for the genotypes with high level of β-carotene compared to those with less level of β-carotene. PPD development was also associated to change in gene expression where many genes get altered during the process (Huang et al. 2001; Cortés et al. 2002). Zidenga et al. (2012) suggested that mechanical damage that occurs during harvesting is initiated cyanogenesis by bringing linamarin and linamarase in contact and subsequently the release of cyanide. The cyanide (HCN) released inhibits mitochondrial respiration by inhibiting complex IV in the mitochondrial electron transfer chain. Inhibition of complex IV causes a burst of ROS production at complexes I and III. It is this oxidative burst that causes PPD. Recent studies about metabolome analyses showed increases in carotenoids, flavonoids, anthocyanins, phen-olics, reactive scavenging species, and enzymes (superoxide dismutase family, hydrogen peroxide, and catalase) under PPD (Uarrota et al. 2014). In the same study, a positive correlation was observed between PPD and antocyanins and flavonoids while a negative correlation was observed with phenolics compounds and carotenoids. Several proteins were up- or down-regulated during the process (Owiti et al. 2011). Plant responds to various stresses such as pathogen attacks, harsh growing conditions, wounding by inducing the expression of a large number of genes that encode diverse proteins. The response of plant tissues to wounding has been studied for a very long time and more recently it has been demonstrated that several genes are wound-inducible. The proteomic approach is a very powerful tool to study the proteins patterns that result from differential gene expression as well as from post-translational modifications (Gray and Heath 2005; Lee et al. 2007; Timperio et al. 2008). Proteome profile of CSR at harvest and during PPD onset revealed 300 proteins showing significant abundance regulation during PPD (Vanderschuren et al. 2014). The identified proteins were mostly associated with oxidative stress, phenylpropanoid biosynthesis (including scopoletin), the glutathione cycle, fatty acid-oxidation, folate transformation, and the sulfate reduction II pathway in which glutathione peroxidase was identified as a possible candidate for reducing PPD. All the information clearly showed that PPD is an active and complex phenomenon involving the possible interaction/crosstalk between the pathways.

The role of ROS in plants

Plants and other living organisms in the oxidizing environment constantly produce ROS from chloroplasts, mitochondria, peroxisomes and other subcellular organelles because of biological metabolic processes such as photosynthesis and respiration. Overproduction of ROS is triggered by a pathogen attack and stress exerted by environmental conditions. ROS or active oxygen species (AOS) or reactive oxygen intermediates (ROI) include the superoxide radical (O_2^-), hydroxyl radical (OH·), hydroperoxyl radical ($HO_2^·$), hydrogen peroxide (H_2O_2), alkoxy radical (RO·), peroxy radical (ROO·) and singlet oxygen ($^1O_2$) (Vellosillo et al. 2010). Plants have either enzymatic or non-enzymatic defense systems to scavenge ROS toxicity and protect against oxidative damage (Vranova and Inze 2002). The enzymatic scavenging system includes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase and glutathione reductase while the non-enzymatic scavenging system includes ascorbic acid (AsA), glutathione and proline. In response to environmental stresses, plants produce increased levels of ROS and SOD which provides the first line of defense is, thus, important in plant stress tolerance (Apel and Hirt 2004). The increase of glutathione reductase activity confers stress tolerance and has the ability to alter the redox state of important components of the electron transport chain (Foyer and Noctor 2011). The organism equilibrium is determined by the ROS homeostasis, including ROS producing in combination with its scavenging. Contact with invading microbe as well as wounding in many plants elevates plant plasma membrane-bound NADPH activity, leading to the rapid accumulation of ROS including hydrogen peroxide (H_2O_2), which diffuses into cells and activates defenses (Apel and Hirt 2004). Increasing evidence indicates that ROS has a dual role as cytotoxic damage, and as a signaling molecule involved in the regulation of response during pathogen attack or various physiological processes (Foyer and Noctor 2005; Mittler et al. 2004; Corpas et al. 2015). The accumulation of ROS in the site of infection during hypersensitive response may contribute to limit the spread of the pathogens or induce signals for establishment of further defense (Mur et al. 2008). Feedback, or feed-forward interaction between ROS and many hormones such as ethylene, jasmonic acid, abscisic acid, gibberellic acid and salicylic acid in response to biotic and abiotic stresses has also been presented (Mittler et al. 2004; Mur et al. 2008). Since ROS is ideally suited to act as signaling molecules because of its small size and ability to diffuse over short distances (Mittler et al. 2004; Pei et al. 2000; Overmyer et al. 2000), it is not surprisingly that numerous researchers paid close attention to the oxidative burst in damage cassava storage roots.

Features of ROS associated to PPD syndrome

Cassava storage root is inevitable predisposed to cell damage due to mechanical injury caused by harvest. Therefore, constitutive defense mechanisms are activated upon harvest as in intact plant subjected to abiotic stress. The response of cell damage produces ROS including superoxide anion and hydrogen peroxide with both local and systemic action (Apel and Hirt 2004). In the case of CSR, studies have been focused on the ROS production and their scavenging induced by cell damage during PPD syndrome (Xu et al. 2013; Zidenga et al. 2012). A burst of superoxide anion and the increased activities of ROS scavenging enzymes such as superoxide dismutase and catalase were observed after oxidative burst caused by harvest damage (Iyer et al. 2010; Reilly et al. 2007). The shortage of scavenge of the overproduced ROS results in accelerate PPD. Previously, Zidenga et al. (2012) suggested that the cyanide produce immediately when cassava is mechanically damaged may trigger the oxidative burst responsible of PPD onset. In fact cassava produces potentially toxic levels of cyanogenic glucosides which break down to release cyanide following cellular disruption and release cyanogens from the vacuole (Siritunga and Sayre 2003; Siritunga et al. 2004). Therefore, increasing the production of ROS scavenges is presented as a key regulator of PPD. The induction of the overexpression of mitochondrial alternative oxidase (AOX) in transgenic cassava was followed by extending of the shelf-life of storage root (SR) for two weeks (Zidenga et al. 2012). Parallel experiments showed that the co-overexpression of Me/Cu/Zn-SOD and MeCAT1 induced in transgenic cassava could also enhance scavenging ROS in CSR after tissue damage (Xu et al. 2013). In addition, the regulation of the activity of glutathione-associated enzymes, including glutathione reductases, glutaredoxins, and glutathione S-transferases have been reported as potential modulator for the onset of PPD (Vanderschuren et al. 2014). Recently, increase in APX, GPX, AsA, and CAT subsequently detoxify the hydrogen peroxide was recently reported in cassava roots during PPD (Uarrota and Maraschin 2015). Taken together these observations strongly support the implication of ROS during PPD process and present many pathways which may trigger their production.

