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Facteurs agronomiques et moléculaires influençant la sensibilité des bananes (*Musa acuminata*, AAA, cv 'Grande-Naine') aux pourritures de la couronne

Agronomical and molecular factors influencing bananas (*Musa acuminata*, AAA, cv 'Grande-Naine') susceptibility to crown rot disease

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Essai présenté en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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Summary

Ludivine Lassois (2009): Agronomical and molecular factors influencing bananas (*Musa acuminata*, AAA, cv 'Grande-Naine') susceptibility to crown rot disease. University of Liege, Gembloux Agro-Bio Tech, Belgium. 162 pages, 15 tables, 19 figures.

Crown rot affects export bananas in all producing countries and is considered to be one of the main export banana post-harvest disease. Variations are observed in the expression of crown rot symptoms. An original approach of the disease is proposed and consists on presenting the fruit quality potential at harvest as a key factor in crown rot development. This potential develops during growth of bananas in the field and depends on a physiological and a parasitical component. The physiological component refers here to the level of fruit susceptibility to crown rot and reflects the physiological state of the fruit. The aim of this study was to clarify the role of the fruit physiological component at harvest in the post-harvest crown rot development.

It appears that the fruit physiological component at harvest greatly influence the post-harvest disease development and thus the fruit susceptibility. Seasonal variations in disease severity were shown in two production area and are related to a variation of the fruit physiological component. In Guadeloupian conditions, the internal necrotic surface of the crown was nearly multiplied by 4 during 11 successive weeks. Two pre-harvest factors that could influence the fruit physiological component by modifying their susceptibility to crown rot, were identified: (i) hand position on the bunch and (ii) source-sink ratio of the banana plant (hand considered as sink and leaves as source). It was shown that within a bunch, there is a gradient of susceptibility to crown rot (r= -0.95), the hands initiated first (the upper ones) being more susceptible than those initiated last (the lower ones). These results also confirmed that source-sink ratio changes have a significant effect on fruit morphology and demonstrated that there is also an effect on fruit susceptibility to crown rot disease. When the sink is decreased by artificial removal of many hands, the level of fruit susceptibility to crown rot decreases.

However, the molecular mechanisms underlying these quantitative host-pathogen relationships were still unknown. A study was designed to compare gene expression, by cDNA-AFLP, between crowns of bananas showing a high susceptibility (S^+) and crowns of bananas showing a low susceptibility (S^-) to *Colletotrichum musae* responsible for crown rot disease. This comparison was performed at two situation time: (i) between crowns (S^+ and S^-) collected one hour before infection and (ii) between crowns (S^+ and S^-) collected 13 days after infection. Genes implied in signaling pathway and proteolytic machinery were identified. It also appears that a cellulose synthase, a CAF1 gene, 2 glycolipid transfer protein and a dopamine-β-monooxygenase were differently expressed between bananas showing different levels of susceptibility.

This is the first study of the characterization of the banana physiological component at harvest which influences the crown rot post-harvest disease development. In addition, to our knowledge, this work is the first to address both pre- and post-infection gene expression with the same host-pathogen combination and different susceptibility levels.

Ludivine Lassois (2009): Facteurs agronomiques et moléculaires influençant la sensibilité des bananes (*Musa acuminata*, AAA, cv 'Grande-Naine') aux pourritures de la couronne. Université de Liège, Gembloux Agro-Bio Tech, Belgique. 162 pages, 15 tableaux, 19 figures.

La maladie des pourritures de la couronne est considérée comme l'une des principales maladies post-récolte des bananes d'exportation. Elle se rencontre dans toutes les zones de production et des variations du niveau d'expression des symptômes sont observées. Une approche originale de l'étude du développement de la maladie est proposée dans ce travail et consiste à présenter le potentiel de qualité du fruit à la récolte comme un facteur déterminant dans le développement post-récolte de la maladie. Le potentiel de qualité s'élabore durant la phase de croissance du bananier et dépend d'une composante physiologique et parasitaire. La composante physiologique caractérise ici le niveau de sensibilité du fruit aux pourritures de la couronne. L'objectif de cette thèse est de clarifier le rôle de la composante physiologique du fruit à la récolte sur le développement post-récolte de la maladie.

Au terme de cette étude, il apparaît que la composante physiologique du fruit à la récolte influence le développement post-récolte de la maladie et donc la sensibilité des fruits aux pourritures de la couronne. L'existence de variations saisonnières de sensibilité des fruits a été démontrée dans deux zones de production. En Guadeloupe, la surface de nécroses internes des couronnes a été presque multipliée par 4 durant 11 semaines successives. Deux facteurs pré-récolte pouvant influencer la composante physiologique du fruit en modifiant leur sensibilité aux pourritures ont été identifiés : (i) le niveau d'insertion de la main de banane sur le régime et (ii) le ratio source-puits du bananier (les mains étant considérées comme des puits et les feuilles comme des sources d'assimilats). En effet, une relation linéaire (r=-0.95) a été établie entre le niveau d'insertion du fruit sur le régime et sa sensibilité aux pourritures de la couronne. Les mains initiées en premier sont plus sensibles que les dernières sorties. Les essais menés ont confirmé que le ratio source-puits du bananier avait un effet significatif sur la morphologie des fruits mais ont également démontré un effet significatif sur la sensibilité des fruits aux pourritures de la couronne. Lorsque le nombre de puits diminue, suite à l'ablation de mains, la sensibilité des fruits restant sur le régime diminue également.

Néanmoins, les processus sous-jacents ne sont pas connus et une approche moléculaire a été envisagée afin d'identifier les déterminants génétiques qui sous-tendent les réactions de sensibilité des fruits aux pourritures de la couronne. L'expression différentielle des gènes a été comparée, via cDNA-AFLP, entre des couronnes de bananes présentant une sensibilité élevée (S^+) et des couronnes de bananes présentant une faible sensibilité (S^-) à *Colletotrichum musae*, agent responsable des pourritures de la couronne. Cette comparaison a été réalisée à deux moments différents : (i) entre couronnes (S^+ et S^-) collectées une heure avant l'infection et, (ii) entre couronnes (S^+ et S^-) collectées 13 jours après l'infection. Des gènes impliqués dans la transduction du signal et dans la protéolyse ont été identifiés. D'autres gènes ont également été mis en évidence: une cellulose synthase, un gène CAF1, deux protéines de transfert de glycolipides et une dopamine-β-monooxygenase.

Ce travail constitue la première étude concernant l'incidence de la composante physiologique des bananes à la récolte sur le développement post-récolte des pourritures de la couronne. De plus, à notre connaissance, ce travail est le premier qui évalue l'expression des gènes en situation de pré- et post-infection avec la même combinaison hôte-pathogène présentant des niveaux de sensibilité différents.

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Ludivine

List of abbreviations

ABA	Abscissic acid
ACP	Afrique, Caraïbes, Pacifique
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
AtGLTP	*Arabidopsis thaliana* Glycolipid transfer protein
BASE	Biotechnologie, agronomie, société et environnement
BORAX	Sodium borate decahydrate
°C	Celsius degree
CA	Controlled atmosphere
CAF	CCR4-associated factor
CARBAP	Centre Africain de Recherche sur Bananiers et Plantains
CB	Cytochrome B561
cDNA-AFLP	Complementary DNA-Amplified Fragment Length Polymorphism
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
cm	Centimeter
C_t	Treshold cycle
CTAB	Cetyl Trimethyl Ammonium Bromide
dATP	Deoxyadenosine triphosphate
Dd	Degree day
DDV	Durée de Vie Verte
DEPC	Diethylpyrocarbonate
Df	Degrees of freedom
DIECA	Diethyldithiocarbamate
DNA	Deoxyribonucleic acid
DoH	Dopamine-β-hydroxylase
DSP	Dual specificity phosphatase
DTT	Dithiothreitol
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
Ef1-α	Elongation factor 1-alpha
EGTA	Ethylene glycol tetraacetic acid
FHIA	Fundacion Hondurena de Investigation Agricola

FUSAGx	Faculté Universiraire des Sciences Agronomiques de Gembloux
FW	Fresh weight
g	Gram
GHCL	Guanidium hydrochloride
GITC	Guanidium isothiocyanate
GO	Gene ontology
GSLs	Glycosphingolipids
H	Hand
Ha	Hectare
HMW-PEG	High molecular weight polyethylene glycol
HTS	High-throughput sequencing
i.e.	id est
IFC	Intervalle fleur-coupe
INS	Internal necrotic surface
Kg	Kilograms
kGy	Kilogray
L	Leaf
LTP	Lipid transfer protein
MA	Modified atmosphere
Min	Minute
ml	Milliliter
mM	Milimolar
mm	Millimeter
mm^2	Square millimeter
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
Nm	Nanometer
OCMB	Organisation Commune du Marché de la Banane
OMC	Organisation Mondiale du Commerce
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PHP	Plantations du Haut Penja
PR	Pathogenesis-related
PVP	Polyvinylpyrolidone
RING	Really interesting new gene

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Reverse transcription
So-Si ratio	Source-sink ratio
ss cDNA	Single-strand cDNA
Td	Daily average temperature
TDF	Transcription-derived fragments
Tmax	Maximum temperature
Tmin	Minimum temperature
UE	Union Européenne
UK	United Kingdom
USD	United states dollar
UV	Ultra-violet
W	Watt
µ	Micron
µl	Microlitre
µM	Micromolar
µg	Microgram
1hbi	1 hour before inoculation
13dpi	13 days post-inoculation

Introduction

Crown rot is a major post-harvest disease of export bananas (Krauss and Johanson, 2000; Reyes *et al.*, 1998). This rot develops during transport, ripening, and storage of bananas, and constitutes a fatal defect when it comes to selling them. It results from the development of several non-specific fungi, including *Colletotrichum musae*, which is often considered as the most highly pathogenic (Finlay and Brown, 1993; Greene and Goos, 1963; Lassois *et al.*, 2008; Lukezic *et al.*, 1967; Shillingford, 1976).

For six years, the Plant Pathology Unit of the University of Gembloux has been conducting research on crown rot disease of bananas in collaboration with two partners. The first is the CIRAD (Centre de coopération internationale en recherche agronomique pour le développement), where crown rot and other diseases involving *Colletotrichum musae* have been a research focus for many years. The second partner is CARBAP (Centre Africain de Recherche sur Bananiers et Plantains), based in Cameroon. This thesis is part of this research program aimed at a better understanding of the conditions and mechanisms determining the post-harvest crown rot development.

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CHAPTER I

Literature Review

1. La banane : de son origine à sa commercialisation

Lassois L., Busogoro JP. and Jijakli H. 2009. *La banane : de son origine à sa commercialisation.* Biotechnologie, Agronomie, Société et Environnement, **13** : 575-586.

La banane: de son origine à sa commercialisation

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Abstract

Cultivated bananas are giant herbaceous plants within the genus *Musa*. They are both sterile and parthenocarpic. There are well over a thousand domesticated *Musa* cultivars, they are mostly triploid (a few are diploid or tetraploid) and are derived from crosses between two wild species, *Musa acuminata* and *Musa balbisiana*. In terms of production, bananas are the fourth agricultural product after rice, wheat, and maize. They constitute the basis of food security for many people. Cropping systems vary widely around the world and contrasting objectives are encountered: consumption by the producer, sale on local or national markets, export, etc. Cooking bananas, including plantains, must be distinguished from dessert bananas, which constitute a major international trade. This international trade started only in the early 1900s but it has since grown continuously. Banana is currently the most exported fruit, in terms of both value and quantity. Despite the high genetic diversity found within the genus *Musa*, the export market is mainly based on single Cavendish. There are major challenges to banana production from biotic or abiotic stresses to continue to meet the criteria of sustainability, quality and yield that are imposed.

Keywords: Banana, origin, genetic diversity, production, international trade, Cavendish

1.1. Origine et classification des bananiers

Le bananier est originaire de l'Asie du Sud-Est, où il est retrouvé de l'Inde à la Polynésie (Simmonds, 1962), et son centre de diversification semble être la Malaysie ou l'Indonésie (Daniells *et al.*, 2001). Il s'est propagé vers l'Afrique de l'Ouest il y a au mois 2500 ans (Mbida Mindzie *et al.*, 2001). Son implantation aux Amériques s'est d'abord faite par la République Dominicaine, en 1516 grâce à des plants en provenance des îles Canaries, et s'est poursuivie vers l'Amérique Centrale et du Sud. Ainsi, depuis des millénaires, les migrations humaines et les échanges de matériel végétal ont introduit le bananier dans des situations écologiques très différentes sur tous les continents (Lassoudière, 2007).

Les bananiers appartiennent à l'ordre des scitaminales, ou zingibérales, et à la famille des *Musaceae*. Ce sont des monocotylédones aux pièces florales par 3 ou multiple de 3, asymétriques zygomorphes avec nervation secondaire des limbes parallèles, absence de formation vasculaire secondaire au sein de la tige et des racines. La famille des *Musaceae* comporte 3 genres à savoir (i) *Musella*, très peu représenté et localisé en Asie , (ii) *Ensete*, ne comportant pas d'espèces parthénocarpiques et (iii) le genre *Musa*, présentant une forte variabilité et caractérisé par des inflorescences avec des bractées insérées séparément des fleurs, à l'inverse du genre *Ensete*. Les premières classifications du genre *Musa* sont apparues à la fin du 19ème siècle. Le genre *Musa* a été divisé en 4 sections (Cheesman, 1947 cité par Heslop-Harrison *et al*., 2007) sur base du nombre de chromosomes et de caractéristiques morphologiques : Les *Australimusa* (n=10) ; les *Callimusa* (n=10) ; les *Rhodochlamys* (n=11) et enfin les *Eumusa* (n=11) avec 10 à 12 espèces qui constituent le genre le plus diversifié et comprend plus de 1000 variétés dont les plantains (Lassoudière, 2007). La section *Eumusa* regroupe presque tous les bananiers cultivés et se caractérise par des bractées sillonnées longitudinalement sur leur face externe et de nombreuses fleurs par bractées disposées en deux rangées. Une étude récente basée sur l'AFLP (Amplified fragment length polymorphism) propose de réduire de 4 à 2 groupes le genre *Musa* en regroupant les *Rhodochlamys* avec les *Eumusa* et les *Australimusa* avec les *Callimusa* (Wong *et al*., 2002). Cependant d'autres regroupements ont été proposés et la classification précise des espèces et sous-espèces reste toujours débattue (Heslop-Harrison *et al*., 2007). De plus, de nombreuses régions du centre de diversification du genre *Musa* en Asie du Sud-Est n'ont pas encore été explorées et de nouvelles variétés et espèces continuent d'être découvertes (Häkkinen, 2009).

D'un point de vue botanique, le genre *Musa* se divise en deux grands types : les variétés comestibles à fruits charnus et les espèces sauvages. Ces dernières, séminifères à fruits non

comestibles sont toutes diploïdes (AA et BB). Actuellement, on en compte environ 180, toutes originaires d'Asie du Sud-Est , mais leur recensement n'est pas encore définitif surtout pour BB (Cirad-Flhor, 2003). Ces variétés fertiles sont cependant importantes car elles présentent différents niveaux de résistance aux maladies et ravageurs. De plus, en se croisant naturellement entre eux, ces bananiers sauvages et séminifères ont contribué à l'élargissement de la diversité génétique. Ils sont donc la base des différents programmes d'amélioration génétique et de créations variétales actuels et futurs. C'est à partir de croisements entre ces espèces que sont apparues des variétés sans graine. Ces bananes qui possèdent des qualités alimentaires ont rapidement intéressé l'homme qui les a intégrées dans son agriculture en utilisant leur potentiel de multiplication végétative par enracinement de leurs ramifications latérales. Le nombre de cultivars ou de variétés comestibles à fruits charnus stériles et parthénocarpiques de part le monde est estimé à 1200 (Cirad-Flhor, 2003) et représente une diversité génétique non négligeable. Aujourd'hui, les variétés cultivées sont classées en groupes selon leur constitution génétique et leur niveau de ploïdie, puis en sous groupes en rassemblant les différents cultivars dérivant les uns des autres par mutations naturelles à partir d'un ancêtre génétiquement commun (Cirad-Flhor, 2003).

Si les bananiers sauvages sont tous diploïdes, les variétés cultivées actuellement sont généralement des clones triploïdes stériles et aspermes (AAB et ABB), issus soit de croisements interspécifiques entre les 2 espèces séminifères sauvages diploïdes principales *Musa acuminata* et *Musa balbisiana*, soit de la seule espèce *M. acuminata* (AAA) (Figure 1).On rencontre plus rarement des variétés diploïdes (AA et AB) et des clones tétraploïdes de nature interspécifique (Lassoudière, 2007). La contribution haploïde de *M. acuminata* et *M. balbisiana* aux bananiers cultivés est indiquée respectivement par A et B (Simmonds *et al.*, 1955).

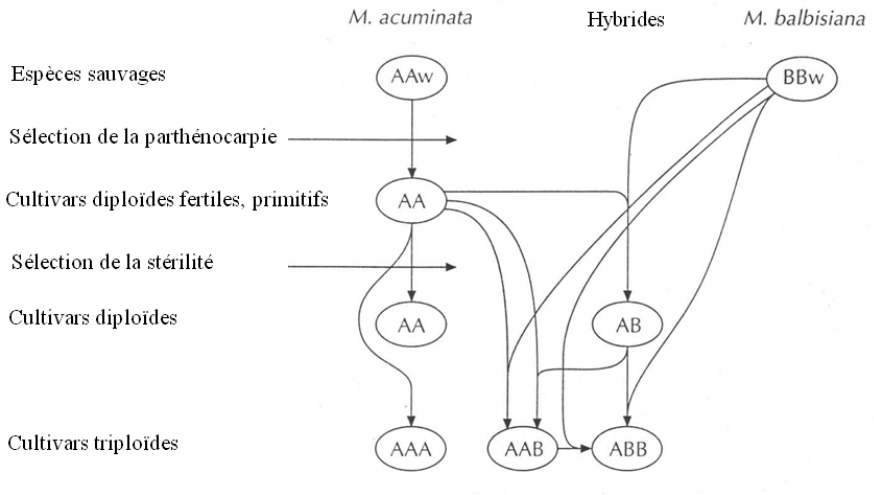

Figure 1 : Evolution des principaux groupes génomiques de la série *Eumusa* (Tiré de Jones, 2000). W : Sauvage.

Le tableau 1 reprend la classification et la répartition géographique des principaux bananiers cultivés. Au sein des bananiers cultivés, il faut différencier deux grands types de bananes comestibles : les bananes qui se consomment à l'état frais, dites « dessert » et les bananes consommées cuites dites « à cuire » comprenant notamment les plantains. Ces derniers (AAB) comprennent de nombreux cultivars variant par leur forme, leur taille, leur couleur, leur goût etc... Produits de manière traditionnelle, leur productivité n'est pas très élevée (10T/ha) mais la culture nécessite peu de soins (Lescot, 2004). Les bananes à cuire constituent souvent l'un des produits essentiels de l'alimentation de base des populations de la zone tropicale humide. Source de carbo-hydrates, elles sont l'équivalent de la pomme de terre en pays tempérés (Lescot, 2004).

Tableau 1 : Classification et répartition géographique des principaux bananiers cultivés (Bakry *et al.*, 1997).

Sous groupe	Cultivars	Type de fruit	Distribution
Groupe AA			
Sucrier	Pisang Mas, Fayssinette, Figue sucrée	dessert sucré	Tous continents
Psang Lilin	-	dessert	Indonésie, Malaisie
Pisang Berangan	-	dessert	Indonésie, Malaisie
Lakatan	-	dessert	Philippines
Groupe AAA			
Cavendish	Lacatan, Poyo, Williams, Grande Naine, Petite Naine	dessert	Pays exportateurs
Gros Michel	Gros Michel, Highgate, Cocos	dessert	Tous continents Philippines
Figue Rose	Figue Rose rose, Figue Rose verte	dessert	Pacifique, Antilles, Afrique de l'Est
Lujugira	Intuntu, Mujuba	à bière, à cuire	Indonésie, Afrique
Ibota	Yangambi km5	dessert	
Groupe AB			
Ney Poovan	Sait Velchi, Sukari	dessert acide	Inde, Afrique de l'Est
Groupe AAB			
Figue Pomme	Maça, Silk	dessert acide	Tous continents
Pome	Prata	dessert acide	Inde, Malaisie, Australie, Brésil, Afrique de l'Ouest
Mysore	Pisang Ceylan	dessert acide	Inde
Pisang Kelat	Pisang Kelat	dessert	Inde, Malaisie
Pisang Rajah	Pisang Rajah Bulu	à cuire	Malaisie, Indonésie
Plantains	French corne, Faux corne	à cuire	Afrique du Centre et de l'Ouest, Caraïbe, Amérique latine
Popoulou	Popoulou	à cuire	Pacifique
Laknao	Laknao	à cuire	Philippines
Pisang Nangka	Pisang Nangka	à cuire	Malaisie
Groupe ABB			
Bluggoe	Bluggoe, Matavia, Poteau, Cacambou	à cuire	Philippines, Caraïbe, Amérique latine
Pelipita	Pelipita	à cuire	Philippines, Amérique latine
Pisang Awak	Fougamou	dessert	Thaïlande, Inde, Philippines, Afrique de l'Est
Peyan	-	à cuire	Philippines, Thaïlande
Saba	Saba	à cuire	Philippines, Indonésie, Malaisie

1.2. La production mondiale de banane

Les bananiers sont cultivés dans plus de 120 pays sur les 5 continents (Bakry *et al.*, 1997) et sur plus de 10 millions d'hectares (Lassoudière, 2007). Les bananes offrent de multiples usages. Elles sont consommées principalement sous forme de fruit frais ou comme légume cuit ou frit mais font également l'objet de nombreuses transformations : chips, frites, beignets, purée, confiture, ketchup, alcool, vin, bière etc.... D'autres parties de la plante sont utilisées comme fibre textile, pour la construction d'abris, la fabrication de couvertures ou comme emballages de cuisson. En termes de production mondiale, la banane est le quatrième produit agricole après le riz, le blé et le maïs (Lassoudière, 2007). Elle occupe le premier rang de la production fruitière, avec un peu plus de 106 millions de tonnes produites annuellement à l'échelle mondiale (Lescot, 2006). Les systèmes culturaux sont très diversifiés dans le monde et les objectifs très contrastés : autoconsommation, ventes sur les marchés locaux ou nationaux, exportation vers des régions proches ou vers les pays industrialisés du Nord. Près de 90% de la production sont issus de petits agriculteurs, produisant pour la consommation domestique et les marchés locaux. Seuls un peu plus de 10% de la production mondiale est destinée à l'exportation. On distingue cependant deux grandes filières de production : celle des bananiers en culture pure, dont une partie des fruits est destinée à l'exportation et celle des bananiers en polyculture, destinés à l'approvisionnement des marchés locaux ou à l'autoconsommation familiale.