Features of calcium signaling associated to PPD syndrome

Plant cells could trigger their elaborate defense systems while perceiving signal messenger coming from their environment, and eventually produce proper physiological responses. In plants, calcium ion (Ca^{2+}) is a ubiquitous second messenger molecule coupling with physiological

response to external and developmental signals (Reddy and Reddy 2004). It plays a key role in the integrity of the cell wall and the membrane systems and acts as an intracellular regulator in many aspects of plant growth and development including stress responses (White and Breadly 2003). Changes in cytosolic free Ca^{2+} concentration were observed during transduction of abiotic stimuli including high light, low and high temperature, hyperosmotic and oxidative stresses and also in the biotic stimuli including fungal elicitors and nodulation factors (Rudd and Franklin-Tong 2001). These Ca^{2+} signatures are recognized by several types of Ca^{2+}-sensor proteins. Ca^{2+}-binding sensory proteins include calmodulins (CaMs), calmodulin-like proteins, calcineurin B-like proteins (CBL), and Ca^{2+}-dependent protein kinases (CDPKs) (Sanders et al. 2002; Snedden and Fromm 2003).

There is an ample evidence to show the involvement of Ca^{2+} signaling in abiotic stress responses. Intracellular changes of Ca^{2+} levels were reported as the first response to diverse abiotic signals and biotic stresses (Beneloujaephajri et al. 2013; Ranty et al. 2006). Numerous data supported that Ca^{2+} and its sensor proteins, CaM, CDPKs and CBLs and downstream elements played an important role in plant adaptation to abiotic stress. Several studies indicated Ca^{2+}-CaM complex triggered the activation of target proteins to produce cellular physiological responses (Bouché et al. 2005; Nookaraju et al. 2011). An example was found in tobacco stressed by wounding, in which three CaM isoforms at different Ca^{2+} concentrations activate the target enzymes NO synthase and NAD kinase (Karita et al. 2004). Similar effect of Ca^{2+} on NO or NAD kinase during PPD cannot be ruled out.

In the case of cell injuries such as the one occurring in PPD syndrome of CSR, a significant up-regulation of CaM observed at the early events of PPD (Owiti et al. 2011) can be associated with a rapid increase in Ca^{2+} which resulted in the oxidative burst as observed in *Arabidopsis thaliana* (Kaplan et al. 2006). This phenomenon was similar to the expression of heat shock proteins under heat stress attributed to the accumulation of CaM in plants (Liu et al. 2003; Zhang et al. 2009). Ca^{2+} seems to be involved in signaling transduction to trigger the activation of programed cell death (PCD) (Zhang et al. 2009; Hoeberichts and Woltering 2002; Levine et al. 1996). It was suggested that CDPKs, in response to elevated cytosolic Ca^{2+} levels, could induce NADPH oxidase activity which is one of the key points of an oxidative burst and PCD process (Hoeberichts and Woltering 2002). In roots of *Arabidopsis thaliana*, mechanical stimulation triggered the rapid and transient increase of cytoplasmic Ca^{2+} concentration; this mechanical stimulation likewise elicits apoplastic ROS production with the same kinetics (Monshausen et al. 2009).

Programmed cell death features in plants

Programmed cell death (PCD) is an active and genetically controlled process aimed at eliminating redundant or harmful cells from healthy tissues during the life cycle of the multicellular organism. It is a highly regulated cellular suicide process and essential for growth and survival in eukaryotes. The biochemical and morphological hallmarks of PCD recorded to be common to plants and animals are compaction and shrinkage of the cytoplasm and nucleus, DNA and nuclear fragmentation into large (50–300 kb) and subsequently small nucleosomal fragment (200 bp, DNA laddering) and calcium influx (De Jong et al. 2000; Wang et al. 1996). PCD was reported in many biological processes in plants including embryogenesis, flower petal senescence and vegetative development such as xylogenesis, and parenchyma formation. Various stress conditions such as cold, nutrient deprivation, salts or D-mannose stresses, pathogen or pathogen toxin (Wang et al. 1996) have been found to induced DNA laddering and subsequently death in plants. PCD is also a process involving new protein synthesis and distinct from necrosis, cell death caused by extrinsic factors, and independent of specific genetic control and cellular activities (Van Breusegem and Dat 2006).

The core component of the apoptotic machinery found in animals is a proteolytic cascade involving a family of cysteine proteases named caspases, which specifically cleave at aspartic acid residues of their substrates (Shi 2002; Zhang et al. 2009). This cleavage may be involved in the detachment of the dying cell making it easy to ingest (Shi 2002). Even if no homologs of animal's caspases have been identified in plants, accumulated evidence in the recent years suggests caspases-like activity playing a pivotal role in plant PCD (He et al. 2008; Woltering et al. 2002). The expression of caspase-3-like has been detected from barley embryonic suspension cells and TMV-infected tobacco leaves (Lam and Zhang 2012). Parallel experiments were performed to show the cleavage of Poly (ADP Ribose) polymerase, a specific substrate of caspase-3 during the apoptotic process in animal by extracting from fungus-infected cowpea. Comparing to the animal apoptotic pathway, this degradation was dependent on the release of cytochrome c into the cytosol and could be inhibited by specifics caspase 3 inhibitors. Using the synthetic fluorogenic caspase-1 substrate (Ac-DEVD-AMC), caspase-like activity was also detected during UV- or heat shock-induced apoptosis of plant cells, and this could be inhibited by caspase-3 inhibitors but not by caspase-unrelated protease (Vacca et al. 2006). All of these experiments evidenced that the functional caspase-like proteolytic activity was detected and its functional involvement in plant cells undergoing PCD.

Recently, the metacaspase, a homolog of caspase, implicated in plant PCD was also detected (Koonin and Aravind 2002; Uren et al. 2000). Even if it was unable to cleave caspase substrates, it was found to be involved in PCD in *Arabidopsis* and yeast (He et al. 2008; Madeo et al. 2002). The level of cell death was increased in transgenic plant by overexpressing some *Arabidopsis* metacaspases (AtMC4, AtMC5) upon treatment with ROS inducing agents and the loss of those genes resulted in a decrease or delay of cell death (Lam and Zhang 2012). PCD following oxidative burst has been demonstrated in many plants (Woltering et al. 2002; Vacca et al. 2006; Danon et al. 2000). Several calcium-binding proteins were induced in response to the stresses. CDPKs, in response to elevated cytosolic Ca^{2+} level, can induce NADPH oxidase, which catalyzes the production of ROS such as superoxides (Sagi and Fluhr 2006). Even if the oxidative burst induced by wound in cassava storage root after harvest has been studied, the linkages between oxidative burst and PCD are poorly understood. In fact, high concentrations of ROS are highly harmful to organisms, and when the symptoms persist, irreversible damage may occur to the cells, resulting in loss of physiological capacity and eventual cell death.

Regarding cassava storage root PPD, PCD process was marked by the down-regulation of cysteine protease which may enhance protease activity leading to PCD as well as the down regulation of many peptides of the phospholipase D α-group of the enzymes during the early and late PPD times points were also presented (Owiti et al. 2011). However, further studies need to be carried out to describe how this pathway works in a PPD process. Biochemical detection of caspase-3-like activity and its inhibitors in plant PCD can be useful to elucidate the mechanism of PPD. Apart from this further functional characterization, understanding of the mechanism on which this pathway interacts with other pathways such as Ca^{2+} and ROS like mentioned in others plants during stress can increase the knowledge about the PPD process in cassava.

Crosstalk among Ca^{2+} signaling, ROS and PCD integration network in cassava to fine-tune PPD syndrome

In cassava, the events that trigger the production of ROS relevant to PPD under abiotic and biotic stresses are poorly understood. Information is rising about the implication of Ca^{2+} in the release of ROS in wound-induced resistance in plant (Beneloujaephajri et al. 2013; Sagi and Fluhr 2006; Bargmann and Munnik 2006). In response to wound induced by *Botrytis cinerea*, it was found that non-wounded leaves of *Arabidopsis thaliana* treated with Ca^{2+} inhibitors were more susceptible to pathogen, suggesting the importance of Ca^{2+} in the induction of basic resistance (Beneloujaephajri et al. 2013). Interestingly, a co-localization of the changes in Ca^{2+} and a burst of ROS were observed after pathogenic or environmental stresses were exerted (Monshausen et al. 2009; Ranf et al. 2011). The transient changes in Ca^{2+} concentration were detected for a few seconds after wounding, followed by the increase of ROS concentration in *A. thaliana* leaves after wounding (Beneloujaephajri et al. 2013). Indeed, in plants a positive feedback mechanism involving NADPH oxidase, ROS and Ca^{2+} was (Sagi and Fluhr 2006). Various Ca^{2+} binding proteins such as CDPKs in response to elevated cytosolic Ca^{2+} levels could induce NADPH oxidase activity, leading to the increase of ROS under stresses (Sagi and Fluhr 2006), and potentially trigger other downstream responses such as apoptosis. CDPK6, a Ca^{2+} related protein of *A. thaliana*, was reported to be involved in the regulation of ROS (Boudsocq and Sheen 2010). An ortholog of this protein found in potato was involved in the phosphorylation of the membrane-bound NADPH oxido-reductase RBOH-D to stimulate its activity for ROS production in response to pathogen attacks (Kobayashi et al. 2007). However, ROS production from the initial Ca^{2+}-dependent activation can subsequently trigger a larger Ca^{2+} influx (Pei et al. 2000; Kadota et al. 2004). Thus, it is possible that Ca^{2+} cytosolic elevation is crucial for ROS accumulation, which in turn contributes to Ca^{2+} signaling in positive feedback loop (Sagi and Fluhr 2006; Bargmann and Munnik 2006). The crosstalk between the second messengers Ca^{2+} and ROS is now recognized in the modulation of the activity of specific proteins that act at the nuclear level to control the expression of determinate defense genes. In plants, Ca^{2+} fluxes were also recognized as an important signaling mediator of the activation of PCD and NADPH oxidase complexes may be stimulated by caspase (-like) activity (Hoeberichts and Woltering 2002). They may trigger the reduction of oxygen to $^{\cdot}O_2^-$ followed by dismutation of $^{\cdot}O_2^-$ to H_2O_2. Subsequently, caspase inhibitors can act to prevent cell death and the preceding accumulation of ROS. Increasing evidence indicated that an interaction may exist between elevated cytosolic Ca^{2+}, accumulation of ROS and subsequent cell damage. Zidenga et al. (2012) showed that the accumulation of ROS during PPD can be the consequence of cyanide released during cyanogenesis and did not observe a substantial reduction of ROS production with diphenyl iodonium chloride an inhibitor of membrane NADPH oxidase. The up-regulation of CaM one of the most important calcium sensors proteins coupled to the increase of cysteine protease during early PPD suggest the implication of others pathways on PPD process recognize as an active process. Growth regulators can interact in coordination under various stress conditions in order to control the downstream stress response or with others pathways to fine-tune the defenses (Verma et al.

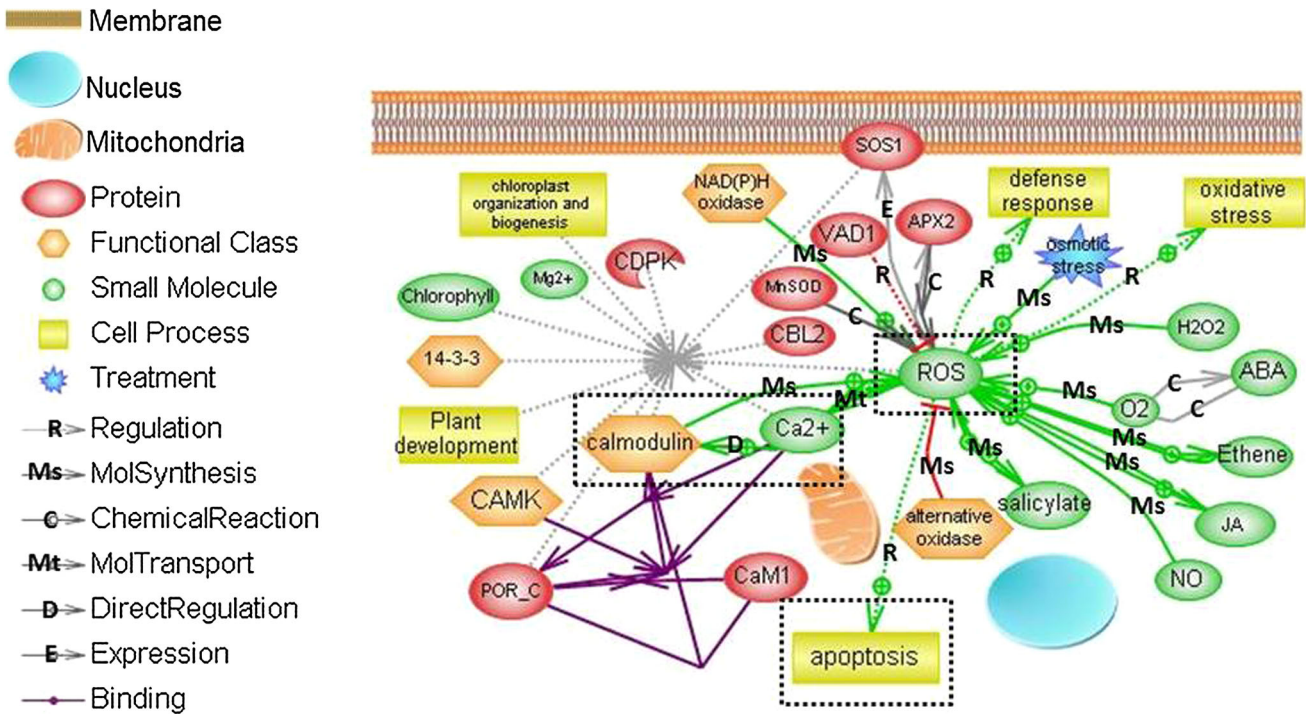

Fig. 3 Putative crosstalk among Ca^{2+}, ROS and apoptosis in cassava storage roots during PPD. The network was generated with Pathway Studio software default. Regulation is marked as an *arrow with R*, MolSynthesis as an *arrow with Ms*, Chemical Reaction as an *arrow with C*, MolTransport as an *arrow with Mt*, Direct Regulation as an *arrow with D*, Expression as an *arrow with E* and Binding as an *arrow without any marks*

2016). The response of plants imposed by abiotic stress such as wound in cassava roots could be mainly controlled by growth regulators even if it remains poorly understood. Taking all information together PPD induced in CSR seems to reflect an integrative crosstalk between signaling molecules, including Ca^{2+}-CaM/CDPK, ROS, hormones such as jasmonic acid, salicylic acid, ethylene, gibberellin acid, cysteine protease and still unknown members of PCD pathway.