Dans les statistiques, il faut distinguer :

Les bananes à cuire comprenant notamment les plantains (AAB) séparés des autres types de bananes à cuire.

Les bananes dessert dominées par les variétés du sous-groupe Cavendish (AAA) séparées des autres bananes dessert pouvant appartenir au groupe AAB (Prata), AA (Figue sucrée) ou AAA (Gros Michel,…).

Les bananes à cuire correspondent à 43% de la production mondiale des bananes et les plantains (AAB) représentent 40% de bananes à cuire.

Le reste de la production mondiale (57%) concerne les bananes dessert, avec la majorité de leur production issue du groupe des Cavendish. L'Inde et le Brésil en sont les deux plus gros producteur et écoulent la quasi-totalité de leur récolte sur les marchés intérieurs (Lassoudière, 2007). En termes de production, ils sont suivis par l'Equateur, la Chine, la

Colombie et le Costa Rica. De 1985 à 2000, la production est passée de 42.5 à 63.4 millions de tonnes, les surfaces ayant augmenté corrélativement de 1 million d'hectares (Lassoudière, 2007). Les études d'impact sur la production bananière sont peu nombreuses. Toutefois, cette industrie est d'une importance vitale pour l'ensemble des pays producteurs. Elle joue non seulement un rôle important dans l'alimentation, mais aussi aux niveaux social, économique et écologique.

1.3. Le commerce international de la banane

La culture de la banane pour l'exportation n'a vraiment débuté qu'à la fin du XIXème siècle. Dès 1870, la Jamaïque organise les premières exportations de bananes Gros-Michel vers les marchés d'Amérique du Nord. Quelques années plus tard, une filière en provenance des Canaries approvisionne le marché anglais avec une autre variété, Petite Naine, du sous groupe Cavendish (Bakry *et al.*, 1997). Ce n'est qu'au début du XXème siècle que des exportations sur de plus longues distances ont débuté grâce aux premiers navires réfrigérés. C'est au cours de cette période pionnière que les méthodes de cultures industrielles et d'exportation massive d'un fruit fragile ont été mises en place. Depuis lors, la banane, qu'il s'agisse de production, d'exportation ou d'importation, n'a eu sur le long terme qu'une croissance continue et constitue à l'heure actuelle le 4ème produit d'exportation mondiale (Wilson *et al.*, 2004).

Malgré la grande diversité existant au niveau des variétés de bananier, le commerce international repose essentiellement sur un seul groupe variétal : les bananes Cavendish dont plus de 30% de la production sont destinés à l'exportation. Les Cavendish fournissent 97% du marché international (Loeillet, 2005). Pourtant, l'offre de bananes de par le monde est riche de variétés quasi totalement inconnues sur les grands marchés d'importations. Seuls 2% de la production de bananes à cuire sont destinés à un commerce international. Cela concerne principalement les bananes plantains (Lassoudière, 2007). Ces dernières sont présentes sur les marchés d'importation depuis des décennies mais les volumes sont limités et leur croissance minime. L'UE a importé environ 23 000 tonnes de plantains en 2000 (EUROSTAT, 2000). En un peu moins de 10 ans, les quantités importées sont restées quasiment inchangées (ODEADOM/Cirad-Flhor, 2000).

Alors que de très nombreux pays produisent la banane, très peu participent de manière substantielle au marché international. Pour ces derniers, la dépendance vis-à-vis de la filière banane est grande. C'est une activité qui occupe toute l'année une main d'œuvre nombreuse et relativement peu qualifiée, jouant ainsi un rôle crucial dans la lutte contre la pauvreté

(Loeillet, 2005). Grâce aux exportations hebdomadaires régulières, des services de fret maritime réguliers ont été créés. Ils ont favorisé les importations de marchandises nécessaires au développement de ces pays et à la vie quotidienne de leurs habitants. Ces exportations régulières ont aussi permis de stabiliser des lignes maritimes sur lesquelles peuvent se construire d'autres filières d'exportation dans les domaines agricole et industriel (Loeillet, 2005).

Sur les 10 exportateurs mondiaux, 7 sont situés en Amérique Latine, 2 en Afrique et un en Asie (Loeillet, 2005). Ils totalisent 95% de l'offre mondiale (Loeillet, 2005). Les exportations américaines sont très largement dominantes, les Philippines s'intercalant au 4ème rang (Lassoudière, 2007). L'Equateur, le Costa Rica et la Colombie fournissent environ 65% du marché international, ce qui illustre le poids de la filière banane dans ces pays, tant au niveau économique que dans la vie sociale et politique (Lassoudière, 2007). Le premier producteur mondial, l'Equateur, exporte chaque année l'équivalent de la consommation de bananes de l'Union Européenne (4,5 millions de tonnes) (Loeillet, 2005). Les pays ACP (Afrique, Caraïbes, Pacifique) et l'Europe ne pèsent que 15% dans le commerce mondial (Lassoudière, 2007). La zone Europe, constituée de l'Espagne et du Portugal, participe au commerce mondial à hauteur de 3% des exportations.

Les exportations en provenance des Caraïbes décroissent alors que celles d'Afrique augmentent, notamment en provenance du Cameroun et de la Côte d'Ivoire. Depuis 1990 la Côte d'Ivoire a multiplié par deux ses exportations et celles du Cameroun ont plus que triplé (Lassoudière, 2007).

Le commerce mondial de la banane dessert est estimé à 14 millions de tonnes (Loeillet, 2005), pour un chiffre d'affaire à l'exportation de plus de 4.9 milliards d'USD (Lescot *et al.*, 2008). Sur les 40 dernières années le marché s'est fortement développé. La production mondiale de bananes dessert à plus que doublé mais cette croissance est principalement due à l'augmentation des surfaces cultivées et non à une meilleure productivité (Picq *et al.*, 2002). Sur la même période, les exportations ont été multipliées par 3,5 (Loeillet, 2005) et la valeur de ces exportations multipliée par 11. La croissance du marché a été de 7% par an entre 1985 et 1995 mais a ralenti ces dernières années (Loeillet, 2005).

La banane est le fruit le plus exporté aussi bien en valeur qu'en quantité. Cinq compagnies aux structures très intégrées contrôlent les ¾ des exportations du marché mondial : Chiquita Brands International (22%), Dole Food Company (21%), Del Monte Fresh Produce (16%), Noboa (7%) et Fyffes (7%) (Lassoudière, 2007).

Les 4 marchés d'importation mondiaux que sont l'Union Européenne, les Etats-Unis, le Japon et la Russie captent 78% de l'offre mondiale de banane dessert (Loeillet, 2005). A noter que certains marchés émergent en Afrique du Nord et au Moyen-Orient (6% de la production mondiale) et en Chine (4% de la production mondiale) (Lassoudière, 2007). Avec 4.6 millions de tonnes d'importation et une consommation moyenne de bananes aux alentours de 10.1kg/hab/an, le marché européen est le premier marché mondial d'importation (Loeillet, 2005). La structure de l'approvisionnement se répartit comme suit : en 2004, l'UE a reçu des bananes de trois origines différentes à savoir (i) Communautaires (16%), (ii) ACP (17%) et (iii) latino-américaine dite « dollar » (67%) (Loeillet, 2005).

Le commerce international de la banane est très complexe et on ne peut en parler sans évoquer le différend qui oppose quelques pays européens, particulièrement la France, et les Etats-Unis. En effet, depuis des années, les Européens et les Américains se livrent une guerre commerciale autour de la banane. Avant la mise en place du marché unique européen, l'approvisionnement en bananes résultait d'une gestion nationale au cas par cas. Les pays ayant des attaches avec des zones de productions, comme la France avec la zone Antillaise et Africaine ou l'Espagne avec les Canaries, privilégiaient ces productions. Les autres pays, sans attache à une zone de production particulière, s'approvisionnaient en bananes « dollars » qui étaient importées sans frais de douane à travers les filières intégrées des sociétés américaines. Cette exonération de droits permit aux dites entreprises de réaliser des bénéfices colossaux, le fruit étant produit à très bas prix en Amérique Latine (Loeillet, 2005). Lors de la mise en place du marché unique Européen (1er janvier 1993), l'approvisionnement en bananes devait passer à une gestion commune à douze membres. Effective depuis le 1er juillet 1993, l'Organisation Commune du Marché de la Banane (OCMB) créée dans le cadre de la mise en place du marché unique européen instaure des quotas spécifiques d'importation et institue un régime d'aides compensatoires destiné à assurer un revenu minimum aux producteurs européens et de la zone ACP. Les bananes européennes et celles des pays ACP, plus chères, bénéficient alors d'importantes aides de l'Union Européenne. Mais la commission dut immédiatement faire face à une double pression: celle des multinationales américaines et celle du front de refus des principaux importateurs de bananes "dollars": l'Allemagne et le Benelux (Loeillet, 2005). En avril 1994, l'Organisation Mondiale du Commerce (OMC) est créée et dénonce rapidement le principe des quotas spécifiques mis en place par l'Union Européenne. Le système de l'OCMB privilégiant les bananes communautaires et ACP est jugé discriminatoire et non conforme aux règles du commerce international par l'OMC (Maillard, 2002). Aussi, malgré une première modification de l'OCMB, le 1er janvier 1999 sous la pression des producteurs de « bananes dollars » et une diminution consécutive des droits de

douane pour ces pays, le nouveau régime européen d'importation est à nouveau dénoncé par l'OMC qui conteste, en particulier, le principe des quotas spécifiques. Depuis le 1er janvier 2006, bien que toutes les négociations ne soient pas achevées, les nouvelles règles sont mises en application dans les grandes lignes. Les quotas d'importation sont abandonnés au profit d'un système uniquement tarifaire, c'est-à-dire fondé sur un droit de douane et sur le principe « premier arrivé = premier servi ». Des clauses particulières sont mises en place pour les pays ACP qui bénéficient de l'absence de droit de douane pour un quota donné. Le volet interne de l'OCMB qui régit l'aide aux producteurs de bananes européens est en cours de réexamen (Lassoudière, 2007).

1.4. La variété Cavendish

1.4.1. Morphologie de la plante

1.4.1.1. Description de l'appareil végétatif

Le bananier est une herbe géante dont le pseudo-tronc est formé par l'emboîtement des gaines foliaires (Champion, 1963) (Figure 2). Les feuilles sont émises par le méristème terminal de la tige vraie souterraine improprement appelée « bulbe ». Les nouvelles feuilles se déroulent au sommet du pseudo-tronc et sont donc de plus en plus jeunes en se rapprochant du sommet. Par convention, elles sont numérotées de la plus jeune à la plus âgée (Bakry *et al.*, 1997). Le nombre de feuilles varie selon le cultivar et les conditions environnementales (Jones, 2000). Les feuilles, dont la durée de vie varie entre 70 et 200 jours, présentent une surface pouvant aller jusqu'à 2 m^2 fournissant ainsi à la plante une surface foliaire importante au moment de la floraison et permettant de canaliser les eaux de pluie (Stover *et al.*, 1987). Toutefois, la longueur et la largeur du limbe s'accroissent au cours du cycle. Au moment de la sortie de l'inflorescence, il reste 11 à 15 feuilles fonctionnelles (Lassoudière, 2007). Pour un développement correct des fruits jusqu'à la récolte, il faut au minimum 8 feuilles fonctionnelles à la floraison et au moins 4 à la récolte. Le bourgeon situé à l'aisselle de chaque feuille donne éventuellement naissance à un rejet. A la fin de la phase végétative, le changement de fonctionnement du méristème central provoque la croissance et l'allongement de la tige vraie au cœur du pseudo-tronc puis l'émergence de l'inflorescence.

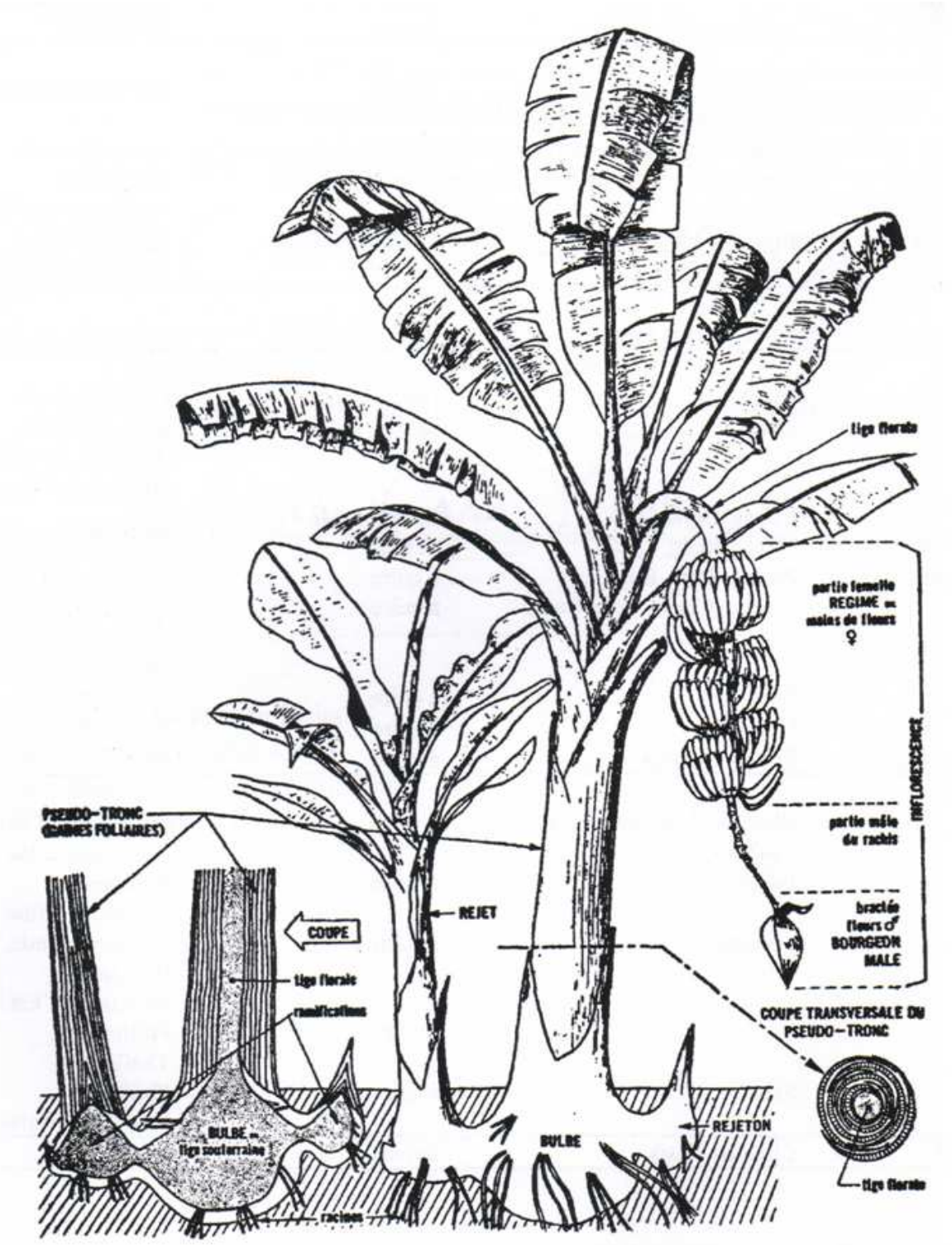

Figure 2: Représentation de l'organisation du bananier et de ses rejets (tiré de Champion, 1963).

1.4.1.2. **L'inflorescence**

Les étapes du développement végétatif ont des répercussions capitales sur la croissance et le développement de l'inflorescence (Lassoudière, 2007). Dans le cas des variétés Cavendish comme la Grande-Naine, la floraison intervient dès qu'une trentaine de feuilles ont été émises. Les premières phases du développement de l'inflorescence ont lieu à l'intérieur du pseudo-tronc pendant la montée de la tige. L'inflorescence du bananier (appelée régime) se caractérise par un pédoncule robuste d'environ 1m recourbé vers le bas (Figure 2). Elle est constituée de spathes pourpres, déhiscentes, imbriquées, disposées selon 3 hélices qui se soulèvent avant de tomber rapidement et à l'aisselle desquelles naissent les rangées simples ou doubles de fleurs. Ce sont les premières rangées de fleurs, appelées mains, qui forment les régimes de fruits. Ces premières rangées sont constituées de fleurs femelles avec un ovaire infère comprenant trois loges carpellaires à l'intérieur desquelles deux rangées d'ovules sont insérées sur un placenta axilaire et des étamines non fonctionnelles. Les ovaires se remplissent de pulpe pour former le fruit sans pollinisation ni formation de graines. Les mains sont composées de 10 à 30 fleurs ou doigts insérés sur le coussinet selon deux rangées et sont numérotées à partir de la première main dégagée. A l'anthèse, les doigts sont dirigés vers le bas et se redressent progressivement pour atteindre, en plus ou moins 15 jours, le stade appelé « stade doigts horizontaux ».

Après les fleurs femelles, apparaissent deux à trois mains de fleurs neutres avec toutes les pièces florales avortées, suivies par les mains de fleurs mâles constituées d'ovaires réduits et d'étamines bien développées. Les fleurs mâles tombent au fur et à mesure de leur libération, dénudant ainsi la partie inférieure de la hampe. La croissance de l'inflorescence se poursuit indéfiniment pour former le bourgeon mâle, constitué de la superposition des bractées. S'il n'est pas coupé, ce bourgeon mâle prolongera sa croissance jusqu'à la maturité des fruits et la fanaison de la tige. Pour le groupe des AAA notamment, une disproportion entre le nombre de mains femelles (4 à 18 mains) et le nombre de mains mâles (200 à 500 mains) est observée (Lassoudière, 2007).

1.4.2. **Le fruit**[1]

1.4.2.1. **Développement du fruit**

Deux grandes phases peuvent-être distinguées dans le développement des fruits : une première phase qui se déroule à l'intérieur du pseudo-tronc et au cours de laquelle les différentes structures du futur fruit se mettent en place.

La deuxième phase se déroule après la sortie de l'inflorescence et correspond essentiellement au développement de la pulpe. Les bananes se développent de manière parthénocarpique à partir des fleurs femelles et sont formées de la peau (le péricarpe) et de la pulpe (endocarpe). Le doigt est relié au coussinet par un pédicelle. Le péricarpe est composé d'un épiderme stomatifère avec cuticule ne permettant que peu d'échanges gazeux, d'une couche parenchymatique sous-épidermique et d'une zone profonde à parenchyme lâche (Omoaka, 2000). La couche parenchymatique sous-épidermique contient des chloroplastes. Les fruits possèdent une grande proportion de peau qui diminue avec leur maturité. Les ovules avortés se retrouvent dans l'endocarpe et les grosses cellules ovoïdes amylifères des 3 carpelles constituent l'essentiel de la pulpe (Lassoudière, 2007).

Quatre périodes essentielles de croissance du fruit sont à retenir pour les variétés Cavendish (Lassoudière, 2007) :

- Croissance faible jusqu'au début de l'allongement de la hampe florale ;
- Divisions cellulaires très actives de 10 jours avant à 30 jours après la sortie de l'inflorescence à l'extérieur. Les divisions cellulaires sont à l'origine du développement de la pulpe et correspondent à une phase de forte élongation du fruit et de faible augmentation du poids sec de la pulpe ;
- Accroissement cellulaire de 30 à 80 jours après la sortie de l'inflorescence à l'extérieur. Cette période correspond au remplissage des cellules de la pulpe par accumulation d'amidon qui est la forme principale de stockage. Les assimilats sont amenés jusqu'aux fruits par le pédoncule dont le rôle est uniquement conducteur ;
- Phase finale de maturation et d'hydrolyse de l'amidon.

[1] L'entièreté de ce paragraphe (1.4.2. le fruit) a été rajouté à la thèse après acceptation de la publication « La banane : de son origine à sa commercialisation » par la revue B.A.S.E. et ne se retrouve donc pas inséré dans la publication.

La phase finale de maturation commence avant la récolte et avant le remplissage maximal du fruit.

Au sein d'un même régime, des différences de développement sont observées. Les fruits initiés les premiers sont par exemple de 30 à 40% plus gros que ceux initiés les derniers (Robinson, 1996). Les écarts de longueur entre la deuxième et la dernière main s'accroissent du début de relèvement des doigts jusqu'à la récolte mais la valeur relative reste constante (20%) (Lassoudière, 2007). En revanche, pour le grade, les écarts ne deviennent importants qu'au cours du mois précédant la récolte (Lassoudière, 2007). Il a été suggéré que le développement différentiel des fruits résulterait des différences au niveau des divisions cellulaires et des caractéristiques de remplissage des fruits causées par la différence d'âge observée entre les fruits. Lassoudière (2007) précise que si la différence d'âge entre les fleurs d'une même main n'est que de deux jours en moyenne, elle est de plus de 15 jours entre les mains 1 et 8. Jullien *et al*. (2001) ont estimé que la différence entre les premières et dernières mains était de 70 degrés.jour.