Based on the data described above, we would propose a mechanism of PPD in cassava. All impact factors associated with PPD were used to generate a biological interaction network using Pathway Studio. This network includes cell process, functional class, protein, small molecule and osmotic stress treatment. The relationship among chemical reaction, expression, regulation, and binding was established (Fig. 3) responding to defense response and oxidative stresses caused from wound damage and microbial infection. Mechanical damage causes by wound induces oxidative burst and stimulates Ca^{2+} influx. This flux is sensed by Ca^{2+} binding proteins such as CaM which has several Ca^{2+}-dependent in vitro activities. It is involved in regulating various cellular and biochemical processes, such as PPD. Ca^{2+} signaling was essential for activating the NO and ROS production induced by mechanical damage. Therefore, Ca^{2+}-CaM complex is a key center to regulate ROS homeostasis. Other key points are ROS and apoptosis. ROS are responsible for mediating cellular defense responses in cassava. The production of ROS, mediated through NADPH oxidase, increases under stress conditions such as wound damage, causing oxidative burst and impairment of normal metabolism. ROS are also key elements in cassava PCD which is essential for microbial infection. In the model, a negative control on cell death-dependent ROS accumulation, promoted by SA and ethylene is limited by jasmonic acid (Fig. 3). In addition, CAT and SOD can interfere with hormones to scavenge the flux of ROS produced restore the equilibrium and PPD can be delayed. In contrary, the increasing production of ROS can trigger both a second peak of $Ca^{2+}{}_{cyt}$ which interfere with mitochondrion and the production of MAPK. MAPK can act with heat shock proteins and others cell death pathway inducing cell wall degradation and subsequently PPD and PCD. All together, we consider PPD in cassava as a complex process in which the crosstalk among Ca^{2+} signaling, ROS and PCD is integrated to fine-tune.

Conclusion

Cassava storage roots is an essential part of the daily diet for millions people and income generation. However, its potential as food and industrial crop is still limited by PPD.

The study of PPD mechanism response is important in increasing our molecular knowledge and for their potential in developing an effective approach to control PPD losses. The recent data revealed the implication of Ca^{2+}-CaM, ROS and PCD pathways in response to PPD. The crosstalk between these pathways during PPD process is proposed in this review. However, more functional studies are needed to better understand when, where and how the proteins involved in these pathways talk or interact to fine-tune the PPD response. Progress in proteomic, cell image technology and molecular genetic analysis will be helpful to drive future research and provide a worthwhile approach to control PPD in cassava storage roots.

Author contribution statement SC made the major contributions to this study in the conception, design, drafting part of manuscript, and final revision. ASMD contributed to part of the conception and design, and drafting manuscript. LJCBC worked at Fig. 2 and revision of manuscript. QXL contributed to part of conception, and critical revision of manuscript. NN worked at part of design and critical revision of manuscript. All authors read and approved the final manuscript.

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RESEARCH

Effects of Calcium and Magnesium Fertilization on Antioxidant Activities during Cassava Postharvest Physiological Deterioration

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ABSTRACT

The current research addressed the effect of fertilizers containing Ca^{2+} and Mg^{2+} on cassava (*Manihot esculenta* Crantz) storage roots (CSRs) during postharvest physiological deterioration (PPD). Cassava PPD tolerant and susceptible genotypes were grown in pots and treated with fertilizers containing Ca^{2+} or Mg^{2+}. Eight months after planting, CSRs were harvested and stored at room temperature for 30 d. Morphological changes, antioxidant activities, mineral content, and starch content were determined. The results indicated that application of Ca^{2+} and Mg^{2+} fertilizers delayed PPD for >10 d in the susceptible genotype (SC5), but Mg^{2+} fertilizer elevated PPD in the tolerant genotype (QZ1). The Ca^{2+} fertilizer significantly increased fresh weight. A significant decrease in starch content in CSRs of SC5 treated with Mg^{2+} fertilizer was observed. The Mg^{2+} fertilizer slightly enhanced Ca^{2+}, Mg^{2+}, and K^{+} contents in both genotypes. In addition, Ca^{2+} fertilization led to a significant increase in H_2O_2 content and subsequent elevation of activities of catalase and peroxidase in both genotypes, especially at harvest. Our findings suggest that soil fertilization with fertilizers containing Ca^{2+} and Mg^{2+} may play a distinct role in PPD delay in CSRs.

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Abbreviations: APX, ascorbate peroxidase; CSR, cassava storage root; DAH, days after harvest; POD, peroxidase; PPD, postharvest physiological deterioration; ROS, reactive oxygen species; SOD, superoxide dismutase.

Cassava (*Manihot esculenta* Crantz) postharvest physiological deterioration (PPD) is the major constraint for cassava storage root (CSR) shelf life duration and directly affects cassava production and its value as a raw material for industrial purposes. Symptoms of PPD include blue-black discolorations, which usually appear on storage roots within 24 to 72 h of harvest, thereby rendering the roots unpalatable and unmarketable (Beeching et al., 1998; Reilly et al., 2004). Postharvest physiological deterioration is also a complex process involving changes not only in enzymatic and protein activities, but also in gene expression and metabolites (Reilly et al., 2004; Iyer et al., 2010; Xu et al., 2013; Vanderschuren et al., 2014; Uarrota and Maraschin, 2015; Zainuddin et al., 2017). In addition, other factors such as genotype, environmental conditions, and soil preparation

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and composition are of high importance when evaluating PPD (Kawano and Rojanaridpiched, 1983; Hirose, 1984).