1.4.2.2. **Physiologie du fruit**

Il existe deux groupes de fruits : (i) les fruits climactériques, comme la banane, la pomme et l'avocat et (ii) les fruits non-climactériques qui comprennent les agrumes, les fraises ou les cerises. Le processus de maturation des fruits climactériques se caractérise par une forte augmentation du taux de respiration (appelé pic climactérique) et par une production endogène d'éthylène. La période post-récolte des bananes d'exportation comprend trois étapes principales (John & Marchal, 1995): premièrement, la phase pré-climactérique au cours de laquelle le fruit reste immature ; deuxièmement, la phase de maturation accompagnée d'une intense activité respiratoire et enfin, la phase de sénescence du fruit.

La phase pré-climactérique, aussi appelée la durée de vie verte, est particulièrement importante pour les mûrisseurs qui recherchent une durée de vie verte la plus longue possible afin de commercialiser des fruits de qualité. Durant cette période, les fruits verts matures présentent une faible activité catabolique et métabolique. Le taux de respiration est faible et la production d'éthylène presque indétectable (Marriott & Lancaster, 1983). La durée de vie verte peut être allongée en modifiant certains paramètres environnementaux de stockage tels que la température, l'humidité relative et la composition atmosphérique.

La période climactérique se caractérise par 3 principaux processus (Seymour *et al*., 1993). Premièrement, une augmentation de la respiration du fruit indiquée par une

augmentation de la production de CO_2. Deuxièmement, une diminution du niveau d'oxygène dans la pulpe et finalement une augmentation rapide et transitoire de la production d'éthylène par la pulpe. Ce pic climactérique peut se produire sur le plant mais dans le cas des bananes d'exportation, celui-ci est induit après la récolte par un apport exogène d'éthylène avant la production naturelle. L'éthylène est physiologiquement actif à faible dose (Peacock, 1972). Il s'agit d'une hormone végétale naturelle qui est synthétisée par la pulpe (Dominguez & Vendrell, 1994) à partir de L-méthionine. Celle-ci sera convertie en S-Adenosylméthionine (SAM) qui sera à son tour transformée en acide 1-aminocyclopropane-1-carboxylique (ACC), précurseur immédiat de l'éthylène. Toutes formes de dommages physiologiques au cours de la croissance du fruit, de la récolte ou de la maturation peuvent aboutir à un stress, à une stimulation de la maturation et de la sénescence et donc influencer la qualité des bananes (Omoaka, 2000). De plus, les maladies parasitaires, telles que les pourritures de la couronne, sont reconnues comme réduisant la durée de vie verte et la qualité des fruits (Jones, 2000).

La maturation des bananes se caractérise par de nombreuses modifications de la pulpe et de la peau permettant l'obtention d'un fruit comestible. Les principales modifications sont la transformation des réserves amylacées en sucres, une chute brutale de la teneur en chlorophylle de la peau et un ramollissement de la pulpe.

La dernière phase du développement post-récolte des bananes d'exportation est la phase de sénescence qui se caractérise par une importante perte de fermeté du fruit.

1.4.3. **Itinéraire technique**

De la plantation à la consommation, la banane dessert d'exportation de type Grande Naine (sous-groupe Cavendish AAA) exige de nombreuses opérations techniques pouvant être très différentes en fonction des zones de production et des systèmes de culture. A titre d'exemple, certains aspects de la culture tels que rencontrés dans la région de Njombé au Cameroun sont présentés ci-après.

1.4.3.1. **De la plantation à la floraison**

Le premier cycle de culture est mis en place au champ par la plantation, en ligne ou en touffes, de rejets, de souches ou de plants issus de la culture *in vitro*. L'objectif principal de l'utilisation de vitroplants est de disposer au champ d'un matériel sain, en particulier indemne de nématodes, de virus et de bactéries. Au cours de sa croissance végétative, le bananier émet des rejets latéraux. Un unique rejet sera sélectionné, par une technique appelée œilletonnage,

afin d'assurer le cycle de culture suivant tout en conservant au maximum une structure de population constante. Le rejet successeur sera sélectionné le plus tôt possible pour favoriser son développement. L'objectif est un retour de cycle de durée minimale, afin d'augmenter le nombre de régimes récoltés par bananier et par an.

1.4.3.2. **De la floraison à la récolte**

Dès l'émergence de l'inflorescence commencent les soins aux régimes. Ces soins vont conditionner la qualité des fruits au moment de la récolte. Les feuilles susceptibles de gêner le développement du régime, ou risquant d'abîmer les fruits par frottements, sont dégagées. Cette opération consiste à découper ou écarter les feuilles en contact avec l'inflorescence. Dans la mesure du possible, cette pratique est limitée au maximum afin de ne pas diminuer le potentiel photosynthétique du bananier.

Au stade « doigts horizontaux » le bourgeon mâle et les dernières mains sont supprimés afin de privilégier la croissance des mains supérieures. Seuls deux doigts, appelés « tire-sèves », sont préservés. Ces derniers permettent d'arrêter les remontées de pourritures dans le rachis.

Les restes des pièces florales sénescentes présentes à l'extrémité des fruits sont également supprimés (Figure 3). Cette opération, nommée épistillage, permet d'éviter une source importante d'inoculum pathogène et de limiter les blessures par contact avec les autres doigts.

Les régimes sont ensuite gainés à l'aide d'un film de polyéthylène permettant de tamponner les variations de température, d'assurer une meilleure croissance des fruits, de présenter une barrière mécanique contre les parasites et de protéger les fruits contre les agressions mécaniques dues, par exemple, aux frottements des feuilles (Figure 3).

Le marquage des régimes se fait également au stade « doigts horizontaux » et permet les prévisions de récolte (Figure 3). En effet, les différents régimes arrivés à ce stade sont marqués d'une bande de couleur spécifique dans le but de connaître leur âge et de prévoir la date de récolte à un âge physiologique déterminé. En fonction des plantations, 9 à 12 couleurs de marquage sont utilisées dans une succession hebdomadaire.

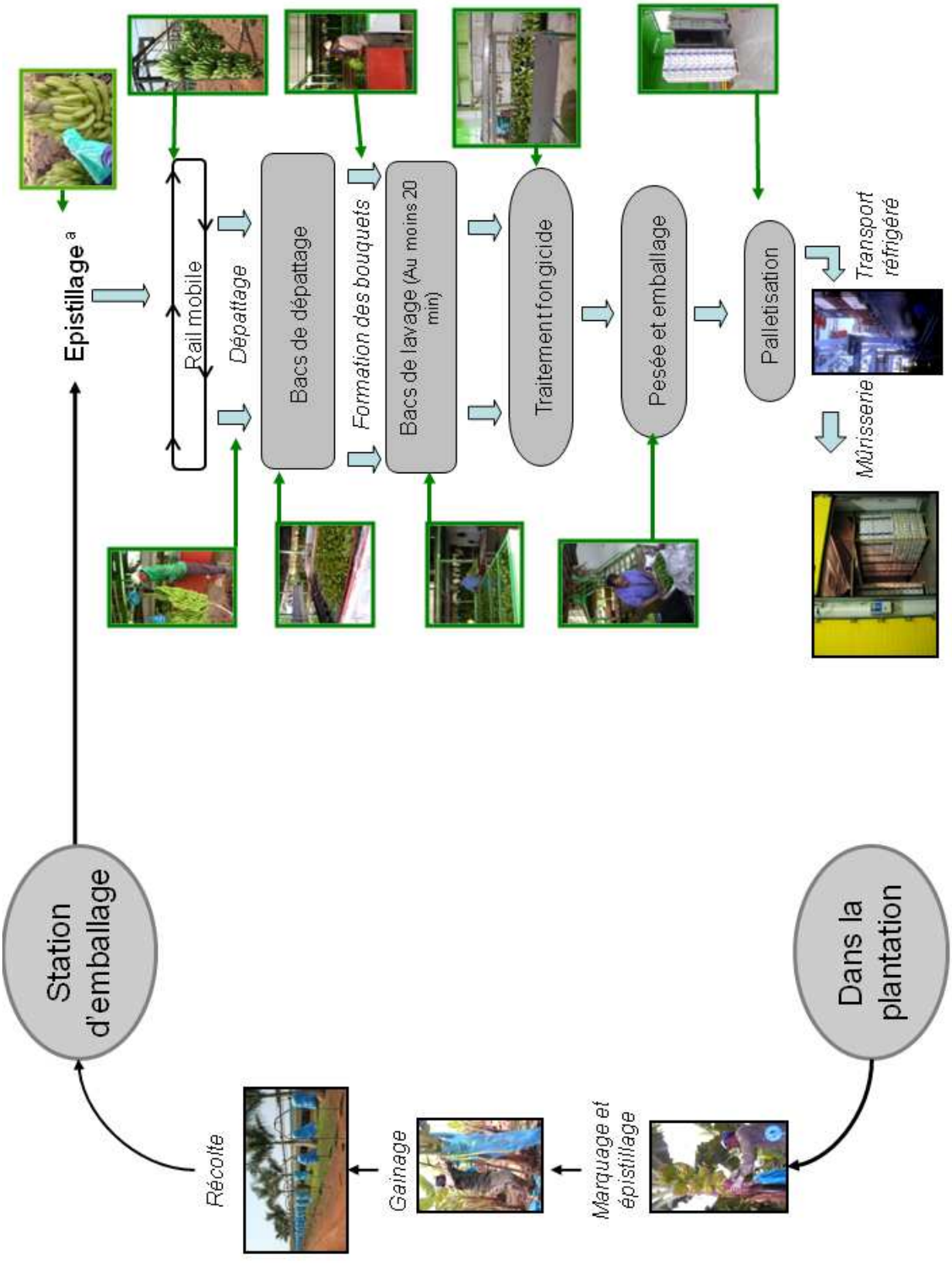

Figure 3: Opérations successives réalisées du champ à la commercialisation (modifié de Lassois *et al.*, accepted). [a] : l'épistillage est réalisé en station de conditionnement lorsqu'il n'a pas été effectué préalablement au champ.

1.4.3.3. **La récolte**

La récolte des régimes ne s'improvise pas. L'objectif est de récolter au grade le plus élevé possible compatible avec l'absence de mûrs d'arrivage à l'entrée en mûrisserie. Le stade de récolte sera donc fonction des délais et des conditions prévalant entre la coupe et l'entrée en mûrisserie. Traditionnellement, la récolte s'effectue lorsque le grade commercial est atteint. C'est-à-dire lorsque le fruit de référence, représenté par le doigt médian du rang externe de la deuxième ou de la quatrième main, a un diamètre de respectivement 36 ou 34 mm. Les fruits sont à ce stade remplis au ¾ et sont encore verts et durs. Le seul critère du grade n'est pas suffisant pour décider du stade optimal de récolte. En l'absence de facteurs limitant, le grade de coupe est atteint lorsque le fruit a accumulé 900°C jours au seuil de 14°C depuis le marquage au stade doigts horizontaux. A cet âge physiologique les fruits ont une durée de vie verte (DVV) qui correspond au temps écoulé entre la coupe des fruits et le début de leur crise climactérique et qui est compatible avec leur transport maritime et leur acheminement vers la mûrisserie. Il est ainsi possible de prévoir la récolte à partir de la date de floraison et de l'utilisation de données météorologiques (Jullien *et al.,* 2008). L'intervalle de temps entre la floraison du bananier et la récolte du régime, appelé « intervalle fleur-coupe » (IFC), est donc théoriquement constant lorsqu'il est exprimé en somme de températures. Il est par contre très variable en jours en fonction de la zone de production, de la saison et surtout des pratiques culturales.

La récolte s'effectue à la machette avec toutes les précautions nécessaires pour éviter les chocs et meurtrissures aux fruits. Les régimes sont portés à l'extérieur des parcelles dans des berceaux matelassées positionnés sur la tête. Le régime est alors déposé avec le berceau dans une remorque ou accroché à un système de câbles qui traverse la bananeraie jusqu'au hangar d'emballage (Figure 3). La récolte du régime marque le début du dépérissement du pied-mère qui est alors coupé. Sa suppression enlève la dominance apicale sur le rejet préalablement sélectionné et permet de poursuivre la culture.

1.4.3.4. **De la récolte au conditionnement**

A la station d'emballage, les régimes sont accrochés à un rail et les mains sont séparées de la hampe florale à l'aide d'un couteau (Figure 3). Les mains sont ensuite plongées dans un bac d'eau enrichi en chlore et en alun appelé bac de dépattage afin de permettre l'écoulement du latex (Figure 3). A la sortie de ces bacs, les mains de bananes sont récupérées, parfois frottées à l'aide d'une éponge savonneuse, et sont découpées en bouquets de 3 à 8 fruits. Ces derniers sont alors placés dans un second bac, appelé bac de lavage, pendant au moins 20

minutes (Figure 3). Ils sont ensuite acheminés sur des tapis roulants vers la zone de traitement fongicide avant d'être pesés et conditionnés dans des emballages plastiques (sacs en polyéthylène perforé ou non, avec ou sans vide d'air) et disposés dans des cartons d'exportation (Figure 3). Les techniques de traitement chimique sont très variées: trempage, tunnel de pulvérisation, pulvérisateurs, cascades, badigeonnage manuel, etc…Mais il semble qu'un bon mouillage des fruits soit essentiel pour assurer une bonne efficacité des traitements fongicides (de Lapeyre de Bellaire *et al.*, 1994).

1.4.3.5. **De la station d'emballage à la mûrisserie**

Les cartons de bananes sont regroupés sur des palettes et sont stockés dans un container refroidi à 13°C (Figure 3). La mise au froid permet d'une part, de minimiser la production d'éthylène et de retarder le processus de maturation et d'autre part, de réduire le développement de champignons éventuellement présents (Krauss *et al.*, 2000). Ces containers sont acheminés par camions vers le port de Douala où les palettes sont débarquées et entreposées dans les cales de navires. Par la maîtrise de la température, de l'hygrométrie et de la composition de l'atmosphère, ces cales assurent la conservation des bananes durant la traversée maritime. Au bout d'une dizaine de jours, les palettes sont débarquées dans le port de destination et sont acheminées par voies terrestres vers les mûrisseries où s'effectuera la maturation artificielle des bananes (Figure 3). Cette maturation est initiée par un apport exogène d'éthylène durant 24h à une température de 20°C. Au terme de ces 24h, les fruits sont ventilés et peuvent être commercialisés.

1.4.4. **Avantages et limites de l'utilisation exclusive de la Cavendish**

Le sous-groupe homogène des Cavendish (AAA) a pu être adopté dans presque toutes les régions tropicales humides pour son énorme potentiel productif (jusqu'à 60 tonnes/ha) associé à une bonne précocité (récolte en 10 mois) et une taille réduite (moins de 3 m) facilitant sa culture (Lescot, 1998). Ainsi, les acteurs de la filière ont fortement investi et se sont organisés exclusivement autour du standard Cavendish. Les efforts de recherche et développement ont été dirigés vers l'optimisation des modes de production, emballage, transport, mûrissage et marketing des bananes Cavendish. A l'heure actuelle, le processus et l'équipement industriel de production et de distribution sont adaptés à la Cavendish. Ce schéma industriel ne laisse que très peu de place à l'introduction d'autres variétés et aux changements. Elle est soumise à une forte pression normative qui pousse à banaliser le produit et à rendre ce marché monolithique : la banane dessert au « format » Cavendish, correspondant aux normes Dole ou Chiquita. Dans l'ensemble des fruits et légumes, il

n'existe pas d'exemple semblable. L'offre du marché pour un fruit est, dans tout les cas, constitué d'au moins deux variétés.

Ainsi, malgré la diversité génétique des bananiers, il n'est pas rare dans certaines régions de production destinée à un commerce d'exportation, de ne rencontrer que de la Cavendish. La diversité des cultivars existant au sein d'une même structure de production est d'autant plus réduite qu'on s'éloigne du centre d'origine du complexe d'espèces pour aller vers des régions ou seuls quelques exemplaires de bananiers ont été introduits. Cette forte spécialisation variétale et géographique s'accompagne d'une concentration du pouvoir de marché entre quelques grandes firmes (Loeillet, 2005).

Il existe évidemment des contraintes liées à la monoculture intensive de type agro-industriel, sans rotation, faisant appel à d'importantes quantités d'intrants et pratiquée durant de nombreuses années (parfois plus de 40 ans) (Lescot, 2004). Ces contraintes sont notamment d'ordre environnemental et phytosanitaire. Ce type de culture peu respectueuse de l'environnement aboutit notamment à une évolution et dégradation des sols. Des déséquilibres biologiques apparaissent rendant la culture plus sujette aux maladies et ravageurs. De plus les populations de pathogènes aériens ou telluriques, inféodés à la culture, ont tendance à s'accroître s'il n'y a pas rupture de leur cycle biologique due à la suppression de l'hôte. Les risques de résistance aux pesticides sont également accrus dans ces conditions et l'apparition de nouvelles maladies est une réalité. Ainsi, le problème majeur des productions bananières sont les lourdes menaces parasitaires qui pèsent sur ces productions face à l'étroitesse de la gamme variétale cultivée à l'heure actuelle en monoculture intensive.

Chez la banane, le premier problème est apparu lorsque la Gros-Michel a été cultivée de manière intensive et que ce premier cultivar commercial a été détruit avec l'apparition de F*usarium oxysporum* cubense ou maladie de Panama. Après 1960 le commerce international de la banane a été dominé par le cultivar de type Cavendish. Plus récemment, une forme extrêmement virulente du pathogène *F. oxysporum*, la race T4, et pouvant s'attaquer aux Cavendish a été décrite (Hwang *et al.*, 2004). D'autres problèmes parasitaires associés aux Cavendish sont apparus et ont été largement développés par divers auteurs (Jones, 2000; Ploetz *et al.*, 2003). Parmi les différentes contraintes liées aux parasites et ravageurs, il faut signaler que les maladies d'origine fongique, très répandues dans les plantations industrielles, constituent la principale perte de rendement et affectent tous les organes de l'hôte (Ploetz *et al.*, 2003). A l'heure actuelle, les plus néfastes, affectant de manière significative la production des Cavendish d'exportation, sont les cercosporioses. D'autres maladies

fongiques, propres aux marchés d'exportation et causant d'importants dégâts et pertes économiques, méritent qu'on les souligne. Il s'agit des maladies de conservation comme les pourritures de la couronne (Lassois *et al.*, accepted) et l'anthracnose.

1.5. Conclusion

Malgré l'importante diversité génétique existant au sein du genre *Musa* le marché d'exportation est dominé par la culture presque exclusive de la variété Cavendish. Cependant, la production de banane d'exportation doit être capable de répondre aux exigences de durabilité, qualité et rendement qui lui sont imposées. Ceci constitue un véritable défi pour les variétés existantes et ce de part l'étroitesse de la gamme variétale utilisée et l'apparition de nouvelles maladies ou de nouvelles souches virulente. Ainsi, d'importantes stratégies de lutte doivent être élaborées.

En bananeraies intensives, le système de culture dominant a longtemps reposé sur une large utilisation systématique des produits phytosanitaires. Au début des années 90, les bananeraies étaient traitées avec des quantités très élevées de matières actives : de 17 à 22 kg/ha/an (Lassoudière, 2007). Actuellement on ne peut plus concevoir la protection phytosanitaire uniquement sous le seul aspect de la lutte chimique. Celle-ci pose de gros problèmes tels que la toxicité des produits, l'accumulation de résidus dans les fruits, la destruction d'organismes non cibles, le risque de pollution des eaux, les pertes d'efficacité des produits. De plus, la protection phytosanitaire doit faire face à une législation de plus en plus restrictive. Pour certaines maladies, comme la maladie de Panama, aucun produit phytosanitaire n'a jamais apporté de réponse satisfaisante.

Ainsi, l'ensemble du système de culture et de l'itinéraire technique est à adapter. Il faut envisager une approche globale de la filière banane dans un but de durabilité de la production. Des solutions techniques ont permis de réduire de plus de 50% l'utilisation des pesticides aux Antilles Françaises au cours de la dernière décennie (Chabrier *et al.*, 2005). Parmi les techniques alternatives à la lutte chimique, on retrouve également la sélection et l'amélioration variétale. C'est aujourd'hui la voie privilégiée pour maintenir à plus ou moins long terme la culture de la banane dans les zones actuelles de production mais il s'agit d'une action de longue haleine. Les premiers travaux d'amélioration des bananiers par croisement ont été amorcés dès les années 20 à la suite de l'extension de la maladie de Panama. Parallèlement à ces activités de croisement, d'autres équipes ont concentré leurs efforts, à partir des années 80, sur la mutagenèse et sur la sélection de variants somaclonaux qui sont apparus à la suite du développement des techniques de cultures *in vitro* pour la multiplication

rapide et industrielle des vitroplants de bananiers. Le comportement de variétés issues de mutations induites par l'application de rayons ionisants sur les bourgeons végétatifs est également évalué. Enfin, l'avènement des techniques de biologie cellulaire et moléculaire a favorisé l'émergence d'équipes qui travaillent sur la transformation génétique des bananiers.

Cependant, les réponses sur la durabilité de la culture bananière ne relèvent pas d'une seule solution miracle, à savoir la recherche d'un produit phytosanitaire plus performant ou même uniquement la mise au point d'un bananier résistant. Elle dépend plutôt d'une mutation des systèmes de culture par l'amélioration d'itinéraires techniques complets basée sur une connaissance approfondie de l'agronomie de la plante, de la biologie des parasites et des relations hôte-pathogènes.