Calcium (Ca^{2+}) and magnesium (Mg^{2+}) are two cationic mineral nutrients that have either structural, physiological, or biochemical functions in growth and stress tolerance in plants. Calcium is an essential component of the plant cell wall that provides mechanical strength for normal transport and retention of other elements (Cakmak and Marschner, 1992). It has been shown to influence protein phosphorylation in plants by affecting the Ca^{2+}–binding modulator proteins such as calmodulin and protein kinases (Budde and Chollet, 1988). When plants are exposed to a stressful situation, a quick rise in cytosolic Ca^{2+} takes place, which is a key factor in the expression of stress-responsive genes and physiological responses of plant cells to such stressful conditions as extreme temperatures, drought, salinity, and pathogenic attack. The changes in cytosolic Ca^{2+} concentrations are often closely related to the severity of the stress. A colocalization of the changes in Ca^{2+} and a burst of reactive oxygen species (ROS) were observed after pathogenic or environmental stress (Ranf et al., 2011). The influence of fertilizers containing Ca^{2+} and Mg^{2+} on the delay of fruit ripening (Aghofack-Nguemezi and Dassie, 2007) and stress tolerance (Jiang and Huang, 2001) was reported in many plants.

Tuber Ca^{2+} concentration may increase through fertilization (Ozgen and Palta, 2004). In potato (*Solanum tuberosum* L.) tubers, Ca^{2+} application can increase tuber Ca^{2+} concentration and reduce internal brown spots (Collier et al., 1978). An effective postharvest control of bacterial rot in potato was achieved by increasing tuber Ca^{2+} concentration through soil fertilization (Conway et al., 1992) and postharvest vacuum infiltration with $CaSO_4$ or $Ca(NO_3)_2$.

Magnesium is one of the most important nutrients involved in plant growth and development. It plays a fundamental role in phloem export of photosynthetic substances from photosynthetic organs to the roots (Esfandiari et al., 2010). Plants under low Mg^{2+} supply are not only very sensitive to high light intensity and heat stress but easily become chlorotic and necrotic, probably due to extensive production of ROS (Hermans et al., 2010; Kobayashi et al., 2013). In plants, when the intracellular Ca^{2+} concentration is submicromolar, the concentration of closely related divalent Mg^{2+} is millimolar (Aghofack-Nguemezi and Tatchago, 2010). It has been observed that the regulation of root hair development under different Mg levels was Ca-signaling-dependent (Niu et al., 2014). Interestingly, Mg-defective root hair produces ROS that stimulate hyperpolarization-activated Ca^{2+} channels, leading to a root-hair-tip-focused Ca^{2+} gradient (Foreman et al., 2003; Niu et al., 2014). Magnesium also plays an important role in activating some enzymes such as ribulose 1,5-bisphosphate (RuBP)-carboxylase and ATPases (Esfandiari et al., 2010). It has been suggested that plant cells compensate for low Ca^{2+} by increasing Mg transport activity, whereas high Ca^{2+} inhibits Mg^{2+} availability to plants (Hermans et al., 2010).

Changes in response to cell damage after harvest include a decrease in starch content and an elevation in cellular respiration and enzymatic activities including regulation of ROS synthetase (Zidenga et al., 2012; Xu et al., 2013). The involvement of Ca^{2+}–calmodulin in PPD development in cassava was reported by Owiti et al. (2011). A possible cross talk among Ca^{2+} signaling, ROS, and programmed cell death to fine-tune the PPD process was recently suggested (Djabou et al., 2017). Moreover, supplying Ca^{2+} and Mg^{2+} fertilizer during crop growth may also influence the mineral content of the harvested storage root or tuber (Palta, 2010). Information on whether soil composition and/or fertilization have an effect on mineral concentrations in cassava roots, as well as on PPD development, is lacking. Investigating the effects of Ca^{2+} and Mg^{2+} fertilization on PPD development will not only provide new insight into the control of this phenomenon but will also contribute to elucidating the cascade of events associated with the establishment of PPD in CSRs. The aim of the study was to assess the effect of augmenting the supply of Ca^{2+} and Mg^{2+} by soil fertilization on the antioxidant activities and mineral and starch contents in CSRs during PPD.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The experiment was conducted at the Cassava Germplasm Pool, Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou City, Hainan Province, from July 2014 to February 2015. Stem cuttings of 15-cm length and 20-mm width with five buds taken from cassava genotypes SC5 and QZ1 were used in the experiment. SC5 and QZ1 are considered cassava genotypes susceptible and tolerant to PPD, respectively. The stem cuttings were planted in 20-L (30-cm diam.) plastic pots containing a composite soil collected from the cassava experiment field of the Chinese Academy of Tropical Agricultural Sciences with the following chemical composition: pH 7.26, 43.38 mg P kg^{-1}, 256.5 mg K kg^{-1}, 19.44% C, 1.6205 cmol Ca kg^{-1}, and 1.0712 cmol Mg kg^{-1}. The experiment was performed in the open air. The climate at the experiment site is tropical with two distinct seasons—the dry season (spring and winter) is warm and windy, whereas the wet season (summer and autumn) is hot and humid. The average annual temperature ranges from 24.5 to 25.28°C, and the average annual rainfall is ~2000 or <1000 mm (Gao et al., 1988). Precipitation varies greatly, with distinct dry and wet seasons; 90% of the rain occurs from May to October, and little rain occurs from November to April.

Application of Calcium and Magnesium Fertilizers

The plants were treated with two different fertilizers containing Ca^{2+} and Mg^{2+} from the Sinochem Shandong Fertilizer Company. Treatment 1 consisted of supplying 6.6 g Ca^{2+} fertilizer (23.5% CaO, 11.7% N), whereas Treatment 2 consisted of supplying 22.47 g Mg^{2+} fertilizer (8.9% Mg, 0.6% B + Fe). The control consisted of growing the plants without any of the Ca^{2+} or Mg^{2+} fertilizers. The fertilizers were applied 90 d after planting, based on the recommendation of CIAT (Howeler, 2002). Each treatment consisted of five plants in three repetitions with one plant per pot.

Eight months after planting, CSRs were harvested, washed, and kept in a controlled environment chamber at 25°C and 60 to 80% humidity for PDD evaluation and biochemical analysis. At each time point, harvested tubers were manually peeled with broad-bladed stainless steel knives. Evaluation of PPD was made by cross-sectioning of the middle of at least three CSRs at 0, 3, 6, 15, and 30 d after harvest (DAH). A sliced section of ~2-cm thickness was photographed for visual observation of PPD development. Onset of PPD was determined by the observation of blue, black, or brownish discoloration on the parenchyma, as is characteristic of PPD (Wheatley, 1982; Salcedo et al., 2010). Slices of flesh sample were snap frozen in liquid N and kept at −80°C for future quantification of H_2O_2 content and evaluation of enzymatic activities. Mineral and starch content were determined after sample drying in an oven at 50°C for 5 d.

Mineral Content Quantification

The sample (0.4 g) was accurately weighed from the dried result of the procedure described above and subjected to various heating grades as follows: 150°C for 30 min until it ceased to smoke, 300°C for 30 min, and 550°C for 3 h to obtain a whitish or grayish ash. The ash was dissolved in 5 mL of 37% HCl and heated for 30 min at 140°C. The mixture was transferred into a 100-mL volumetric flask, and the volume was completed to 100 mL with distilled water. This constituted the basic solution.