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2. Crown rot of bananas: pre-harvest factors involved in post-harvest disease development and integrated control methods

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Crown rot of bananas: pre-harvest factors involved in post-harvest disease development and integrated control methods

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Abstract

Crown rot is a complex disease which affects export bananas in all producing countries. Usually invisible when the fruits are packed for transportation from tropical countries to distant destinations, disease symptoms occur during shipment, ripening, and storage. This disease, characterised by rot and necrosis, affects tissues joining the fingers with each other, called the crown. It may reach the pedicel and ultimately the banana pulp when the crown rot is severe. Losses from 10% to 86% have been recorded for bananas respectively treated and untreated.

In this paper, we proposed to inventory current knowledge on crown rot disease and associated control measures which must be considered throughout the production channel in order to be effective. We suggest a new, approach to consider this post-harvest disease of bananas. In this approach we consider that bananas elaborate in the field a fruit quality potential. We consider that this new concept of preharvest quality potential is a key factor in understanding crown rot development, since is it responsible for most of disease incidence variations observed in the industry. The fruit quality potential depends on both a physiological and a parasitic component, both of which depend on agro-technic and pedo-climatic factors of the crop production area. The physiological component is defined as the sensitivity of the fruits to crown rot, while the parasitic component reflects the capacity of the parasitic complex to induce a level of disease.

The content of this review is divided into two parts. First the fruit quality potential at field level is addressed, with special interest on its physiological and parasitic components. Secondly, the control methods are examined at different steps of the channel, in order to give an overview of a possible integrated control strategy.

2.1. Introduction

Bananas are grown in over 120 countries and rank second in world fruit production, after oranges and before grapes. The banana industry, which is based on a small number of cultivars belonging to the Cavendish subgroup, is a vital source of income, employment, and export revenue for most exporting countries, which are mainly developing countries in Latin America, the West Indies, Southeast Asia, and Africa. However, major losses often occur during shipping of bananas to their final market mainly because of ripening incidents, appearance defects, and storage decay such as anthracnose and crown rot that occur during shipping. Such post-harvest diseases negatively impact the market value of bananas, especially when they are assessed at the port of arrival or in ripening facilities, thus contributing to banana quality depreciation and constraining export trade. Anthony *et al.* (9) reported that post-harvest diseases were responsible for 20% of harvest losses in Sri Lanka in 1997.

Crown rot affects export bananas in all producing countries and is considered to be one of the main export banana post-harvest diseases (61, 83). This became a major problem in the banana industry during varietal reconversion initiatives in the 1960s. Up until 1960, only Gros-Michel subgroup cultivars were cropped for export, and for economic and practical reasons bananas were shipped in complete bunches consisting of double layers of fruits called hands which are arranged helicoidally around a central axis called the stalk. These cultivars were, however, susceptible to Panama disease (*Fusarium oxysporum* f. sp. Cubense) and thus replaced by Cavendish subgroup cultivars because of their resistance to Panama disease. However, Cavendish bananas are more fragile during shipping (33) and this triggered a revolution in the banana industry as bunch shipping was discontinued. Bananas were instead cut into clusters consisting of several banana fruits joined by the crown tissues (Fig. 1) and boxed for shipping.

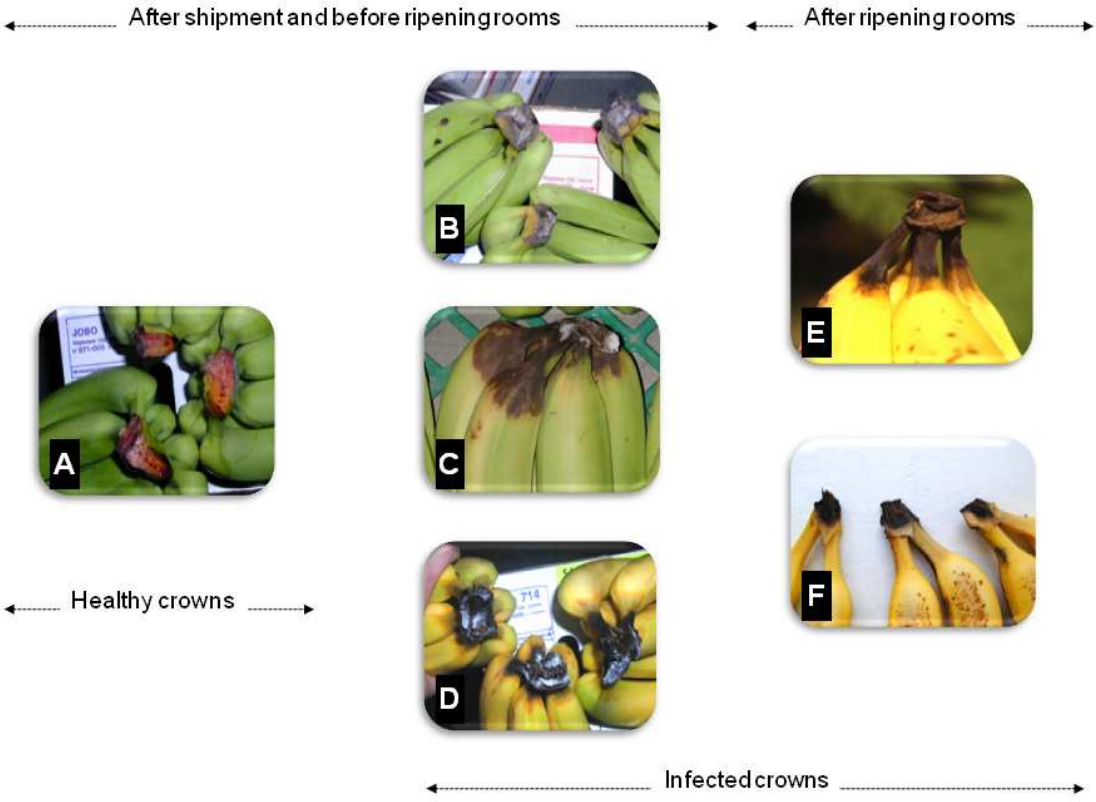

Fig. 1: Crown rot symptoms. (A) Healthy crowns after maritime shipping before being placed in ripening rooms; (B) Crowns diseased with a superficial mycelium after maritime shipping; (C) Rot noted on bananas after maritime shipping; (D) Rot on the peduncles inducing banana ripening upon their arrival after maritime shipping; (E) External crown rot symptoms after fruit ripening; (F) Internal crown rot symptoms after fruit ripening.

The crown became a prime site for infection by different pathogens (41, 52, 64). The incidence of crown rot periodically increases during the rainy season, and losses of over 10% have been recorded in the UK in bananas coming from the Windward Islands that were harvested during this period (61). Losses of up to 86% have also been reported in non-chemically treated bananas from the Philippines (6).

The first studies on crown rot focused on (i) the etiology of this disease, especially on the identification of the most pathogenic fungal species involved; and (ii) on post-harvest control methods, mainly chemical. Nevertheless, these studies provided neither a good understanding of the conditions most conducive for disease expression nor adequate control of this disease at all spatio-temporal scales. There is now some evidence that fruit physiology at harvest influences crown rot development and that all interactions among the microorganisms implicated should be considered. Taking these into account, a banana quality build-up scheme was proposed (Fig. 2).

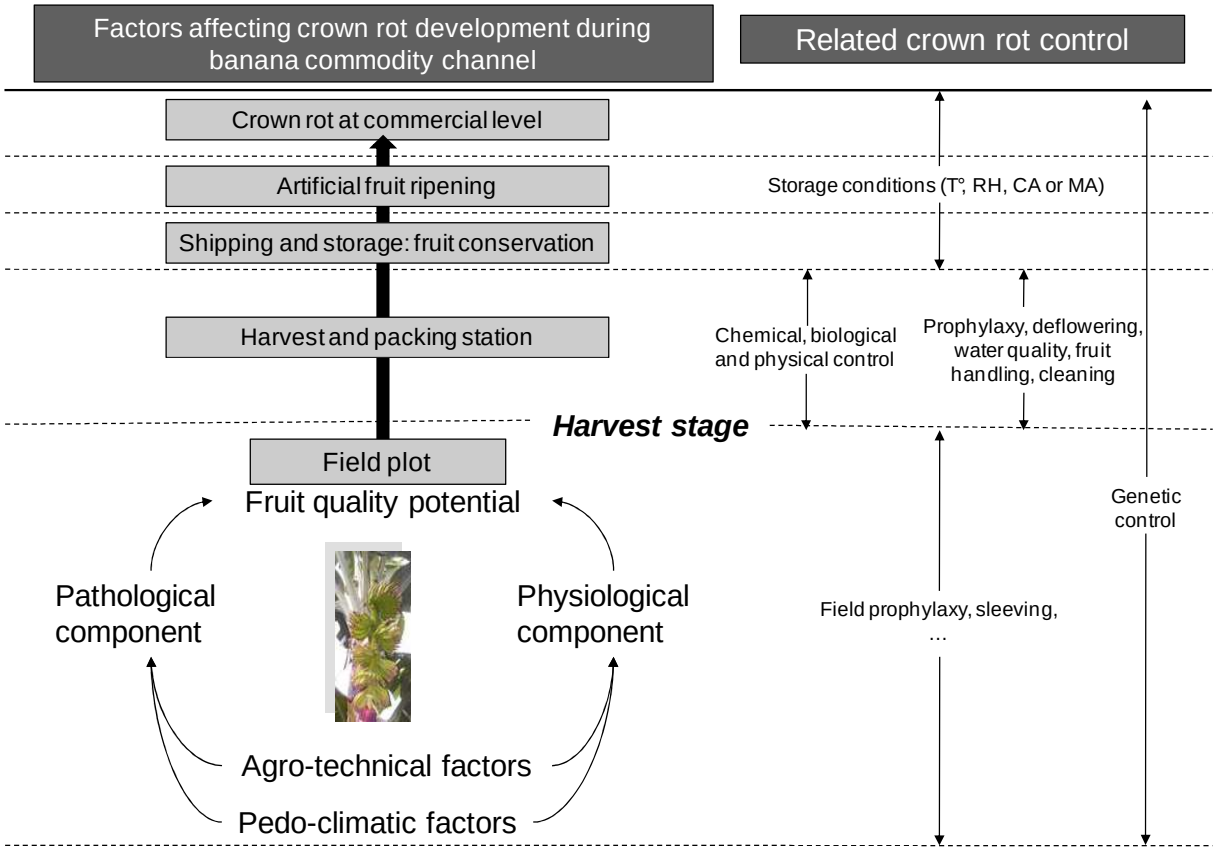

Fig. 2: Diagram representing different key factors that arise throughout the commodity chanel concerning crown rot development at the commercial stage, and related control methods. T°: temperature; RH: relative humidity; CA: controlled atmosphere; MA: modified atmosphere.

In this scheme, the fruit quality potential at harvest is presented as a key factor in crown rot development. The quality potential elaborates during the banana growth phase in the field and depends on two components: (i) a fruit physiological component, which determines the fruit susceptibility to the disease; and (ii) a parasitic component which reflects a level of crown contamination by the fungal complex, as well as the pathogenicity of this complex. The influence of environmental and agrotechnical pre-harvest factors on these two components of the fruit quality potential is a new approach in the post-harvest disease research, and we will therefore discuss their importance in this review.

2.2. Crown rot symptoms

Crown rot affects tissues of the so-called “crown”, which unites the peduncles (Fig. 1). The rot is not visible when the bananas are boxed, and symptoms generally appear only after maritime shipping. The rot begins with mycelial development on the surface of the crown (Fig. 1B), followed by the peduncles (Fig. 1D) and fruit (Fig. 1C). The bananas may detach from the peduncle during severe infections. Crown rot results from the development in the

crown of several common fungi, which constitute a fungal complex, and leads to softening and blackening of tissues at the site of the wound left when the cluster was cut from the bunch. The role of bacteria in the complex (64) has not been confirmed by detailed research. The symptoms develop rapidly during ripening when the physiology of the fruit undergoes modifications that facilitate fungal development (46).

Crown rot affects the fruit quality because of the development of necrosis on the fruit, and it can also trigger early ripening of bananas during shipping (77) (Fig. 1D). Ripening may be caused by ethylene released by stressed and necrotic tissues (31, 65, 89), but also by ethylene produced by mycelia of fungi such as *Colletotrichum musae* (23, 78). Onset of the disease cannot be predicted and it spreads in an irregular pattern on all clusters in a shipping box. The same box may contain both healthy and infected clusters.

2.3. Fruit quality potential as a key factor in crown rot infection patterns

Geographical and seasonal variations have been noted in the incidence of crown rot disease of bananas (61, 64, 87). Lukezic *et al.* (64) showed that the incidence of this disease varied throughout the year in Honduras. It is generally higher during the summer (March–September) and declines during the coldest period (October–February). They also demonstrated that this pattern did not seem to be correlated with variations in the fungal complex isolated from banana crowns. Moreover, in Jamaica, a high disease incidence was found to be correlated with periods during the year when temperatures were highest (87), whereas in the Windward Islands, incidence was reported to be highest during the rainy period (61). These spatiotemporal fluctuations reflect the variations in the banana fruit quality potential that depends both on a parasitic and physiological component (Fig. 2).

2.3.1. Parasitic component of the fruit quality potential

In crown rot, the parasitic component reflects a level of crown contamination by the fungal complex, as well as the pathogenicity of this complex.

2.3.1.1. Etiology of crown rot and pathogenicity of the fungal complex

Crown rot is the result of the activity of a fungal complex. The microorganisms most commonly isolated in crown rot are: *Musicillium theobromae, Colletotrichum musae, Ceratocystis paradoxa, Lasiodoplodia theobromae*, *Nigrospora sphaerica*, *Cladosporium* sp., *Acremonium* sp., *Penicillium* sp. and *Aspergillus* sp., as well as many *Fusarium* spp.,

including *F. semitectum*, *F. verticillioides*, *F. sporotrichoides*, *F. oxysporum*, and *F. solani* (9, 39, 41, 51, 64, 66, 71, 76, 85, 96, 97).

Several organisms may be involved in disease development. Moreover, disease severity and the nature of the complex may vary substantially, depending on the production area (Table 1) and season. Lukezic and Kaiser (63) showed that fungal populations may differ between banana plants and even between crowns.

Fungi of the complex do not all have the same pathogenicity and variations have been reported between regions (Table 1). Knight (58) considered that *F. oxysporum*, *F. verticillioides* and *F. graminearum*, which have been isolated frequently from crowns of Windward Island bananas, are primary pathogens, whereas *L. theobromae*, *M. theobromae*, and *N. sphaerica* are considered to be relatively nonpathogenic species. According to the findings of Marin *et al.* (66) in Costa-Rica, *F. verticillioides* and *F. semitectum* are the most pathogenic species. According to Griffee (40), *C. musae*, *L. theobromae*, *C. paradoxa*, *F. semitectum*, and *F. graminearum* are major pathogens involved in this disease. However, many authors agree on the strong pathogenicity of *C. musae*, which may trigger an infection from a very small amount of inoculum (34, 39, 62, 64, 85). Other pathogens require larger amounts of inocula to induce crown rot symptoms (34, 40, 59, 60, 62).

A wide range of fungal complex compositions have been noted in natural contaminations. The nature of this composition, the specific pathogenicity of the different microorganisms involved as well as interactions among them may even alter the pathogenicity of the complex. *C. musae* was more pathogenic when it was inoculated alone than when it was coinoculated with other species (62); however, Anthony *et al.* (9) found that *L. theobromae*, *F. Verticillioides*, and *C. musae* were more highly pathogenic when coinoculated than when they were inoculated separately.

The respective roles of the different species that could belong to the fungal complex has been thoroughly studied, whereas little information is available on the antagonistic or synergistic relationships among these different species. It is essential to gain greater insight into these interactions so in order to better characterise the pathogenicity of the fungal complex.

Table 1: Bibliographic data on the different main fungal species isolated from crown rot in different banana-producing regions. Percentage values correspond to isolation frequencies when given by the authors. "X" indicates that the pathogen has been identified on crown by the authors, but without providing any isolation frequency. F: frequently isolated; MF: most frequently isolated; R: rarely isolated. "I" indicates that authors have only evaluated the pathogenicity of different know fungal species without information about the isolation frequencies. Informations about the pathogenicity are also showed when given by authors. : Highly pathogenic; : Medium pathogenicity; : Slightly or non pathogenic.

References / Countries		*Colletotrichum musae*	*Fusarium semitectum*	*Fusarium verticillioides*	*Fusarium sporotrichoides*	*Fusarium oxysporum*	*Fusarium solani*	*Lasiodiplodia theobromae*	*Musicillium theobromae*	*Gliocladium roseum*	*Nigrospora sphaerica*	*Acremonium sp.*	*Penicillium sp.*	
Honduras	64	3%	80%	14%					12%	81%			93%	
Central and South America	39	F	MF	X					R	F		R		
	66	0-10%	4-50%	0-28%							0-30%		0-8%	0-33%
Windward Islands (WI)	97	23-33%								5-7%	5%			
	41	36%	27%	6%					2%	8%		<1%		
	96	24%	18%	3%		3%	2%		2%	3%	2%	<1%	2%	<1%
	51	26-44%	7-23%	6-21%					3-9%	4-18%	0-10%	24-26%	0-10%	
	62	I		I									I	
	34	X	X	X					X					
	57	I	I											
	58	I	I	I		I			I	I		I		
	40	I	I	I		I			I	I		I		
Jamaica	85	11%	F	X		X			3%	13%		1%	3%	
Sri Lanka	9	X		X					X					
Nigeria	76	27%	3%	10%		4%	6%		26%	2%				
Somalia, WI, Guatemala	71	38%	X	X	20%					2%			4%	

2.3.1.2. **Factors influencing the level of crown contamination by the fungal complex**

Very little information is available on the epidemiology of crown rot. The fact that the characteristics of each species involved in the disease complex differ complicates studies on the infection cycle. There are nevertheless some general features. Badger (11) showed that the relative humidity generally has to be over 86% for germination of conidia of most fungi involved in the complex. In banana plantations, this group of fungal species sporulates abundantly on all putrescent organs such as leaves (68, 88), floral parts (2, 28), and bracts (26, 63). *C. musae* and some *Fusarium* species are primary colonizers of decomposing leaves (69) and floral parts (26). Banana contamination by spores of *C. musae* and various *Fusarium* spp. mainly occurs during the first 40 days following bunch emergence, and these are gradually replaced by another fungal complex (28). Spores of some species such as *Colletotrichum* spp. are mainly disseminated by rainwater (26, 28, 47), whereas others are airborne (63).

The fungus *C. musae,* which is also a causal agent of anthracnose, establishes quiescent infections in the field during the first month following banana flowering (26). These quiescent infections could also contribute to the onset of crown rot if the pathogen has an opportunity to colonize the crown region (42); however, for the other pathogens, even though field infections cannot be excluded, infections mainly occur during harvest and when clusters are trimmed from bunches (70). Contamination generally takes place when hands are cut with a contaminated knife (35, 39, 94, 96), or when clusters are cleaned with contaminated washing water (86). At harvest, the fruits and senescent floral organs bear high quantities of spores that could potentially contaminate the crowns. Some of these spores could be removed by washing and then accumulate in the washing water (91), while others, such as *C. musae* appressoria, may adhere tightly to the fruit surface (84). The washing tanks are the main source of inocula according to Shillingford (86). After the banana clusters are dipped in the washing water, the spores can penetrate a few millimetres into the vascular vessels of the crown, and disease is then hard to control with a fungicide spray treatment (33). Green and Goos (39) showed that a suspension of *C. musae* spores could penetrate 5–7 mm into the crown tissues within only 3 min. The crowns may also be contaminated by airborne spores before the fruit are placed in boxes in the packing station. The risk is especially high when the facilities are dirty or if debris (pistils, stalks and fruits) is piled up nearby. Conidia can survive for several months under extreme temperature and humidity conditions before germination (70).

2.3.2. **Physiological component of the fruit quality potential**

The physiological component refers to the level of fruit susceptibility to crown rot, which reflects the physiological state of the fruit and is dependent on agrotechnical and pedoclimatic factors during plant growth. Only a few studies have been conducted on factors influencing the level of banana susceptibility to crown rot. In Guadeloupian conditions, it was shown that marked variations in fruit susceptibility could occur at the same production site over a period of 10 successive weeks (62). Variations in crown rot susceptibility between different Guadeloupian production areas during the same period have also been observed (L. Lassois, unpublished).

Fruit age as expressed in accumulated degree day (dd) (53) also seems to affect crown rot development. An intra-bunch banana crown rot susceptibility gradient has been documented. Clusters growing on the first hands of a bunch, which are, on average, 70 dd more advanced than the hands initiated last (54), are more susceptible than clusters developing on the last hands (L. Lassois, H. Bastiaanse, M. Chillet, M.H. Jijakli, A. Jullien, and L. de Lapeyre de Bellaire, unpublished). Moreover, a linear relationship between fruit age (in dd) and the susceptibility of the fruit to crown rot was reported, with the oldest fruit being most susceptible to this disease (36).

Some agricultural practices have a bearing on crown rot development. Modifying the source-sink ratio by trimming leaves and hands (where leaves are considered as sources and fruits as sinks) may induce variations in fruit susceptibility. Severe bunch trimming leads to a sharp drop in banana crown rot susceptibility when the bananas are harvested at a constant physiological age of 900 dd (L. Lassois, H. Bastiaanse, M. Chillet, M.H. Jijakli, A. Jullien, and L. de Lapeyre de Bellaire, unpublished).

The mechanisms underlying variations in fruit crown rot susceptibility have yet to be investigated. The plant mineral status has an effect on banana susceptibility to various diseases (18, 45). Preformed fungitoxic polyphenolic compounds could be involved in banana resistance to post-harvest diseases (1, 4, 19, 72, 74). Nevertheless, all factors potentially involved in plant resistance mechanisms could have an impact on the level of banana crown rot susceptibility. Plants are equipped with a series of defense mechanisms controlled through the expression of different genes. Genes governing observed susceptibility variations could be identified by assessing differences in gene expression between bananas with different levels of susceptibility. Studies are currently under way to identify the underlying mechanisms and key genetic factors involved in crown rot susceptibility variations using a differential

expression analysis technique (L. Lassois, P. Frettinger, L. de Lapeyre de Bellaire, P. Lepoivre and H. Jijakli, unpublished).

2.4. Crown rot control methods

Banana crown rot, like other storage diseases, has an especially detrimental impact on export produce. A routine postharvest treatment with a fungicide is the main method currently used to control this disease. Problems may, however, arise from differences in fungicide efficacy associated with the level of susceptibility of the fruit to crown rot or of the pathogens to the different fungicides. Finally, discharge of fungicidal slurries can also lead to environmental pollution, and residues of fungicides may subsequently be detected in the marketed bananas. Research focused on alternative nonchemical control methods is of considerable interest in the current increasingly prohibitive social and legal setting. It is essential to implement sound integrated control strategies throughout the commodity chain considering the complexity of the disease and the difficulty of its control (33, 91).

2.4.1. Field control methods

We have a set of methods that can usually be implemented in an integrated way to achieve efficient crown rot control (Fig. 2). These are mainly preventive measures aimed at limiting fungal contamination of bananas in the field.

2.4.1.1. Sanitation of banana plantations

Most species involved in the fungal complex are saprophytes that occur on senescent banana organs, especially on decomposing leaves (69). Old leaves present in the banana plantation may harbour inocula that could be responsible for severe contamination of bananas by this fungal complex (90). The inoculum pressure, and thus the disease development rate, may be reduced through regular elimination of senescent leaves around the fruit (91). Banana floral parts are also inoculum sources, especially for *C. musae* and several *Fusarium* species (28). In light of the potential role of bunch stalk contamination in the development of crown rot (35), early elimination of flower parts in the field is also essential for reducing bunch contamination by the pathogens (26).

2.4.1.2. Plastic sleeving to protect bananas

No accurate studies have been carried out to assess the impact of sleeving on the crown rot development rate. It is nevertheless known that bunch sleeving (Fig. 3) with perforated

plastic film protects bunches from fungal contamination, thus especially curbing the development of pitting disease (44) and speckling disease (52). Moreover, it has been shown that sleeving can reduce contamination of banana bunches by *C. musae* by over 80% (26). These findings suggest that sleeving directly reduces crown contamination in the field, or that sleeved fruits release fewer spores in washing water during packing operations.

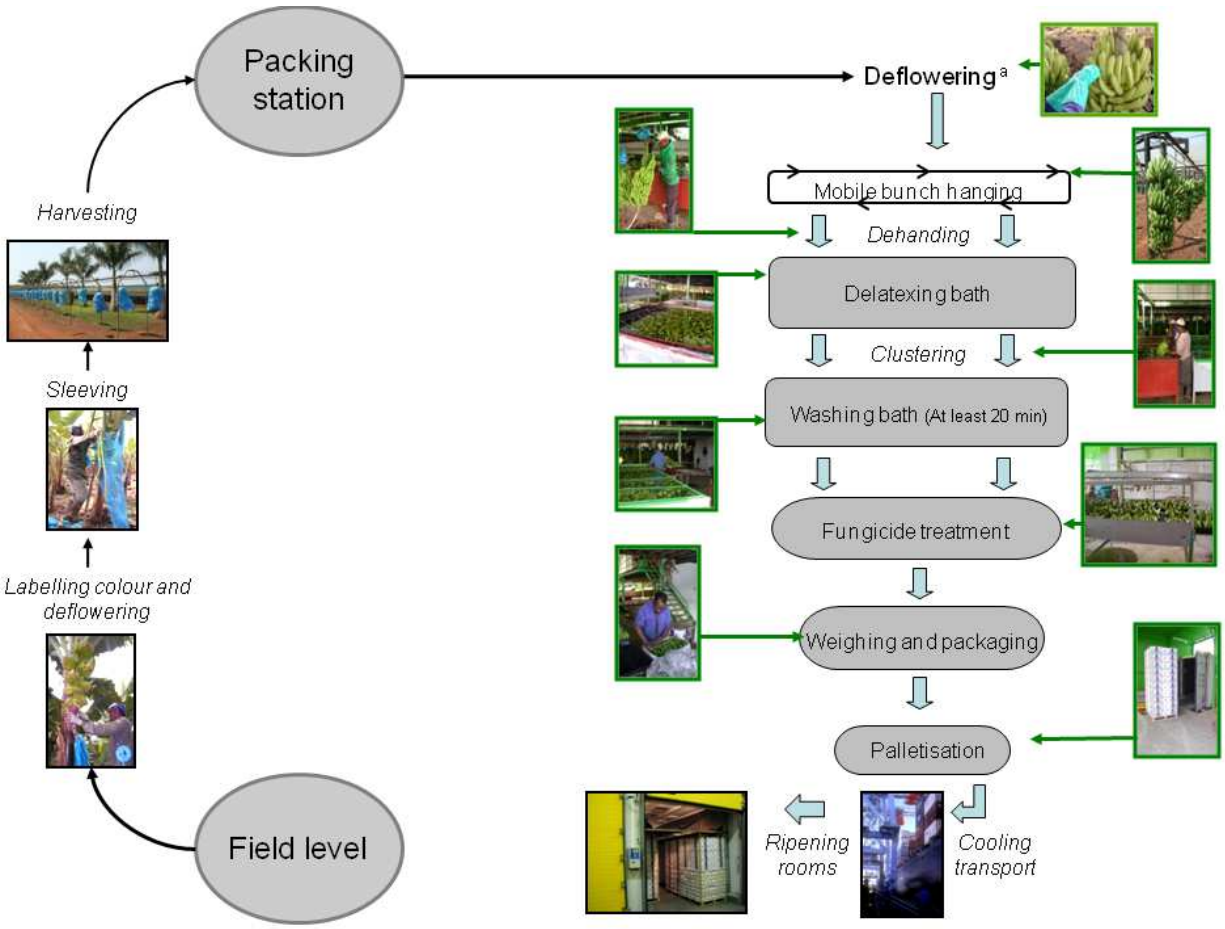

Fig. 3: Successive operations carried out from the field to the banana marketing stage. [a]: deflowering is done in the packing station when it has not been done in the field.

2.4.1.3. **Controlling the banana harvest stage**

Bananas are exported when they have grown to a commercial grade specified by European regulations (Commission Regulation (EC) No 2257/94 of 16 September 1994, setting quality standards for bananas) and market requirements. However, bananas should be harvested at an age that will ensure a sufficient conservation. It has also been shown that the best estimator of fruit shelf life is physiological age, expressed as a sum of accumulated temperatures from flowering to harvest rather than in days (53). It was shown that the physiological age of bananas (expressed in dd) has an impact on crown rot susceptibility (36).

The fruit physiological age should thus be taken into account at harvest. Some practices such as field trimming of false hands, some true hands, male buds and external fruits of hands can accelerate the fruit pulp filling rate. These practices can thus reduce the physiological age that bananas need at harvest to enable them to reach a sufficient commercial grade (61, L. Lassois, H. Bastiaanse, M. Chillet, M.H. Jijakli, A. Jullien and L. de Lapeyre de Bellaire, unpublished).

2.4.1.4. **Genetic control**

The range of varieties grown for dessert banana export is very narrow as all clones belong to the Cavendish subgroup (12). Moreover, breeding is complicated because triploid banana varieties are generally sterile. In the past, banana genetic improvement programs were mainly focused on obtaining varieties resistant to Sigatoka and Panama diseases. Resistance to crown rot of improved varieties has not been considered in any breeding programs to date.

According to Marin *et al.* (66), FHIA-01 and FHIA-02, two hybrids produced by the FHIA breeding program, are partially resistant to crown rot. Conversely, Perez Vicente and Hernandez (79) consider that these two varieties are more susceptible to crown rot caused by *F. semitectum* and *C. musae* as compared with Cavendish varieties. According to these authors, only FHIA-23 is more resistant to crown rot than Grande-Naine. Finally, the first FHIA hybrids were introduced in the late 1980s, but their characteristics differ from those of Cavendish bananas and consumers have shown little interest in them, thus limiting their distribution.

2.4.2. **Postharvest control methods**

2.4.2.1. **Chemical control**

Routine post-harvest fungicide treatment is still the most efficient crown rot control method. This strategy was introduced in the late 1960s with the discovery of systemic benzimidazole derivative fungicides (thiabendazole and benomyl). They are classified as antimitotic compounds. Gradually, other fungicides that inhibit ergosterol biosynthesis, such as imazalil and bitertanol, were also introduced (29, 37). The application methods vary markedly: dipping, spraying (Fig. 3), cascade treatment, etc., but in every case the bananas must be thoroughly wetted to ensure the fungicide treatment efficacy (29, 50). Alum is often combined with fungicide slurries to neutralise latex residue remaining on crowns when the bananas are removed from the washing bath (73); however, this mixture seems to have a negative impact on the performance of some active ingredients such as thiabendazole (50). The

time between crown trimming and fungicide application is critical. Crown rot severity seems to increase when fungicide application is delayed (39). Bananas are nevertheless usually treated in time in packing stations, i.e. just after they are removed from the washing bath. Problems concerning the development of resistant strains may arise with this practice. Postharvest fungicides generally have the same mode of action as those sprayed in the field to control Sigatoka disease. *C. musae* strains resistant to thiabendazole, and generally to all antimitotic compounds, have been detected in many banana-producing countries (27, 46, 51, 61). In Guadeloupe, it has been shown that thiabendazole-resistant *C. musae* strains developed after exclusive foliar applications of benomyl over a decade (1972-1982) for Sigatoka Disease control (27). Postharvest use of chemicals could ultimately be prohibited because the number of active substances registered for postharvest treatment has been reduced by current regulations, and legislation is becoming increasingly restrictive. Moreover, the efficacy of intensive fungicide treatments is not always adequate. In some areas where very few resistant strains are present, the efficacy of fungicides for postharvest disease control was found to vary substantially depending on season and production area (16). Finally, consumers are now highly selective with respect to food safety concerns, and demand is increasing for produce that has not undergone any postharvest chemical treatments. Alternative non-fungicide solutions are therefore being sought.

2.4.2.2. **Preventive measures in packing stations**

Deflowering in packing stations

Deflowering (Fig. 3) involves the removal of all floral parts that could potentially harbour inocula before the dehanding operation. When the floral parts have not been removed in the field, deflowering is done in the packing station before bunch trimming, thus reducing the risk of contamination of the different washing baths (61, 85).

Packing station water quality

Fruit dehanding is a risky operation because the resulting wound is the main portal for infection by crown rot pathogens. Contamination of crowns when the bananas are processed in the dehanding tanks and then in the washing tanks is a key step in the crown-rot development process (Fig. 3). Spore accumulation in the water can be reduced by regularly changing the washing water in the washing and delatexing baths (91) (Fig. 3). These baths can host a high quantity of spores, mainly *Fusarium* sp. and *Verticillum* sp., that detach from the peel surface (86), whereas *C. musae* conidia are less often found (85). Care should be taken to avoid

contaminating the baths with plant debris (pistils, leaves, trimming waste, etc.) and the water should be regularly refreshed.

It is also recommended that the bath water be treated with active chlorine (10, 33, 91) or quaternary ammonium disinfectants in order to hamper contamination (86), but the efficacy of this disinfection procedure is controversial (33, 91). The chlorine concentration should be regularly adjusted to offset the high observed losses by volatilization or through redox reactions with latex or other organic matter in the washing baths (86). It is especially hard to maintain the bath water quality when the water is recirculated in a closed system. In such situations, latex contamination gradually increases in the tanks, so it is hard to control the water quality simply through chlorine treatment. Note also that, due to changes in market standards, it is important to reduce health risks associated with the development of human pathogens in the washing baths.

Packing station sanitation

Protective measures implemented in the packing station are aimed at keeping the crowns of freshly trimmed bananas away from all inoculum sources. To ensure efficient crown rot control, it is thus essential to keep the packing station and the adjoining facilities clean. Waste (stalks, low-grade fruit, etc.) located in the vicinity of the banana packing area, which could contaminate the air in the station, must be eliminated (35, 61). It has been shown that trimming clusters in a clean environment rather than in the field can reduce the crown rot incidence by 50% (35).

The fruits should also be cleaned before being trimmed from the bunch. A contaminated trimming knife could spread inocula from the peel into the crown tissues (64). This may be avoided by washing the bunches in lightly chlorinated water before they are trimmed into clusters (61).

2.4.2.3. **Banana and crown trimming**

Bananas should be trimmed with a clean stainless steel blade. Finlay and Brown (34) showed that roughly trimming the crowns, or ripping them off the hands, significantly increased the level of fruit contamination. Tissue fragments on the surface of the crowns dry out and quickly become senescent, thus providing an ideal site for rot development (34). Moreover, banana trimming knife tips are rounded to avoid banana fruit wounds (61). It is also important to cut wide crown sections containing as much crown tissue as possible, a technique that seems

to enhance crown resistance to rot and seldom leads to the spread of rot into the fruit pedicels (73).

2.4.2.4. **Banana storage techniques**

The temperature, relative humidity and atmospheric composition are the main environmental factors that impact storage disease development. These factors may directly affect the biology of the pathogens, but they can also have an indirect effect by slowing down the fruit metabolism. These environmental parameters can thus be modified, especially to extend the banana greenlife. The greenlife represents the period between the fruit harvest and the beginning of the climacteric phase and seems to be a prime factor in the development of storage diseases since a direct relationship has been noted between the degree of banana ripeness and susceptibility to disease (73). Fruit resistance also seems to decrease at the onset of the ripening process. It is therefore crucial to carefully manage these factors to ensure efficient banana crown rot control (61).

Storage temperature

One way to slow down the banana metabolism, and thus to delay crown rot development, is to refrigerate the fruit in boxes at the lowest possible temperature without changing fruit physiology. Cooling should be continuous throughout the shipping phase, which in turn should be as short as possible. Containers designed for maritime shipping of bananas are climate controlled at 13-14 °C, because temperatures below 12 °C are detrimental to fruit quality (73). Fungal growth is slowed down at 13 °C (34), which is a good tradeoff with respect to preserving the bananas and hampering fungal colonization of the crowns. However, this is much lower than the optimal temperatures for germination and growth of fungi responsible for crown rot, but it is not low enough to slow down the activity of these fungi, which can take place within a broad temperature range, i.e. 8 to 36 °C (90). As a result, crown rot incidence and severity are higher in bananas exposed to temperatures over 16°C (91).

Relative humidity

Although most pathogens require a high relative humidity for their *in vitro* development (11), bananas are less susceptible to crown rot under these conditions. A high relative humidity seems to hinder transpiratory water loss from the fruit, which is essential to ensure a long greenlife. Indeed, banana greenlife is markedly reduced under low relative humidity atmospheric conditions (30 to 40%) as a result of ethylene production from the fruit peel (77).

The senescence of banana crown tissues, which is conducive to crown rot development, can be hampered by maintaining their turgidity (73).

Atmosphere composition

The composition of the atmosphere around bananas during shipping can be manipulated to slow down metabolic activity. Modified atmospheres (MA) can be achieved by packing bananas in sealed plastic bags (polybags), and controlled atmospheres (CA) are obtained by injecting nitrogen in the storage rooms. It was shown that crown rot can be partially controlled by packing bananas in MA (H. Bastiaanse, L. de Lapeyre de Bellaire, L. Lassois, C. Misson, and M. H. Jijakli, unpublished). For MA, the balance of an atmosphere with a lower O_2 and a higher CO_2 content depends on the extent of fruit respiration, bag permeability, and the composition of the air outside the bag (95). The O_2 and CO_2 contents generally range from 1 to 10% and 2 to 14%, respectively, depending on the quality and thickness of the plastic packaging (67, 95). This modification in the gas composition reduces the fruit respiratory intensity and hampers endogenous ethylene synthesis, which can considerably increase the length of the preclimacteric phase. MA also inhibits the metabolism of some pathogenic agents. These plastic bags must also have a high mechanical strength because even small punctures will upset the MA (16).

High CO_2 (>15%) and low O_2 (<1%) levels are toxic to many fungi (3, 38). Unfortunately, bananas cannot be stored under these conditions because major alterations generally occur during ripening when the atmospheric CO_2 level is above 7 to 12% or when the O_2 level is below 1 to 2% (22, 95, 100).

Another way to modify gas exchange is to coat the peel with a wax composed of sucrose esters combined with cellulose or fatty acids. This wax blocks the stomatal pores, thus reducing gas exchange (14). The fruits have higher permeability to CO_2 than to O_2, and wax coatings accentuate this difference. This leads to a substantial decrease in the fruit internal O_2 content without increasing the CO_2 to an excessively high level, thus creating an ideal atmosphere for preserving bananas without alterations (13).

2.4.2.5. **Physical control methods**

Hot water treatments

Hot water treatments destroy the pathogens (15) and modify the fruit environment by activating antimicrobial compounds in the peel (24). de Costa and Erabadupitiya (24) showed that the optimal temperature and exposure time for controlling crown rot was 50 °C for 3 min. Higher temperatures lead to pale fruit, and exposure times of over 5 min reduce Brix values (24) and damage the fruit peel (101). A 20 min longer treatment at a temperature under 45°C is effective for controlling *C. paradoxa*, with the percentage of infected bananas decreasing from 100% to less than 15% (83). However, commercial tests on naturally infected fruit have not achieved crown rot control, and ripening delays have also been noted. There has been no commercial adaptation of this technique to date, except in other tropical fruits such as papaya (21, 75) and mango (20, 92). Hot water treatments have also been combined with the application of antagonistic bacteria in order to increase the biological control efficacy (24).

UV and gamma radiation treatment

Ultraviolet light (UV) treatments have considerable potential for fruit and vegetable post-harvest diseases control. Stevens *et al.* (93) discussed the possibility of inducing apple resistance to *Colletotrichum gloeosporioides* through UV-C treatments. In bananas, however, the peel seems to be too sensitive to UV for the use of this technique for postharvest control of some pathogens of the complex like *C. musae* (49).

Kanapathipillai *et al.* (55) pointed out that gamma ray treatment (38 min at 4 kGy) inhibited spore germination, the formation of *C. musae* appressoria, and all fungal development on the surface of fruit pieces. However, these authors did not investigate the effects of gamma rays on whole fruit, or on their complex components. Although dosages of around 0.5 kGy can extend banana greenlife (67, 99), Marriott and Palmer (67) noted that irradiation can alter the banana peel; the maximum dose tolerated by the fruits is likely around 0.5 kGy (99). Finally, the development of postharvest fruit irradiation has been hampered especially by the high cost of such treatments and their low consumer acceptance (99).

2.4.2.6. **Biological control**

Postharvest biological control is very promising because the crown rot infection site on the fruit is limited, the environmental conditions during storage are clearly defined and stable,

and bananas have a high added value (48). The results of many studies have suggested that the use of microorganisms such as fungi, bacteria and yeasts could provide partial crown rot control (7, 24, 25, 32, 43, 60, 62, 80, 98). It has also been shown that biological control efficacy increases with the antagonistic agent concentration and with the incubation time between the application of the antagonist and crown contamination by the fungal complex (62, 80). Antagonists can be used to significantly reduce lesions induced by the fungal complex that causes crown rot, but the control efficacy is limited and variable (62). This type of control, when used alone, cannot provide total crown rot control, and so should be combined with other control tactics such as calcium additives and MA packaging (H. Bastiaanse, L. de Lapeyre de Bellaire, L. Lassois, C. Misson, and M. H. Jijakli, unpublished).

The impact of several natural substances or nonsynthetic fungicides, such as preparations of calcium, plant extracts or organic acids, on crown rot development has also been evaluated. *Allium sativum* extracts (61), and essential oils of *Cinnamomum zeylanicum*, *Syzygium aromaticum* (81, 82), *Cymbopogon nardus*, and *Ocimum basilicum* (9) have also been found to have fungicidal activity. Win *et al.* (101) showed that cinnamon extracts reduced crown rot, increased greenlife, and had no negative effects on postharvest banana quality. These plant extracts have fungistatic and fungitoxic activity and inhibit conidial germination and mycelial growth of *C. musae*, *Fusarium* spp., and *L. theobromae*. However, cases of phytotoxicity have been reported and the level of control provided by these natural substances is not sufficient to meet market requirements. The use of antioxidants (56) and organic salts (5), sometimes combined with surfactants (8), can also enhance crown rot control. Finally, treatments with Biocto 6 (seed extract from citrus) combined with a wax-based additive (Verdiol), was found to provide the same level of crown rot control as fungicide treatments of export bananas (30).

2.5. Conclusion

Crown rot studies and control are especially complex because of the observed diversity and variability in the composition and pathogenicity of the complex involved in the development of this disease. The broad range of possible situations complicates studies on both the parasitic and physiologic components of fruit quality. Further studies are thus required to gain better insight into this disease, especially since little documentation is available on certain aspects such as the epidemiology of crown rot. It is also very important to enhance the overall understanding of banana physiological mechanisms involved in the induction of fruit resistance to crown rot pathogens with the aim of improving control.