The mineral content was determined by atomic absorption spectrophotometry according to García and Báez (2012) with a Varian atomic absorption spectrophotometer (AA-7000, Shimadzu) with an air-acetylene flame and wavelengths set to 422.7, 285.2, 766.5, and 589 nm for Ca, Mg, K, and Na, respectively. The samples were aspirated into the flame, and the corresponding absorption of characteristic radiation by the atomic vapor of the element was recorded. Calcium carbonate, $MgSO_4$, KNO_3, and NaCl were used as the respective standards for Ca, Mg, K, and Na. For Na and K determination, 1 mL of the basic solution was dissolved in 9 mL of distilled water; for Ca and Mg determination, 1 mL of the basic solution was dissolved in 3.5 mL of distilled water. To eliminate P interference, 1% La was added to the sample ash solution of Ca and the standard solution of $CaCO_3$. For each ash solution, the analyses were performed in triplicate.

Starch Content Quantification

Precisely 0.1 g of dry CSR flesh was used for the determination of starch content, according to the International Association for Cereal Science and Technology Method 122 (ICC, 1994). Ten milliliters of 70% ethanol were added to the sample; the mixture was heated at 60°C for 20 min and centrifuged at 5000*g* for 5 min. The supernatant was discarded and the pellet was collected. The operation was repeated three times. The remaining starch was dissolved in a boiling solution of $CaCl_2$ at 119°C for 20 min. After cooling at room temperature, 1 mL of 15% $K_4[Fe(CN)_6]\cdot 3H_2O$ and 1 mL of 30% $ZnSO_4\cdot 7H_2O$ were added to each sample, and the mixture was transferred into a 100-mL volumetric flask. The final volume was adjusted to 100 mL with distilled water. The solution was then filtered using Whatman filter paper. The optical activity of the filtrate was measured using a polarimeter (WZZ1), and the starch content was calculated using the following equation: starch content (%) = $(100A \times 100)/[(2 \times 184)m]$, where A is the absorbance of the solute, 2 is the length of the polarimeter tube in decimeters, 184 is the specific rotation of cassava starch, and m is the weight of the sample.

Hydrogen Peroxide Quantification

The flesh of CSRs was ground into powder with liquid N using a mortar and pestle. About 100 mg of the frozen powder was rapidly homogenized in 900 μL of 15 mM of ice-cold physiological saline solution. The homogenate was centrifuged at 10,000 rpm for 10 min at 25°C, and the supernatant was recorded. The supernatant (500 μL) was used for the determination of H_2O_2 content using a kit (Nanjing Jiancheng Bioengeneering Institute, China). The fluorescence was quantified using an Ultraspec 2100 Pro (Amersham Healthcare Biosciences) at 405 nm. The samples were analyzed in triplicate.

Evaluation of Antioxidant Enzyme Activities

Assay of Superoxide Dismutase and Peroxidase Activities

The flesh of CSRs was ground into powder with liquid N. About 100 mg of the powder was homogenized in 900 μL of 0.1 M phosphate saline buffer (pH 7.4) for peroxidase (POD) and superoxide dismutase (SOD) evaluation. The homogenate was centrifuged at 3500 rpm for 10 min at 25°C. The supernatant was collected to determine the activity of each enzyme using spectrophotometric diagnostic kits (Nanjing Jiancheng Bioengeneering Institute) in three replicates. The activities of SOD and POD were detected according to the manufacturer's instructions, as described by Hu et al. (2016).

Assay of Catalase Activity

The flesh of CSRs was ground into powder with liquid N. About 100 mg of the frozen powder was homogenized in 900 μL of 15 mM of ice-cold physiological saline solution. The homogenate was centrifuged at 2500 rpm for 10 min at 25°C. The supernatant was collected to evaluate the activity of the enzyme using kits (Nanjing Jiancheng Bioengeneering Institute) according to the manufacturer's instructions, as described by Hu et al. (2016).

Assay of Ascorbate Peroxidase Activity

Ascorbate peroxidase (APX) activity was measured via a commercial assay kit (Beijing Solarbio Technology Company, China) according to the supplier's protocol.

Statistical Analysis

The experiments using pots were arranged in a completely randomized design. All of the experiments were performed in triplicate. One-way ANOVA was performed to test the variance among the means of different treatments, and Tukey's test was used to compare treatment means. Significance was determined at $p < 0.05$. The results were expressed as the mean of three biological replicates. All statistical analyses were performed with the software SPSS version 20 (IBM Corporation, 2011).

RESULTS

Effect of Calcium and Magnesium Soil Fertilizers on Morphological Changes of Storage Roots during Storage

Morphological symptoms of PPD in storage roots were recorded as shown in Fig. 1. The first symptom of PPD was observed in the SC5 control at 6 DAH while in Mg-treated plants. Discoloration related to PPD was observed at 30 DAH in storage roots. Delay of PPD onset was observed in SC5 in tubers from plants treated with Ca^{2+} fertilizer (Fig. 1A). In QZ1, the first sign of PPD was observed in the treatment with Mg^{2+} fertilizer at 6 DAH, followed by the Ca^{2+} fertilizer treatment at 15 DAH (Fig. 1B).

Effect of Calcium and Magnesium Fertilizers on the Number and Fresh Weight of Tubers at Harvest

Eight months after planting, CSRs were harvested, and the numbers of tubers per plant as well as the average fresh weight of storage roots per plant were recorded. The soil fertilization did not exert a significant effect on the number of tubers per plant. However, when comparing genotypes, SC5 produced more roots than QZ1 (Table 1). These results could account for why the genotype SC5 presented a higher fresh weight of roots than QZ1.

Table 1. Effect of Ca^{2+} and Mg^{2+} fertilization on the number and weight of cassava tubers. Each value is the mean of three replicates.

Genotypes	Treatments	No. of tubers per plant	Weight
			g plant^{-1}
SC5	Control	5.8	928.7b†
	Ca^{2+}	4.0	1163.6a
	Mg^{2+}	5.0	845.8c
QZ1	Control	2.0	412.1f
	Ca^{2+}	3.3	675.7d
	Mg^{2+}	3.2	535.7e
LSD at 0.05			23.9

† Values in same column followed by different letters are significantly different ($p < 0.05$) by Tukey's test.

Mineral Content Analysis of Cassava Storage Roots

The mineral contents of the CSRs are presented in Table 2. Compared with the control, the amounts of Ca^{2+} and Mg^{2+} in CSRs of QZ1 slightly increased after the application of Ca^{2+} fertilizer, whereas in SC5, the Ca^{2+} content was slightly diminished. On the other hand, concentrations of Ca^{2+} and Mg^{2+} in CSRs of SC5 were slightly increased by the application of Mg^{2+} fertilizer. The K^+ and Na^+ contents were also affected by Ca^{2+} and Mg^{2+} fertilization. A slight increase of K^+ was observed in both genotypes treated with Ca^{2+} and Mg^{2+} fertilizers compared with the control, whereas the Na^+ content decreased in QZ1 and increased in SC5 under Mg^{2+} fertilization.