There is considerable growing interest in alternative methods to reduce or even completely eliminate fungicide treatments. To be efficient, these alternative control methods should not be too specific considering the broad spectrum of pathogens involved in the complex. No alternative methods to chemical control are currently efficient enough by themselves to match the efficacy of fungicide treatments. However, the results of some experimental trials have shown that crown rot can actually be managed by combining different nonchemical control methods. Crown rot research should enhance the overall understanding of this disease and thus lead to the development of an effective integrated control strategy.

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3. Contributions of molecular biology to understanding the mechanisms involved in variation of banana susceptibility to crown rot disease

3.1. Introduction

Biological responses of fruits, and notably physiological changes and susceptibility to fungi, are controlled and regulated by the expression of certain genes associated with specific metabolic pathways. Although the genome is identical in every cell of an organism, genes may be expressed differently over time (development-stage-specific expression), in space (cell-type-specific expression), and according to the state of the fruit (which may be, for example, more or less susceptible to a disease). One way to understand the phenomena and reactions involved in variation of banana susceptibility to crown rot disease in relation to the physiological state of the fruit at harvest is to identify genes involved in these processes via the study of their expression. Transcriptomic analysis is an important tool for evaluating gene expression. Both molecular biology and genetic engineering have spawned a wide range of techniques for studying the differential expression of genes in a given tissue on the basis of transcript levels.

3.2. cDNA-AFLP analysis

3.2.1. Introduction

cDNA-AFLP derives from AFLP (amplified fragment length polymorphism) analysis, based on PCR amplification and developed by Vos *et al.* (1995) primarily for mapping genomic DNA. AFLP was adapted for analysis of mRNA levels in plants, and cDNA-AFLP was first described by Bachem *et al.* (1996). Since its introduction in 1996, this technique has become a reference tool for the study of differently expressed genes (Botton *et al.*, 2008). The technique consists in using RT-PCR to compare mRNA populations produced by different populations of cells. This wide-range screening has revealed differential transcription of genes whose function is "*a priori*" unknown, by comparison of the transcriptomes of similar cells or tissues (Bachem *et al.* 1998; Bachem *et al.* 2001; Van der Biezen *et al.* 2000; Durrant *et al.*, 2000).

3.2.2. Principle

After reverse transcription of the mRNA, the synthesized double-stranded cDNA is digested by a pair of restriction enzymes, one with a 4-basepair recognition site and one with

a 6-basepair recognition site. The ends of the digested fragments are then ligated to specific adapters. All digested, ligated cDNAs are amplified with a pair of primers, one hybridizing with each adapter. This step, called pre-amplification, yields many different fragments that cannot be separated on a polyacrylamide gel. A second amplification, called selective amplification, is performed with selective primers containing, in addition to the sequence of the adapter, one, two, or three additional bases at the 3' end and allowing selective amplification of cDNA fragments with complementary 5' nucleotides. Thus, only a sub-population of fragments, separated on a polyacrylamide gel, are amplified. Radiolabeling of one of the two primers used makes it possible to visualize the fragments by autoradiography.

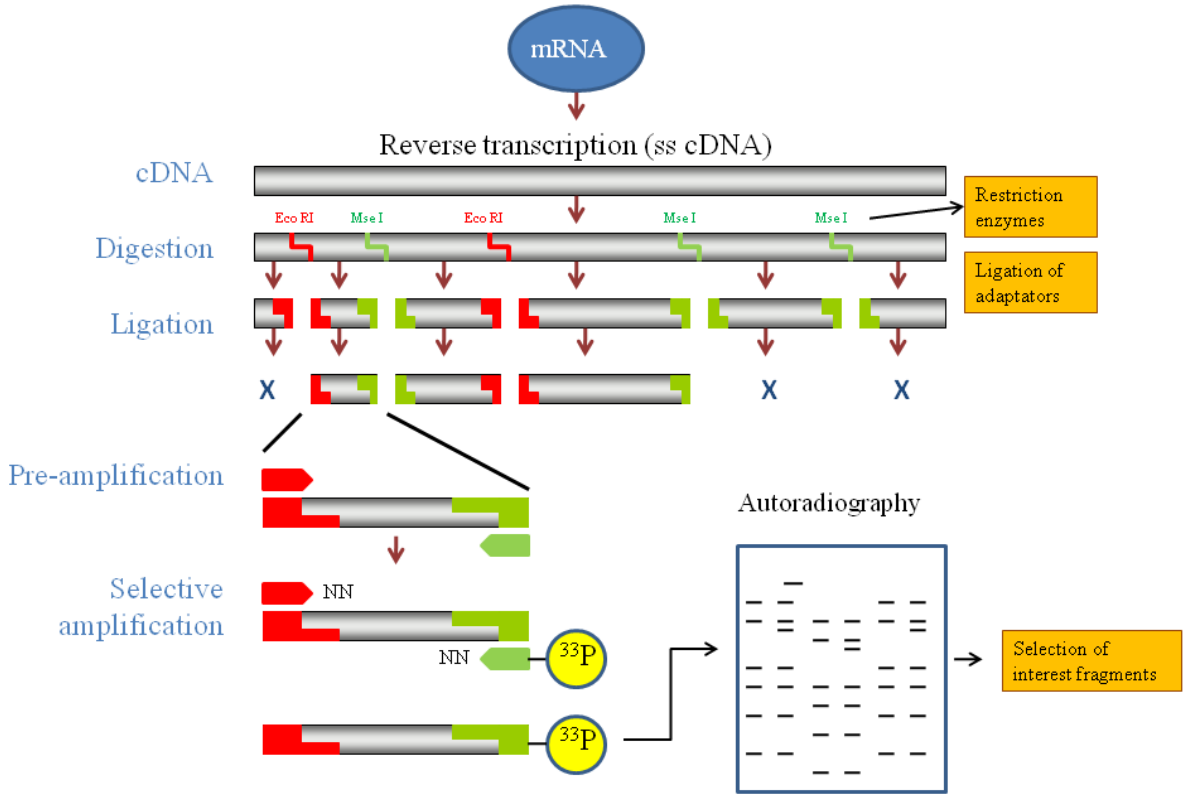

Figure 1 : Schematic representation of the cDNA-AFLP applied with Eco RI and Mse I like restriction enzymes and radiolabelled with ^{33}P. The digestion of cDNA by Eco RI and Mse I and the adaptator ligation result in the generation of fragments flanked by (i) identical adaptators and by (ii) non-identical adaptators. Fragments that are flanked by identical adaptators are excluded from further amplification due the formation of a hairpin structure.

3.2.3. **Advantages**

The cDNA-AFLP method does not require prior sequence information and can thus be used with any biological system, especially when genomic resources are lacking (Bachem *et*

al., 1996 ; Ditt *et al.*, 2001). Secondly, the technique allows identification of new genes (Botton *et al.*, 2008; Cappelli *et al.*, 2007; Ramonell and Somerville, 2002; Reijans *et al.*, 2003). These features are particularly interesting because the factors responsible for quantitative variation of symptoms in the case of compatible interactions linked to infection are still poorly understood and most likely remain to be discovered. More than two populations of mRNAs can be compared simultaneously, and by using all combinations of specific primers, the cDNA-AFLP makes it possible in principle to amplify all cDNAs (Donson *et al.*, 2002). Its reproducibility is very high (Campalans *et al.* 2001; Lassois *et al.*, 2009; Matz and Luckyanov, 1998), having been estimated at 100% (Khun, 2001). Its sensitivity is comparable to that obtained with microarrays (Reijans *et al.*, 2003), allowing detection of transcripts present in very low quantity (Decorosi *et al.*, 2005; Lievens *et al.*, 2001; Ramonell and Somerville, 2002; Reijans *et al.*, 2003; Savelkoul, 1999) and even of transcripts that are considered rare (Fukumura *et al.* 2003). Bachem *et al.* (1998) evaluated the sensitivity of cDNA-AFLP at one copy of mRNA per cell. In addition, the expression profiles obtained correlate well with those obtained by Northern blotting (Albertini *et al.*, 2004; Bachem *et al.* 1996; Breyne *et al.*, 2003; Cnudde *et al.*, 2003; Donson *et al.*, 2002; Durrant *et al.* 2000, Jones *et al.*, 2000). The false positive rate is low, lower than with other similar techniques such as differential display (Matz and Luckyanov 1998, Kuhn, 2001; Campalans *et al.*, 2001). Moreover, cDNA-AFLP analysis requires only a very small amount of sample (Cappelli, 2005) and allows discrimination of homologous genes (Decorosi *et al.*, 2005). As its cost is low and it does not require specific hardware, it is affordable for smaller laboratories (Ramonell and Somerville, 2002).

3.2.4. **Disadvantages**

Despite its many advantages, cDNA-AFLP analysis has a few disadvantages. Firstly, the interpretation of results can be complicated by the fact that a single initial mRNA can give rise to amplification of several fragments (Matz and Luckyanov, 1998; Lorkowski and Cullen, 2004). Secondly, as it is tedious to use all primer combinations, cDNA-AFLP is generally performed with only a few primer pairs. Thus, cDNAs that doe not have the restriction sites recognized by the enzymes used are not analyzed by the method (Habu *et al.*, 1997; Lorkowski and Cullen, 2004). In addition, information on differently expressed genes is limited to bands that are sequenced, and the technique does not provide quantitative data on the abundance of transcripts (Ramonell and Somerville, 2002). Since cDNA-AFLP analysis requires radiolabeling PCR primers, safety precautions must be taken and the work must be carried out in a laboratory accredited for work with radioisotopes (Decorosi *et al.*, 2005).

Finally, a weakness of the technique lies in the possibility of obtaining false positives due to co-isolation of sequences of the same size as the fragment of interest. This is why it is imperative to confirm the results obtained by means of an independent technique such as real-time RT-PCR.

3.2.5. **Application of the method**

The cDNA-AFLP technique and its application to plants were first described by Bachem *et al.* (1996), who analyzed the differential expression of genes in *Solanum tuberosum in vitro* tuberization systems. Other authors subsequently applied the cDNA-AFLP analysis to the same model (Trindade *et al.*, 2003, Trindade *et al.*, 2004) and since then, the technique has been applied to many other models, among which cotton plants (Ma *et al.*, 2007), apple (Yao *et al.*, 2007), rice (Akihiro *et al.*, 2006), *Arabidopsis thaliana* (de Diego *et al.*, 2006), and sugarcane (Borras-Hidalgo *et al.*, 2005) are just a few examples. The technique has revealed interesting parallels between the responses of plants to pathogens and environmental constraints and the expression of genes that establish these responses (Lin *et al.*, 2003, Zheng *et al.*, 2009; Ramonell and Somerville, 2002; Simoes-Araujo *et al.*, 2002; Nimbalkar *et al.*, 2006; Ditt *et al.*, 2001; Ditt *et al.*, 2005; Chen *et al.*, 2003). cDNA-AFLP analysis has also been applied to other models besides plants: to mammals such as horses (Cappelli *et al.*, 2005; Capelli *et al.*, 2007), rats (Fukuda *et al.*, 1999), dogs (Vandeput *et al.*, 2005), and humans (Egert *et al.*, 2006), but and also to fungi (Avrova *et al.*, 2003; Botton *et al.* , 2008), yeast (Massart and Jijakli, 2006; Reijans *et al.*, 2003), bacteria (Decorosi *et al.*, 2005; Dellagi *et al.*, 2000), and nematodes (Qin *et al.*, 2000).

3.3. High-throughput sequencing for transcriptomics

Recently developed high-throughput sequencing (HTS) technology has made possible powerful strategies for comprehensively interrogating nucleic-acid-based information in a cell at unparalleled resolution and depth (Qui *et al.*, 2009; Lister *et al.*, 2009). The availability of relatively low-cost sequencing techniques rapidly producing huge amounts of sequence information has triggered a paradigm shift in genomics (Lister *et al.*, 2009). The diversity of applications to which such techniques have been applied demonstrates the immense range of cell processes and properties that can now be studied at single-base resolution. Transcriptome sequencing is among these applications, as high-throughput sequencing has become a real alternative to other classical techniques for studying the transcriptome. It offers many advantages: quantitative detection of messengers, detection of rare transcripts and small RNAs, and no *a priori* dependence on annotation of the genome. This approach thus offers

the plant biologist unprecedented opportunities to probe the functions and dynamics of plant cells and populations. Currently and in planning future research, these techniques cannot be ignored. They will notably make it possible to obtain information that will help to elucidate plant responses to pathogen attack.

3.4. References

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CHAPTER II

Aims and proposed research

As show in figure 1 hereafter, crown rot development at commercial level depends on several post-harvest factors. The first researches on crown rot focused on (i) the etiology of this disease, especially on the identification of the most pathogenic fungal species involved; and (ii) on post-harvest control methods, mainly chemical. Nevertheless, these researches did not provide neither a good understanding of the most conducive conditions for disease expression, nor a perfect control of this disease at all spatio-temporal scales. Variations were observed in the expression of crown rot symptoms when post-harvest conditions were assumed to have been reasonably constant, although not actively controlled (Griffee, 1976; Lukezic *et al.*, 1967; Shillingford, 1978).

We suggest a new, interesting and original approach to consider the crown rot post-harvest development. It consists to present a fruit quality potential at harvest as a key factor in crown rot development (Lassois *et al.*, accepted) (chapter 1.2). This potential develops during growth of bananas in the field and depends on a physiological and a parasitical component which both are function on agro-technical and pedo-climatic factors of the crop production area.

The parasitic component reflects both a level of crown contamination by the parasitic complex and the pathogenicity of this parasitic complex.

The physiological component refers here to the level of fruit susceptibility to crown rot. This level of banana crown rot susceptibility reflects the physiological state of the fruit. Yet little is known about the influence of the fruit physiological component in post-harvest development of the disease and on the pre-harvest factors influencing the level of banana susceptibility to crown rot. Although pedo-climatic conditions and agro-technical factors are known to influence the development of this post-harvest disease (Krauss and Johanson, 2000; Lukezic *et al.* 1967; Shillingford, 1978), there are few studies linking such fluctuations to the field susceptibility level (Lassois *et al.*, 2008). In addition, the cellular mechanisms and genetic determinants underlying the susceptibility to crown rot disease variation are not yet known.

The aim of this study was to clarify the role of the fruit physiological component at harvest in the observed crown rot disease incidence variations on *Musa acuminata*, AAA, cv ‘Grande-Naine’. To evaluate the influence of the fruit physiological component in post-harvest disease development it is necessary to overcome the influence of the parasitic component. To do this, fruit susceptibility is measured by lesion size after standardized artificial inoculations (de Lapeyre de Bellaire *et al.*, 2008). In figure 1 the general research

outline of the study is depicted. Specific aims were first to evaluate the influence of the fruit physiological component at harvest on the crown rot post-harvest development (chapter 3.1) and to characterize the importance of some pre-harvest factors in determining fruit susceptibility to crown rot (chapter 3.2). Then, the genetic determinants of fruit (*Musa acuminata*, AAA, cv 'Grande-Naine') susceptibility to crown rot caused by *C. musae* were studied. For this part of the work, the molecular techniques necessary from sample conservation to differential gene expression analyses had to be developed, tested and compared (chapter 4.1). The cDNA-AFLP technique was applied in order to identify the pre- and post-infection genes differently expressed between bananas with different levels of susceptibility to crown rot (chapter 4.2).

Finally, in chapter 5, conclusions and perspectives for further studies have been formulated.

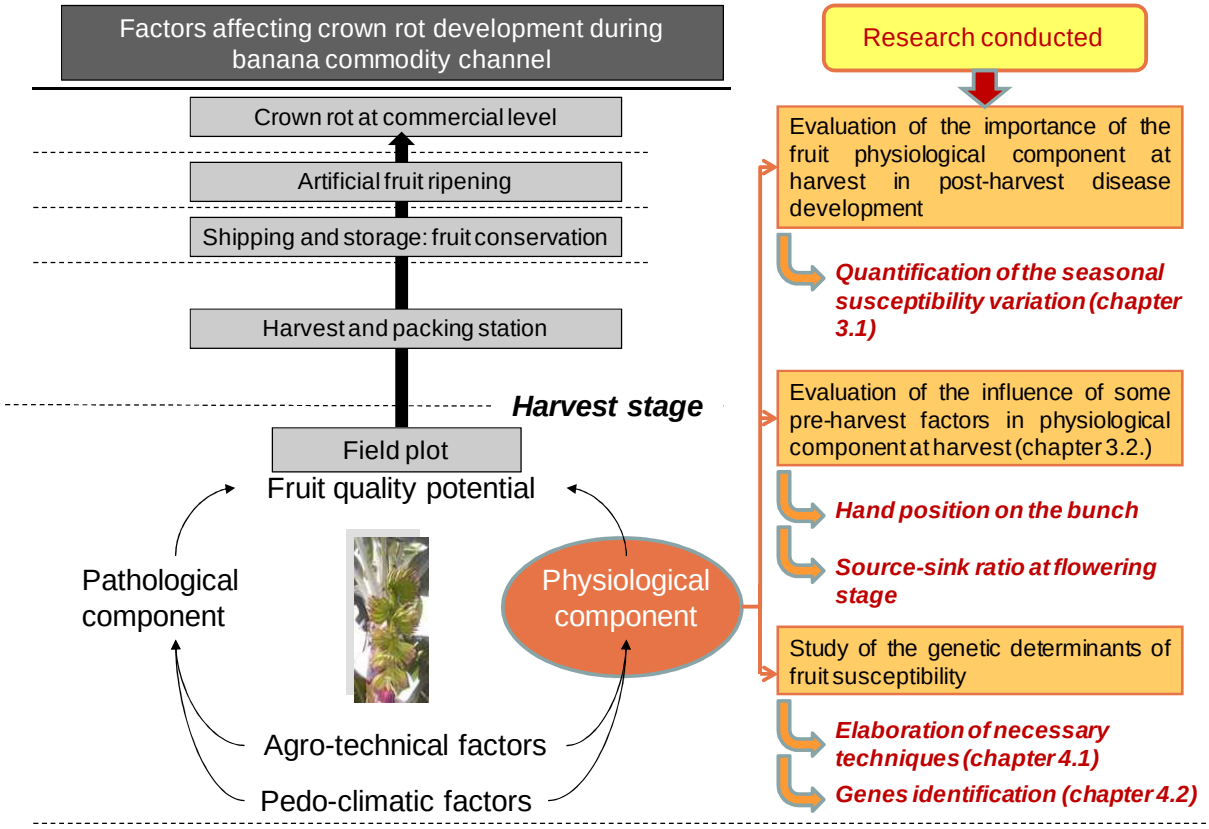

Figure 1: Diagram representing different key factors that arise throughout the commodity chain concerning crown rot development at commercial stage and research outline of the study on the physiological component of the fruit quality potential at harvest.

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CHAPTER III

Significance of the fruit physiological component at harvest on the post-harvest crown rot development and identification of pre-harvest factors influencing the fruit susceptibility

1. Temporal variation of fruit susceptibility

1.1. Introduction

As explained in the above review of the literature, a banana quality build-up scheme has been designed for the anthracnose model (Chillet and de Lapeyre de Bellaire, 1996a). In the light of the similarities between anthracnose and crown rot, this scheme was transposed to the crown rot model (Lassois *et al.*, accepted), postulating that crown rot disease development depends on a physiological and a parasitic component. In crown rot disease, however, the relative importance of the physiological and parasitical components in disease development is unknown. Seasonal fluctuations in crown rot severity have been reported in situations of natural contamination (Griffee, 1976; Lukezic *et al.*, 1967; Shillingford, 1978) and it have been suggest that fruit susceptibility variation could occur but this has never been confirmed by detailed research conducted in controlled inoculation situation. In the present study, fruit susceptibility to crown rot disease was monitored for 11 weeks in order to identify the role of the physiological component in disease development. As in our other experiments and to avoid dealing with the complex parasitic component, the susceptibility level of the fruit was measured by the size of the lesions after standardized artificial inoculations (de Lapeyre de Bellaire *et al.*, 2008). For the first time in crown rot disease, we have demonstrated that fruit physiological component at harvest greatly influence the post-harvest crown rot development. The results obtained are included in the following publication and are presented hereafter the way they have been published.

Lassois L., de Lapeyre de Bellaire L. and Jijakli H. 2008. *Biological control of crown rot of bananas with Pichia anomala strain K and Candida oleophila strain O*, Biological Control, **45**: 410-418.

1.2. Materials and methods

1.2.1. Fruit sampling and assessment of banana susceptibility to crown rot disease

The banana cultivar used was Grande-Naine (*Musa acuminata* AAA, Cavendish group). As susceptibility to some postharvest diseases depends on banana physiological age (Chillet *et al.*, 2006), all fruits were harvested at the same physiological age of 900°C.days according to the method described by Ganry (1978). One cluster of 4 bananas from either the 2nd or 3rd hand of each of 5 homogenous bunches was harvested in the morning on the day each experiment began in order to evaluate the average susceptibility of the bananas at this harvest

time. Thirteen experiments like this were conducted over 11 weeks from March to June 2003 at the CIRAD experimental station (Neufchâteau) in Guadeloupe, France. Assessment of rot progression was carried out 13 days after artificial inoculation (i.e. 3 days after ethylene treatment). The internal progression of rot from the original inoculation point was determined by cutting the crown longitudinally and measuring the rot area in the crown. This "internal necrotic surface" (INS) was measured and expressed in mm^2. The INS average of 5 banana clusters was calculated for each experiment.

1.2.2. **Artificial inoculation of crown rot agents**

The clusters of 4 bananas were placed in tap water for 20 minutes, for latex elimination, before refreshing the crown surface with a knife. These cuttings were square, with regular and clean-cut sections in order to obtain similar crowns. The crowns were surface-sterilized by immersion in 50% ethanol. Three fungal pathogens frequently observed in the complex from Guadeloupe were used to inoculate the clusters, namely *C. musae*, *F. moniliforme*, and *Cephalosporium* sp. These pathogens had been isolated in Guadeloupe from different organs of the banana plant (crown rots, floral remnants) and identified as being frequently involved in the development of crown rot. They were conserved at -80°C in glycerol solution (50%). Before use, they were grown at 25°C on Potato Dextrose Agar (PDA) (BioMérieux, Lyon, France) for 7 to 10 days. Conidia were removed by flooding the plates with sterile distilled water and filtered through a 40-µm sieve. Conidia concentrations were determined with a Mallassez cell. *F. moniliforme*, *Cephalosporium* sp, and *C. musae* were mixed and the respective final concentrations of these species were 10^4, 10^4, and to only 10^3 conidia/ml for *C. musae* because of its strong pathogenicity. One-hundred microlitres of conidial suspension was applied to the centre of the freshly exposed crown tissue and covered with a small filter paper, which was withdrawn 15 minutes later. The 5 clusters of 4 bananas were packed in punched polyfilms normally used in the industry and placed in a small cardboard box (24*23*23cm) in order to simulate commercial packing. To simulate shipment, the boxes were stored on shelves in a conditioned room ($15m^3$) at 13°C for 10 days. Then artificial ripening was initiated by exposing the bananas to 1000 ppm ethylene (Azethyl, AIR LIQUIDE, France) for 24 h at 20°C. After strong ventilation they remained at 20°C for another two days before the assessment of crown rot.

1.3. Results and discussion

As shown in Figure 1, the INS of the bananas inoculated with the complex increased regularly from 112.6 mm^2 to 426.6 mm^2 in the course of all experiments. These values represent respectively 31% and 100% of the total crown surface.

Seasonal fluctuations in crown rot severity have been reported for situations of natural contamination (Griffee, 1976; Lukezic *et al.*, 1967; Shillingford, 1978), but the present investigation is the first report of such fluctuations in the case of artificial contamination. The present results confirm our postulate that the development of crown rot at the commercial level does not depend solely on the parasitic component. It also depends on the physiological state of the fruit at harvest, which determines its susceptibility to the fungal complex. On the other hand, the present results demonstrate that the physiological component is quite variable, allowing considerable variation of disease incidence over a short period of time. In the present work the parasitic component was constant and controlled, and the edaphic conditions can be assumed to have been reasonably constant, although not actively controlled. Climate variations were thus probably involved in the observed fluctuations of fruit susceptibility to crown rot. The mechanisms underlying the susceptibility level are still unknown, however.

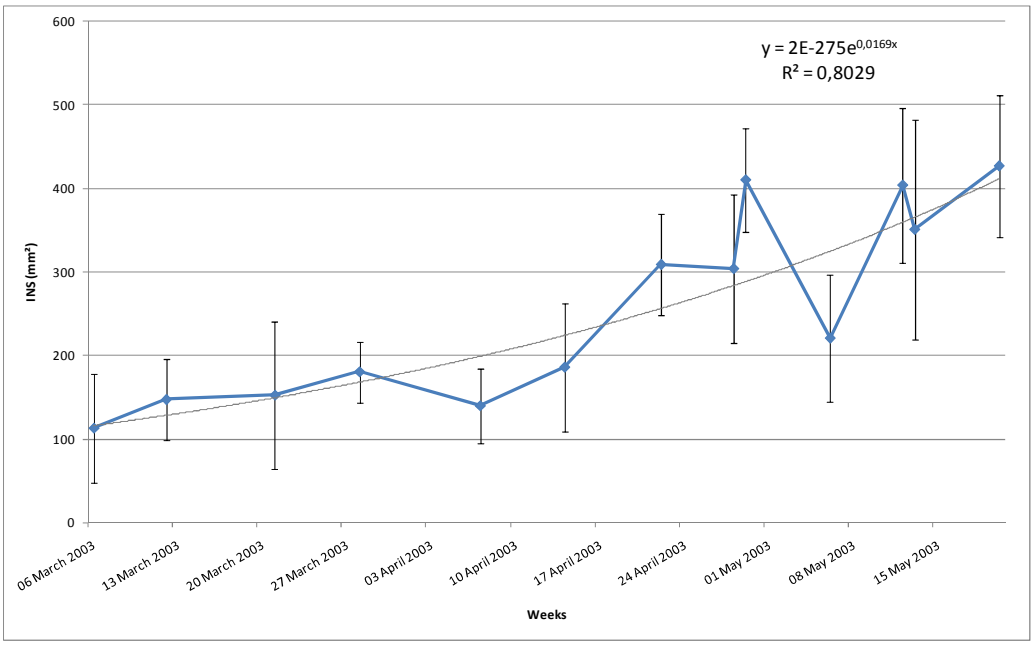

Figure 1: Variation of the Internal Necrotic Surface (INS) assessed on clusters inoculated with the fungal complex composed by *Colletotrichum musae* (10^3 conidia/ml), *Fusarium moniliforme* (10^4 conidia/ml) and *Cephalosporium* sp. (10^4 conidia/ml) in the course of all experiments carried out between 06/03/03 and 22/05/03.

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2. Hand position on the bunch and source-sink ratio influence the banana susceptibility to crown rot disease

It was showed there was a lack of information and researches about the role of the fruit physiological component that influence the fruit quality potential at harvest. In the same way, no information was available about the pre-harvest factors implicate in the observed variation in susceptibility. We first tried, but without success, to identify simultaneously parameters that influencing the fruit susceptibility to crown rot in field conditions (results not show). It quickly became apparent that the environment-plant relationships determining susceptibility of fruit were very complex. We therefore concentrated on the study of 2 individual pre-harvest factors. We identified that fruit position on the bunch and source-sink ratio at flowering stage have an impact on the fruit susceptibility level.

The results obtained were accepted for publication.

Lassois L., Bastiaanse H., Chillet M., Jullien A., Jijakli M.H. and de Lapeyre de Bellaire. *Hand position on the bunch and source-sink ratio influence the banana susceptibility to crown rot disease*. Annals of Applied Biology. DOI: 10.1111/j.1744-7348.2009.00381.x

Hand position on the bunch and source-sink ratio influence the banana fruit susceptibility to crown rot disease

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Running title: Banana susceptibility to crown rot disease

Abstract

The post-harvest development of crown rot of bananas depends notably on the fruit susceptibility to this disease at harvest. It has been shown that fruit susceptibility to crown rot is variable and it was suggested that this depends on environmental pre-harvest factors. However, little is known about the pre-harvest factors influencing this susceptibility. The aim of this work was to evaluate the extent to which fruit filling characteristics during growth and the fruit development stage influence the banana susceptibility to crown rot. This involved evaluating the influence of (i) the fruit position at different levels of the banana bunch (hands) and (ii) changing the source-sink ratio, on the fruit susceptibility to crown rot. The fruit susceptibility was determined by measuring the internal necrotic surface (INS) after artificial inoculation of *Colletotrichum musae*. A linear correlation (r = -0.95) was found between the hand position on the bunch and the INS. The source-sink ratio was found to influence the pomological characteristics of the fruits and their susceptibility to crown rot. Fruits of bunches from which six hands were removed (2 hands remaining on the bunch) proved to be significantly less susceptible to crown rot (INS = 138.3 mm^2) than those from bunches with 8 hands (INS = 237.9 mm^2). The banana susceptibility to crown rot is thus likely to be influenced by the fruit development stage and filling characteristics. The present results highlight the importance of standardizing hand sampling on a bunch when testing fruit susceptibility to crown rot. They also show that hand removal in the field has advantages in the context of integrated pest management, making it possible to reduce fruit susceptibility to crown rot while increasing fruit size.

Keywords: *Colletotrichum musae*, *Musa* sp., plant-pathogen interactions, post-harvest disease, fruit quality.

2.1. Introduction

Crown rot disease affects export bananas in all producing countries and is viewed as one of the main post-harvest diseases of bananas. The disease develops during shipping, ripening, and storage and has a negative impact on the market value of bananas (Slabaugh and Grove, 1982). It results from the development of several relatively nonspecific pathogens, but many authors agree on the high pathogenicity of *Colletotrichum musae*, which can trigger an infection from a very small inoculum (Finlay and Brown, 1993; Lassois *et al.*, 2008).

Geographic and seasonal variations have been noted in the incidence of banana post-harvest diseases (Chillet and de Lapeyre de Bellaire, 1996; Chillet *et al.*, 2007; Krauss and Johanson, 2000; Lassois *et al.*, 2008; Lukezic *et al.*, 1967; Shillingford, 1978). It has been suggested that these spatiotemporal fluctuations may reflect variations in the banana fruit quality potential that develops in the field and which determines the post-harvest onset or absence of diseases (Chillet and de Lapeyre de Bellaire, 1996; Lassois *et al.*, accepted). The quality potential comprises a physiological and parasitic component, which both depend on agro-technical factors and on soil and climate environment conditions (names pedo-climatic factors).

The physiological component here refers to the fruit susceptibility to crown rot. In order to overcome the parasitic component, this susceptibility level of the fruit is measured by the size of lesions in standardized artificial inoculations (de Lapeyre de Bellaire *et al.*, 2008). In the case of anthracnose it has been shown that fruit susceptibility at harvest is a key factor in the development of this disease and its control (Chillet *et al.*, 2007; Chillet *et al.*, 2006). Little is known, however, about the factors influencing the banana susceptibility to crown rot. Although pedo-climatic conditions and agro-technical factors are known to influence the development of this post-harvest disease (Krauss and Johanson, 2000; Lukezic *et al.* 1967; Shillingford, 1978), there are few studies linking such fluctuations to the field susceptibility (Lassois *et al.*, 2008).

The aim of this work was to characterize the importance of some pre-harvest factors in determining fruit susceptibility to crown rot. In particular, we were interested in the influence of the fruit development stage and fruit filling characteristics. First we examined whether the position of a hand in a banana bunch influences the susceptibility of its fruits to crown rot, taking advantage of the fact that the hands of a bunch differ as regards to both their development stage and filling status (Jullien *et al.* 2001a). Next we evaluated how a change in

the source-sink ratio during growth of the bunch affects fruit susceptibility to crown rot (leaves being the source and fruit, the sink) considering that source-sink ratio modification leads, among others, to changes in the rate of fruit filling (Jullien *et al.*, 2001b). The source-sink terms are commonly used in plant characterization. Leaves were considered as source tissues because they produce excess of assimilate, while fruits were sink organs. With respect to metabolism, plant organs are generally divided into source and sink tissues. Source tissues like mature leaves produce excess of assimilates which are transported via the phloem to the different sink tissues not able to produce themselves sufficient amounts of assimilates.

2.2. Materials and Methods

A banana plant produces an inflorescence called a bunch. Fruits on a bunch are grouped into female and male hands arranged helicoidally around a central axis called the stalk. The hand at the top of the bunch is the first to be initiated and hands are traditionally numbered from this one downward. Each hand can be divided into clusters consisting of several banana fruits called fingers (Figure 1).

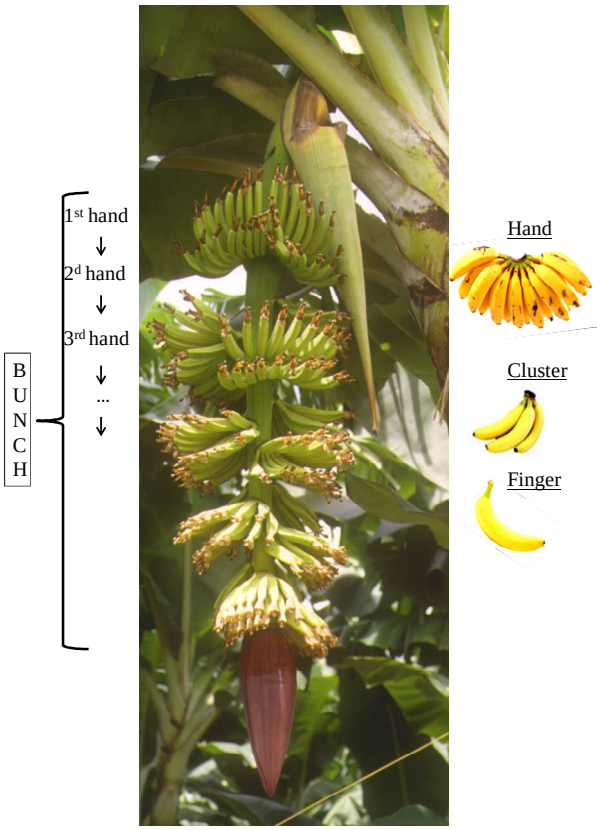

Figure 1: Organization of a banana bunch

2.2.1. **Plant material**

For each performed test, homogeneous sets of banana plants (*Musa acuminata* [AAA group, Cavendish subgroup] cv Grand Nain) which have grown under the same agro-technical practices were randomly selected at the Dia-Dia commercial plantation (PHP, Njombe, Cameroon (altitude: 80 m; annual mean temperature: 26.5°C; annual mean rainfall: 3500 mm). Non-systemic fungicide applications were used to control foliar diseases. The crown tissues have never been in contact with fungicides. The date of flowering was indicated by tying a colored belt to each bunch at the horizontal finger stage in order to predict the time of harvest. Bunches were also covered with a plastic sleeve at this stage. Bunches were harvested at a constant physiological age (Jullien *et al.*, 2008), i.e. when the mean daily temperature sum accumulated by the fruit at the 14°C threshold between flowering and harvest reached 900 degree days (dd). Temperatures were recorded at a weather station on the plantation. The daily average temperature (Td) was estimated from measurements of maximum temperature (Tmax) and minimum temperature (Tmin).

2.2.2. **Evaluation of susceptibility to crown rot**

Hands of bananas collected on the day of the experiment and transported to the laboratory were cut into clusters of 4 fingers without defects. The crown surfaces were refreshed with a knife. These cuttings were square, with regular, clean-cut sections in order to obtain similar crowns. Smoothly cut crowns were obtained with a sharp knife, leaving as much crown tissue as possible. Latex from crown tissues was dried with absorbent paper and the crowns were surface-sterilized by submersion in 50% ethanol. Fifty microlitres of *C. musae* conidial suspension containing 10^4 conidia/ml was applied to the centre of the freshly exposed crown tissue and covered with a small paper filter. The *C. musae* strain was isolated in Njombe, Cameroon. It is sensitive to thiabendazole and was stored at –20°C in a glycerol solution (30%). *C. musae* cultures were grown at 25°C in Mathur medium ($MgSO_4.7H_2O$: 2.5g/l; KH_2PO_4: 2.7g/l; peptone : 1 g/l; yeast extract: 1g/l; saccharose: 10 g/l; agar: 15 g/l) for 10 days. Conidia were removed by flooding the plates with sterile distilled water and filtration through a 45µ sieve. Their concentrations were determined with a Mallassez cell. Two hours after application of the conidial suspension, the clusters were packed in punched polyfilms normally used in the industry, placed in commercial boxes, and stored at 13°C for 10 days to simulate shipment. Artificial ripening was then initiated by dipping the bananas for 5 seconds in an ethrel solution (480 g/l), after which the clusters remained at 20°C for another 3 days before crown rot assessment. The internal progression of rot was determined by cutting the

cluster crown longitudinally in two and measuring the surface of rot spread into the crown, from the original inoculation point. This “internal necrotic surface” (INS), calculated by assuming a rectangular shape, was expressed in mm^2. Its average value was taken as a measure of fruit susceptibility to crown rot.

2.2.3. **Intra-bunch variation of fruit susceptibility to crown rot**

From March to May 2005 and during 10 weeks, 1 bunch per week having reached 900dd was harvested (Figure 2). Hands were separated from the bunch to evaluate the susceptibility to crown rot of each hand and numbered from 1 to 8 by order of appearance on the bunch. Hand 1 being the first to have appeared at the top of the bunch (Figure 1). Each hand was then divided into 3 clusters of 4 fingers. The susceptibility of each cluster to crown rot was assessed as described in section 2.2.2. The average INS values calculated for the 3 clusters of a hand were subjected to two-way crossed-mixed ANOVA (Hand, Bunch) performed with Minitab software. Finally, results were submitted to a linear regression analysis.

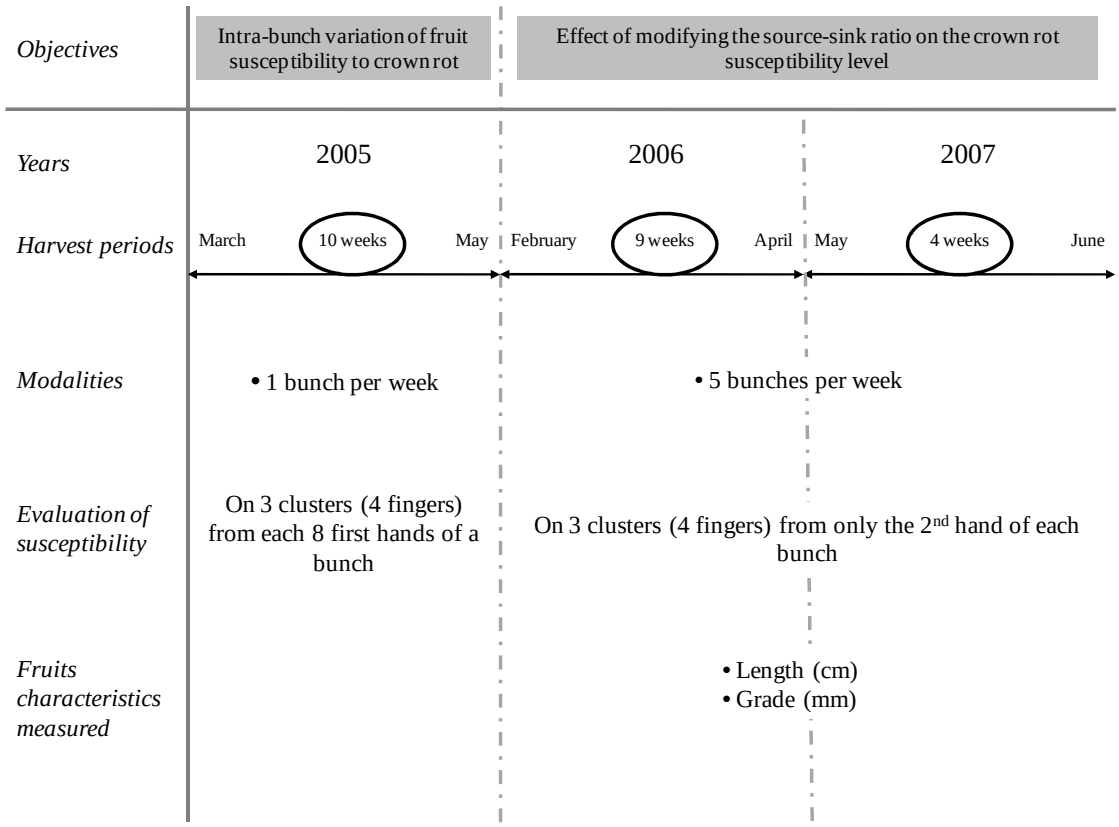

Figure 2: Chronology and modalities of the various tests performed

2.2.4. **Effect of modifying the source-sink ratio on the crown rot susceptibility**

The source-sink ratio was modified as follows during fruit growth by removal of leaves (L) and hands (H) at the flowering stage (horizontal finger stage) so as to obtain the following treatments:

12L/2H: 12 leaves and two hands (the first two to have appeared on the bunch) remaining at the flowering stage;

12L/8H: 12 leaves and eight hands (the first eight to have appeared on the bunch) remaining at the flowering stage;

5L/2H: 5 leaves (the last to have emerged) and two hands (the first two to have appeared on the bunch) remaining at the flowering stage;

5L/8H: 5 leaves (the last to have emerged) and eight hands (the first eight to have appeared on the bunch) remaining at the flowering stage;

An empirical source-sink ratio was also calculated. As the banana plants with 12 leaves and bunches of 8 hands (12L/8H) did not undergo leaf or hand removal, they were considered as reference to calculate an empirical source-sink ratio (So-Si ratio). The empirical So-Si ratios for the other treatments including leaf or hand trimming were estimated with the following formula: [(Number of leaves/number of hands) for the treatment] / [(Number of leaves/number of hands) of the reference treatment]

For example, the empirical source-sink ratio calculated for the plant with the 12L/2H configuration was [(12/2)/(12/8)] = 4.

Five banana plants per week were selected for each ratio, and bunches were harvested when the fruits had accumulated 900 dd (Jullien *et al.*, 2008). Two test series were conducted (Figure 2): 9 successive repetitions (weeks) between February and April 2006 and 4 successive repetitions (weeks) between May and June 2007. Fruit susceptibility was assessed on 3 clusters of 4 fingers on hand 2 of each bunch, as described in 2.2. The average INS values calculated for the 3 clusters were subjected to partially hierarchical mixed four-way ANOVA (Treatment, Bunch, Week, Year), each cluster being taken as an experimental unit. This was followed by the contrast hypothesis testing to separate leaves and hands effects.

In addition to assessment of fruit susceptibility, some fruit pomological characteristics were measured. On each harvested hand 2, the lengths (cm) and diameters name grades (mm) of the fruits were systematically measured. Statistical analysis consisted of two partially hierarchical mixed three-way ANOVAs (Treatment, Week, Year) carried out with Minitab software followed by the contrast hypothesis testing to separate leaves and hands effects.

2.3. Results

2.3.1. Intra-bunch variation of fruit susceptibility to crown rot

The position of fruit on the bunch was found to influence fruit susceptibility to crown rot very highly significantly ($p < 0.001$) (Table 1). Although very highly significant differences were observed between bunches (weeks) ($p < 0.001$), the trend was the same whatever the bunch, as no effect of the interaction 'hand x bunch' was observed ($p = 0.218$).