Analysis of Cassava Storage Roots Starch Content

The starch content in CSRs gradually decreased in both genotypes in response to soil fertilization from 0 to 30 DAH. In the SC5 genotype, a significant decrease was observed in tubers from plants treated with Mg^{2+} fertilizer from 6 to 30 DAH (Table 3). In QZ1, a slight decrease occurred with all the treatments from 0 to 15 DAH. Both genotypes showed considerable decrease of starch content at 30 DAH.

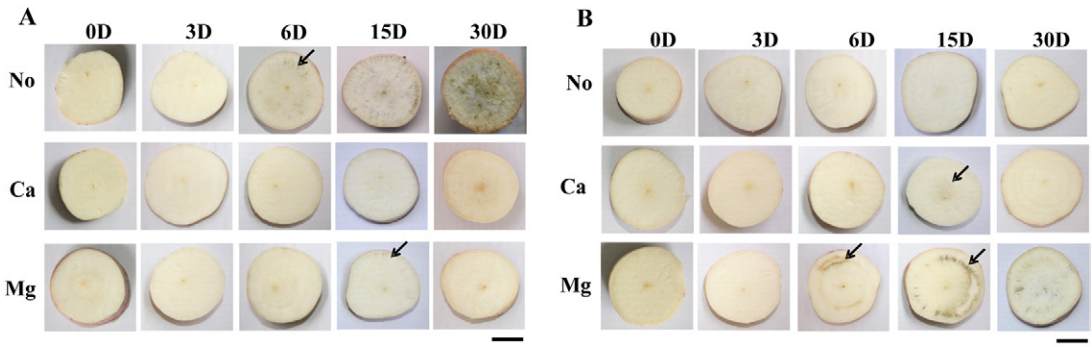

Fig. 1. Visual observation of postharvest physiological deterioration (PPD) onset during storage at 0, 3, 6, 9, 15, and 30 d after harvest in (A) SC5 and (B) QZ1. The first symptoms of PPD were observed at 15 d after harvest. Black arrows show the onset of PPD in the parenchyma during storage.

Table 2. Interactive effect of Ca^{2+} and Mg^{2+} fertilization on mineral content of cassava tubers. Each value is the mean of three replicates.

Genotypes	Treatments	Na	Ca	Mg	K
SC5	Control	0.12a†	0.129a	0.108abc	1.35ab
	Ca^{2+}	0.13a	0.121ab	0.112ab	1.39ab
	Mg^{2+}	0.14a	0.184a	0.117a	1.49a
QZ1	Control	0.12a	0.076b	0.088bc	1.17b
	Ca^{2+}	0.13a	0.118b	0.088bc	1.24b
	Mg^{2+}	0.03a	0.139ab	0.084c	1.29ab
LSD at 0.05		0.07	0.06	0.02	0.05

† Values in same column followed by different letters are significantly different ($p < 0.05$) by Tukey's test.

Table 3. Interactive effect of Ca^{2+} and Mg^{2+} fertilization on tubers starch content during storage. Each value is the mean of three replicates.

		Starch content				
		Day after harvest				
Genotypes	Treatments	0 d	3 d	6 d	15 d	30 d
		%				
SC5	Control	68.97a†	66.50a	54.19d	51.72c	46.80c
	Ca^{2+}	66.50b	61.58c	59.11c	59.11b	51.72a
	Mg^{2+}	64.04c	61.58c	50.90e	36.95d	27.09f
QZ1	Control	66.50b	66.50a	59.11c	59.11b	49.26b
	Ca^{2+}	68.97a	64.04b	61.58b	61.58a	32.02e
	Mg^{2+}	66.50b	66.50a	64.04a	61.58a	41.87d

† Values in same column followed by different letters are significantly different ($p < 0.05$) by Tukey's test.

Changes in Hydrogen Peroxide Content in Cassava Storage Roots

The changes in H_2O_2 content of SC5 and QZ1 are presented in Table 4. A significant decrease of H_2O_2 content was observed at 3 and 6 DAH in both genotypes in tubers from plants treated with Ca^{2+} fertilizer compared with the control. However, high amounts of H_2O_2 were accumulated in the control as compared with the Ca^{2+} and Mg^{2+} fertilizer treatments at 3 and 6 DAH (Table 4).

Activity Changes in Antioxidants in Cassava Storage Roots

The effects of fertilization on antioxidant activities are presented in Table 5. In SC5, the activity of SOD in tubers from plants treated with Ca^{2+} and Mg^{2+} fertilizers was higher than in the control at 3 DAH and then decreased in both treatments at 6 and 15 DAH (Table 5). In QZ1, SOD activity after treatment with Ca^{2+} fertilizers was lower than in the control and after treatment with Mg^{2+} fertilizers at 3 DAH. An increase in activity was observed in CSRs treated with Ca^{2+} at 15 DAH and Mg^{2+} at 30 DAH. Higher activity of POD in the treatment with Ca^{2+} and Mg^{2+} fertilizers in SC5 and QZ1 was observed at 30 DAH as compared with the control. However, a diminution of POD activity was observed in the treatment with Ca^{2+} and Mg^{2+} fertilizers in SC5 and QZ1 at 15 DAH.

Table 4. Interactive effect of Ca^{2+} and Mg^{2+} fertilization on H_2O_2 content of tubers during storage. Each value is the mean of three replicates.

		H_2O_2				
		Day after harvest				
Genotypes	Treatments	0 d	3 d	6 d	15 d	30 d
		μmol g^{-1} fresh wt.				
SC5	Control	245.8c†	326.6a	383.0a	223.3d	246.1b
	Ca^{2+}	255.4b	313.8b	306.0b	288.6b	255.6f
	Mg^{2+}	169.5e	276.4c	190.4d	345.1a	216.3c
QZ1	Control	263.6b	334.5a	304.9b	274.6c	147.7a
	Ca^{2+}	275.0a	225.2d	272.6c	268.5c	158.8e
	Mg^{2+}	215.2d	306.0b	305.1b	353.5a	185.1d
LSD at 0.05		9.2				

† Values in same column followed by different letters are significantly different ($p < 0.05$) by Tukey's test.

The activity of CAT in QZ1 gradually increased from 0 to 30 DAH, and the highest activity of CAT was observed in the CSRs treated with Mg^{2+} fertilizers in SC5 and treated with Ca^{2+} fertilizers in QZ1 at 30 DAH (Table 5). High activity levels of APX in QZ1 were observed in the control and both treatments at 6 DAH. At 15 DAH, the APX activity in SC5 and QZ1 treated with Ca^{2+} and Mg^{2+} fertilizers was greater than that in the control.

DISCUSSION

Soil fertilization with Ca^{2+} and Mg^{2+} delayed the onset of PPD symptoms up to 10 d in SC5, a PPD-susceptible cassava genotype. This is consistent with the report of Aghofack-Nguemezi and Tatchago (2010), who also observed a shelf life prolongation of red-ripe tomatoes (*Solanum lycopersicum* L.) as well as a ripening delay in mature green tomatoes fertilized with Ca^{2+} and Mg^{2+}. In fact, it is well established that the Ca ion can delay ripening and senescence by stabilizing the cell membrane and increasing the rigidification of monolayers (Aghofack-Nguemezi and Tatchago, 2010). Furthermore, the severity and incidence of potato tuber rot was minimized by the application of Ca^{2+} during potato growth (Mantsebo et al., 2014). The Mg ion affects electrostatic cross-linking between membrane components to a lesser extent than the Ca ion (Leshem, 1991; Aghofack-Nguemezi and Tatchago, 2010).

Antioxidant enzymes are one of the important reactive oxygen detoxifier systems in plant cells. Therefore, an increase in antioxidant enzyme activity can be considered an important defense strategy against oxidative stress (Shi et al., 2006). Fertilization with Ca^{2+} and Mg^{2+} had a positive effect on antioxidant production in both genotypes, which is in accordance with Siddiqui et al. (2012). An increase in antioxidant activity was also reported in tomatoes under a high concentration of Mg (Fanasca et al., 2006). Calcium has been shown to influence the production of heat shock proteins that help the

Table 5. Interactive effect of Ca^{2+} and Mg^{2+} fertilization on peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities of tubers during storage. Each value is the mean of three replicates.

		POD					SOD				
		Days after harvest									
Genotype	Treatment	0 d	3 d	6 d	15 d	30 d	0 d	3 d	6 d	15 d	30 d
		U g^{-1}					g mol^{-1}				
SC5	Control	408.1d†	428.4a	405.2c	404.8a	261.9e	234.7a	221.5c	225.9a	216.9b	241.6c
	Ca	388.8e	423.7a	403.7c	386.0c	334.4c	228.5b	228.6b	210.5b	213.1b	248.9b
	Mg	434.4b	407.8c	206.5d	394.1b	294.4d	216.9c	228.5b	178.3c	214.7b	222.4d
QZ1	Control	423.7c	396.7d	418.4b	408.5a	235.6f	225.2b	234.6a	213.4b	215.9b	214.3e
	Ca	566.7a	384.4e	428.9a	376.3d	357.8b	217.3c	189.5d	213.1b	233.0a	207.0f
	Mg	431.5b	417.8b	427.3a	388.5bc	375.0a	217.8c	234.3a	213.2b	204.3c	254.5a
LSD at 0.05				5.82					4.30		
		CAT					APX				
		Days after harvest									
Genotype	Treatment	0 d	3 d	6 d	15 d	30 d	0 d	3 d	6 d	15 d	30 d
		U g^{-1}									
SC5	Control	26.8c	23.9c	34.2a	28.4e	63.2e	8.6c	6.7d	8.3d	5.0f	5.4d
	Ca	24.5a	21.6d	43.9d	28.5d	81.9a	9.6b	5.4f	7.8e	8.2c	6.7b
	Mg	25.9d	29.1d	15.7b	57.2a	106.9b	6.9e	8.5b	4.6f	7.2e	6.1c
QZ1	Control	18.2b	22.4c	79.2d	40.4f	68.2f	6.1f	7.7c	10.5c	7.4d	4.6f
	Ca	37.1b	16.8c	35.2c	46.7f	179.9d	8.2d	6.2e	10.7b	8.7b	5.1e
	Mg	12.1b	57.2b	62.9c	101.4b	141.3c	9.8a	9.9a	12.3a	11.1a	7.2a
LSD at 0.05				3.30					0.09		

† Values in same column followed by different letters are significantly different ($p < 0.05$) by Tukey's test.

plant to tolerate the stress of prolonged heat (Chang et al., 2006). Furthermore, the addition of Ca^{2+} to the tip of the pollen tube caused increased ROS accumulation (Wilkins et al., 2011). On the other hand, a decrease of antioxidant enzyme was observed in wheat (*Triticum aestivum* L.) after Ca^{2+} foliar application (Dolatabadian et al., 2013) suggesting a significant role of Ca in the protection of plants against oxidative stress.

The present study showed that the application of Ca^{2+} enhanced Ca^{2+} and Mg^{2+} contents in CSRs of QZ1. This is in line with previous studies on potatoes, where the Ca^{2+} concentration was significantly increased by supplemental Ca^{2+} application. It has been suggested that the application of Ca^{2+} or Mg^{2+} in the soil may increase the uptake of these minerals differentially in each genotype. This effect was noted where the application of Ca^{2+} and Mg^{2+} fertilizers, either in the soil or on the aerial part of tomato plants, led to an augmentation of Ca concentration in the fruits (Aghofack-Nguemezi and Tatchago, 2010). In SC5, the application of Mg^{2+} fertilizers enhanced Ca^{2+} and Mg^{2+} contents. An increase in the Ca^{2+} level in bananas (*Musa acuminata* Colla) after dipping in $MgSO_4$ solution was previously reported by Aghofack-Nguemezi and Dassie (2007). However, further studies are needed to elucidate the mechanism by which Mg^{2+} fertilizer triggered an increase in Ca^{2+} content in cassava CSRs. The K^+ content increased in CSRs of both genotypes treated with Ca^{2+} and Mg^{2+} fertilizers. The effect of K^+ on the antioxidant level by the activation of protein synthesis osmoregulation, energy transfer, and stress resistance has been addressed by previous reports (Devi et al., 2012; Marschner, 2012).

Application of Ca^{2+} had a significant effect in increasing the average weight of tubers. On the contrary, the lowest number of CSRs per plant was observed in the Ca^{2+} treatment. This result was similar to that reported by Ozgen and Palta (2004), who found both an improvement of tuber grade and a decrease of tuber number in potatoes after soil Ca^{2+} application. An increase in Ca^{2+} concentration in the soil may suppress tuberization signaling by increasing gibberellic acid (Ozgen and Palta, 2004). There has been strong evidence for the critical role of gibberellic acid in tuberization (Xu et al., 1998), which can also be modulated by a Ca^{2+}–calmodulin pathway (Gilroy and Jones, 1993).

In conclusion, Ca and Mg appear to be involved in the modulation of enzymatic activities during PPD in CSRs. The increase of Ca^{2+} content under Ca^{2+} fertilization followed by a delay of PPD onset for 10 d in SC5 has provided an important clue for PPD control by manipulating soil Ca and mineral contents. Furthermore, these results elucidate the implication of Ca^{2+} signaling in the PPD process and its connection with ROS, since an increase of antioxidant activities was observed in the tubers from plants treated with Ca fertilizer.

Conflict of Interest

The authors declare that there is no conflict of interest.

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