Table 1: Results of a two-way crossed-mixed ANOVA (hand, bunch) on Internal Necrotic Surface (mm^2).

		Internal Necrotic Surface	
Source	**d.f.**	***F*-value**	***P*-value**
Hand	7	17.85	<0.001
Bunch	9	6.95	<0.001
Hand * Bunch	63	1.17	0.218

A gradient of susceptibility was observed between hand 8, with an INS average of 73.6 mm^2, and hand 1, with an INS average of 150.9 mm^2 (Figure 3). Hand rank is an equidistant ordinal variable that can be compared to a quantitative value in the calculations. Hence, a linear correlation was found ($r = -0.95$) between the hand position on the bunch and the 8 corresponding INS means.

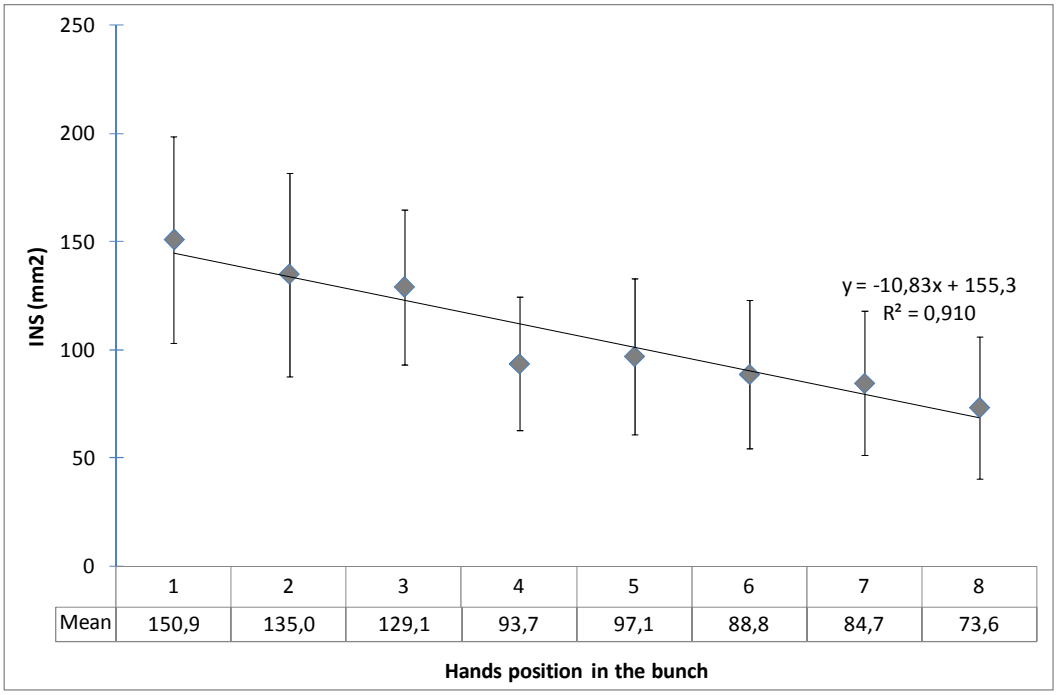

Figure 3: Mean values and standard deviations of internal necrotic surface (INS mm^2) as a function of the hand position on the bunch.

2.3.2. **Effect of the source-sink ratio modification on the crown rot susceptibility**

The change in the source-sink ratio imposed during flowering by removal of both leaves and hands from the bunch had a highly significant effect on the fruit susceptibility to crown rot ($p = 0.004$) (Table 2). Although there was also a highly significant effect of the year factor ($p = 0.004$), the trend was the same in both years because no interaction was observed between the treatments and the years ($p = 0.225$) leading to analyze the results of both years together. The fruit susceptibility to crown rot showed a very highly significant variation ($p<0.001$) from week to week within a year without consequence on the preceding conclusions.

Table 2: Results of a partially hierarchical mixed four-way ANOVA (Bunch, Treatment, Week, Year) on Internal Necrotic Surface (mm^2) and of two partially hierarchical mixed three-way ANOVAs (Treatment, Week, Year) on fruit grade and length.

		Internal Necrotic Surface		Grade		Length	
Source	d.f.	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Treatment	3	57.87	0.004	140.46	<0.001	103.49	<0.001
Year	1	13.29	0.004	10.85	0.001	102.91	<0.001
Treatment * Year	3	1.52	0.225	4.80	0.003	2.44	0.065
Week (Year)	11	10.14	<0.001	7.65	<0.001	2.58	0.005
Treatment * Week (Year)	33	1.77	0.009	1.14	0.283	1.64	0.022
Bunch (Treatment Year Week)	191	2.32	<0.001	/	/	/	/

The ranking of different treatments (Table 3) shows that the fruits of bunches from which 6 hands were removed (treatments 12L/2H and 5L/2H) were significantly less susceptible to crown rot (INS = 138.3 mm^2) than those of bunches with 8 hands (12L/8H treatment and 5L/8H, INS = 237.9 mm^2). There appeared no susceptibility difference, however, between treatments 12L/8H and 5L/8H or between treatments 12L/2H and 5L/2H. Thus, the removal of 6 out of 8 hands had a significant effect on fruit susceptibility to crown rot but the removal of 7 out of 12 leaves did not. Table 3 also shows the classification of the INS by order of decreasing empirical source-sink ratio. It is noteworthy that in trend terms, the crown susceptibility increased as the empirical source-sink ratio decreased.

Table 3: Mean values and standard deviations of Internal Necrotic Surfaces (INS) mm^2, fruit grade (mm), and length (cm) after treatments affecting the source-sink ratio. All fruits were obtained from hand 2. The letters a, b, c, d, e and f represent groups of statistically similar fruits based on contrast testing. 12L/2H: banana plant with 12 leaves and 2 hands; 5L/2H: banana plant with 5 leaves and 2 hands; 12L/8H: banana plant with 12 leaves and 8 hands; 5L/8H: banana plant with 5 leaves and 8 hands. The source-sink ratio was estimated according to the number of leaves and fruits removed in each treatment, as compared to unaltered banana plants (12L/8H).

Treatment	Source-sink ratio	INS (mm^2)	Grade (mm)	Length (cm)
12L/2H	4	130.2 ± 65.4^{a}	37.9 ± 0.3^{c}	22.0 ± 0.2^{e}
5L/2H	1.7	146.4 ± 60.2^{a}	36.8 ± 0.3^{c}	21.3 ± 0.2^{e}
12L/8H	1	227.4 ± 66.7^{b}	33.3 ± 0.2^{d}	19.5 ± 0.2^{f}
5L/8H	0.4	248.4 ± 72.0^{b}	32.0 ± 0.2^{d}	18.6 ± 0.2^{f}

2.3.3. **Effect of the source-sink ratio modification on fruit grade and length**

The source-sink ratio change imposed during flowering by removal of both leaves and hands had a very highly significant effect on fruit length ($p < 0.001$) and grade ($p < 0.001$) (Table 2). The ranking of the different treatments (Table 3) shows that the fruits of bunches from which 75% of the hands were removed (treatments 12L/2H and 5L/2H) were significantly longer and had a significantly higher grade (average length = 21.6 cm and average grade = 37.3 mm) than those of bunches with 8 hands (treatments 12L/8H and 5L/8H, average length = 19 cm and average grade = 32.6 mm). Removal of many hands thus has a significant effect on the morphometric characters of the fruit. No difference in fruit grade or length was observed, however, between treatments 12L/8H and 5L/8H or treatments 12L/2H and 5L/2H. Thus, leaf removal had no significant effect on these morphometric characteristics (Table 3). It is noteworthy that the fruit pomological characteristics and banana yield increased as the empirical source-sink ratio decreased (Table 3). The various changes in the source-sink ratio had an effect on fruit filling.

2.4. Discussion

We show here that fruit development stage and filling characteristics are parameters influencing post-harvest susceptibility of bananas to crown rot. Within a bunch, there is a gradient of susceptibility to crown rot, the hands initiated first (the upper ones) being more susceptible than those initiated last (the lower ones). It has already been established that morphological differences between hands of a same bunch result from differential development associated with cell division and fruit filling characteristics (Jullien *et al.*

2001a). Here it appears that competition within a bunch affects not only the morphological characteristics of the fruits but also the fruit quality potential. Taking into account the observation of Jullien *et al.* (2001a) that the hands initiated first are approximately 70dd ahead those initiated last, the susceptibility gradient might be due to this physiological age gap between fruit in the same bunch. Effectively, in the case of anthracnose of banana, the fruit susceptibility has been shown to increase with the physiological age of the fruit expressed in dd (Chillet *et al.*, 2007; Chillet *et al.*, 2006).

The position of a hand on the bunch has also been shown to influence fruit filling. This results in gradients of pulp dry weight, cell number per fruit, and starch grain number per cell (Jullien *et al.* 2001a) and also in differences in sap concentration and composition within the same bunch (Kurien *et al.*, 2000). Furthermore, it is well known that partitioning of assimilates between various sink organs are complex and not equally distributed (Kozlowski, 1992). These differences in filling characteristics within a bunch may be involved in the observed susceptibility changes.

We confirm here that source-sink ratio changes have a significant effect on fruit morphology, as previously demonstrated in several studies (Chillet *et al.*, 2006, Daniells *et al.*, 1987, Daniells *et al.* 1994; Israeli *et al.*, 1995, Johns 1996, Jullien *et al.* 2001b; Kurien *et al.* 2000; Mouen Bedimo *et al.*, 2003). We reveal, furthermore, a new effect of a source-sink ratio change on the fruit quality potential: when the sink is decreased by removal of many hands, the fruit susceptibility to crown rot decreases. Few studies on various plants have linked the importance of source-sink ratio, and thus photosynthetic assimilate distribution, in plant-pathogen interactions (Barrière, 1985; Barrière *et al*., 1981; Dodd, 1980; Pegg, 1986; Seetharama *et al.*, 1991). However, in the case of bananas, the present results contrast with the previously reported observation that modifying the source-sink ratio has no effect on fruit susceptibility to anthracnose or on the fruit conservation potential (Chillet *et al*., 2006). This suggests that different mechanisms govern the susceptibility of fruit to crown rot and anthracnose. Jullien *et al.* (2001b) have also highlighted the impact of this source-sink ratio on fruit filling characteristics, and notably on the rate of cell filling. In our study, we consider that hand and leaf removal result in a modification of the empirical source-sink ratio. In keeping with the observation of Jullien *et al*. (2001b), we assume that an increased empirical source-sink ratio results in an increased fruit filling rate. One might hypothesize that this increased cell filling rate is involved in the observed reduction of the fruit susceptibility to crown rot. On the other hand, the impact of the source-sink ratio on nutrient availability, distribution, storage, and assimilative transformation has been extensively documented in

many models (Noquet *et al.*, 2004; Savin *et al.*, 2006; Famiani *et al.* 2000; Dordas, 2009; Zhenming *et al.*, 2008). We suggest that a modification of the source-sink ratio lead to a change in the partitioning of assimilates between various sink which influences the formation of secondary metabolites involved in plant-pathogen interaction. When hands are removed, the competition between sinks is reduced and the availability of mobile assimilates for remaining hands is more important.

It is noteworthy that in our study source reduction (leaf removal) had a lesser effect on fruit susceptibility to crown rot than sink reduction, since removal of about 60% of the leaves (7 out of 12) had little effect on fruit susceptibility, in contrast to removal of 75% of the fruits (6 out of 8). It is recognized that the impact of defoliation on the qualitative and quantitative development of bananas is variable and highly dependent on (i) when the defoliation is performed (Arcila *et al.*, 1995), (ii) the intensity of defoliation (Israeli *et al.* 1995, Robinson and Anderson, 1990, Rodriguez *et al.*, 2005), and (iii) how defoliation is done: mechanically or through the action of pathogens (Robinson *et al.*, 1992). Here, mechanical defoliation appears not to have been sufficiently early and/or severe to influence the processes determining fruit susceptibility to crown rot. On the other hand, a compensatory phenomenon reducing the impact of defoliation might also be involved, as the development of a bunch results from the distribution of dry matter not only from the leaves but also from other parts of the plant. In this way, the rachis and pseudostem may partially compensate for late-occurring defoliation (Eckstein *et al.*, 1995). It has also been shown that an increased photosynthetic capacity of the remaining leaves may partially compensate for losses caused by defoliation (Robinson *et al.*, 1992).

2.5. Conclusion

The susceptibility of bananas to crown rot is thus likely to be influenced by the stage of fruit development and by filling characteristics, these parameters being in close interaction and dependent on the soil-climate conditions and agro-technical factors of the production area. It is essential not to lose sight of the fact that the regulation of plant susceptibility is, in all cases, the result of nutritional balance established during plant growth. This balance is the consequences of all physiological relations of the whole plant and the environmental factors and might affect plant-pathogen interaction by two ways. First by influencing the ability of the plant to establish defense mechanisms, notably through changes in secondary metabolism. Secondly by altering the bioavailability of nutrients necessary for pathogen development.

However, the molecular underlying mechanisms implied in the susceptibility variations observed in this study still unknown.

The fact that the fruit susceptibility depends on the hand position in the bunch shows the importance of standardizing the sampling method when measuring the susceptibility of bananas to crown rot. The hands collected for an experiment should be collected systematically from the same position on each bunch. As the hands in the upper portion have more fruits and allow division into more 4-fruit clusters, it has been recommended to use the third hand of the bunch, which is more stable from one bunch to another than the first two hands (Jannoyer, 1995). If more than one hand per bunch is needed to carry out the experiment, it is essential to work with successive hands like demonstrated in this paper.

Lastly, the main method used to control crown rot is a systematic chemical post-harvest treatment. Apart from the environmental, social, and legislative problems resulting from this chemical control strategy, growers also face problems of treatment efficiency which are notably related to the fruit susceptibility in some specific areas. Only a truly integrated pest management strategy applied to the whole chain can provide effective alternatives to chemical treatment (Lassois *et al.*, accepted). We have shown that an increase in the empirical source-sink ratio makes it possible to reduce fruit susceptibility to crown rot while increasing fruit size at harvest. Thus, early hand removal in the field might be used as part of an integrated pest management scheme.

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CHAPTER IV

Identification of genes potentially implied in quantitative banana response to crown rot disease

1. Development of molecular biology techniques for the identification of genes differently expressed

1.1. General introduction

Prior to identify genes which could be implicated in banana quantitative defence-response to crown rot, it was necessary to develop all techniques necessary for the study of differently expressed genes.

The first difficulty was in the crown collected conservation in order to transport sample from Njombé, Cameroun to Gembloux, Belgium without affecting the RNA expression profil and quality. Indeed, for technical reasons, RNA extraction could not be realized in Cameroon. Among the various tested solutions, the most efficient and practical in our conditions was to freeze-dried our freshly collected sample. It is an original method because it has not been used extensively in plant tissues.

Another difficulty was to obtain good quality RNA from banana crown tissues which is a prerequisite to studying gene expression. Because of its high level in polysaccharides and polyphenols, banana RNA could not be extracted with standard methods. We have tested several methods, from simple to more time-consuming, and comparing them to choice the best one.

Finally the cDNA-AFLP was evaluated like method which could be used for studying gene expression between bananas showing various level of susceptibility.

All these results have been published:

Lassois L., de Lapeyre de Bellaire L. and Jijakli H. 2009. *Combining an original method for preserving RNA expression in situ with an effective RNA extraction method makes it possible to study gene expression in any banana fruit tissue*. Fruits, **64**: 127-137.

Combining an original method for preserving RNA expression *in situ* with an effective RNA extraction method makes it possible to study gene expression in any banana fruit tissue

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Running title: RNA extraction from banana fruit tissue

Abstract

Introduction. RNA isolation is a prerequisite to studying gene expression in banana and to understanding changes occurring in response to the environment. Standard extraction methods do not efficiently extract RNA from plants such as banana, with high levels of phenolics, carbohydrates, or other compounds that bind to and/or coprecipitate with RNA. **Materials and methods**. Five to seven RNA extraction methods were compared. Four crown-tissue storage methods were also compared. cDNA-AFLP was used to ensure that the obtained RNA was of sufficient quality for molecular applications and that RNA expression was unaltered by *in situ* storage. **Results and discussion**. The modified hot-borate method proved to be the best RNA extraction method, allowing high yields of good quality, undegraded RNA from the crown, fruit peel and pulp at all stages of ripening. The RNA obtained by this method was of sufficient quality for molecular applications such as cDNA-AFLP that give highly reproducible results. Freeze-drying of fresh tissues and tissue conservation in hot-borate buffer, two original storage methods, appear appropriate for preserving RNA *in situ* without ultra-low temperature. The RNA obtained was of high quality, undegraded, and useful for all downstream applications. The genome expression profile obtained by cDNA-AFLP analysis was unaltered by these methods for storing collected tissues. **Conclusion**. By applying all the suggested procedures in this work, it is possible to store and study gene expression in any banana fruit tissue, whatever the maturity stage, without affecting the RNA expression level.

Keywords: Belgium / *Musa* sp. / bananas / freeze-drying / RNA / storage / extraction

1.2. Introduction

Banana is cultivated in more than 120 countries and holds the second place in world fruit production, just behind orange and in front of grape. Exported dessert bananas belong to the Cavendish subgroup. Gene expression studies are essential to understanding the physiological processes of this plant species [1, 2]. Expression profiling, however, requires adequate sample conservation and high-quality RNA isolation for cDNA library construction and molecular analysis. The total RNA and mRNA obtained must notably be free of protein, genomic DNA and secondary metabolites. RNA extraction is thus a crucial step, but it can be problematic because of the relative instability of RNA, largely due to RNA degradation by ribonucleases [3]. Conservation and isolation of RNA from plant material, particularly from fruit tissues, presents special challenges [4, 5]. First, most plant materials contain relatively high levels of RNase activity, mostly located in the vacuoles [6]. They also contain various plant metabolites, such as polysaccharides and polyphenols. They bind to and/or co-precipitate with RNA, affecting the yield and quality of RNA [7] and rendering it unsuitable for cDNA synthesis. Thus, qualitative and quantitative differences in polysaccharide and polyphenol content among different plant tissues significantly influence the efficiency of nucleic acid extraction and purification procedures [3, 8]. Many procedures [4, 5, 8–14] have been developed to cope with endogenous polysaccharides, phenolics and RNases during RNA isolation from various plants, but the efficiency of an extraction method depends largely on the kind of plant or tissue.

Another potential problem with RNA is its rapid degradation or alteration during tissue storage. In some cases, plant tissues must be preserved as collected, especially when a genome-wide expression profile is to be studied. The most commonly used method to store tissues for preparation of RNA is to remove fresh tissues and to maintain them at a strictly ultra-low temperature [(–50 to –70) °C] [15]. However, other efficient storage methods without ultra-low temperature could be of interest but have not been extensively documented. Freeze-drying is proposed in some cases to conserve animal tissues [16–20], human tissues [21, 22] and bacteria [23] in order to further RNA extraction. However, in plant tissues, freeze-drying has not been used extensively to preserve RNA [4, 24] and contradictory results have been reported [25, 26].

The first aim of our study was to evaluate various techniques of RNA extraction from fresh banana tissues for quantity, quality and integrity, but also its adequacy for downstream molecular analyses such as cDNA-Amplified Fragment Length Polymorphism analysis

(cDNA-AFLP). This RNA fingerprinting technique derives from AFLP (Amplified Fragment Length Polymorphism), a method described by Vos *et al.* [27] for genomic DNA fingerprinting, which was applied for the first time like cDNA-AFLP to plants by Bachem *et al.* [28].

Classical RNA isolation techniques were compared with methods specifically elaborated for banana or other plants rich in polyphenols and polysaccharides.

As collected materials sometimes have to be stored prior to RNA extraction, the second aim of our study was to assess different methods for preserving RNA *in situ*. This means both avoiding RNA degradation between sample collection and RNA extraction and ensuring that nothing happens, during storage, to alter the expression profile obtained. Checking that this latter criterion is met requires obtaining pre- and post-storage differential expression profiles by cDNA-AFLP.

1.3. Materials and methods

1.3.1. Plant materials

Ripe yellow bananas of the Cavendish subgroup were bought on the market. Preclimacteric green bananas of the Cavendish subgroup were obtained from a commercial source in Belgium (Van Damme, Brussels) just before the ethylene treatment that triggers ripening.

1.3.2. RNA extraction methods

RNA extraction methods were tested on various fruit tissues from green and ripe bananas. Samples of peel, pulp and crown tissue were sliced, immediately frozen in liquid nitrogen, and ground to a fine powder with a pre-cooled mortar and pestle before extraction. Five extractions were performed for each tested method. All materials and reagents were treated so as to be RNase-free. Glassware was oven-baked at 240°C overnight. Sterile disposable plastic wares were used when it was possible and non-disposable plastic wares were rinsed with 0.1 M NaOH, 1 mM EDTA, followed by RNase-free water. Solutions (water and other solutions) were treated with 0.1% DEPC (diethyl pyrocarbonate), incubated overnight at 37 °C, then autoclaved to eliminate residual DEPC.

Five RNA extraction methods were applied to peel and pulp from green bananas. Methods based on commercial products were carried out according to the supplied

instructions. Methods taken from the literature were carried out according to the authors' protocols. These methods were:

1. The commercial ready-to-use RNA isolation product TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA), according to the protocol proposed for extraction from plant tissues: this is an improved version of the single-step RNA isolation method developed by Chomczynski and Sacchi [29]. During sample homogenization or lysis, TRIZOL Reagent maintains RNA integrity while disrupting cells and dissolving cell components.

2. The 'SV Total RNA Isolation System' developed by Promega (Madison, WI, USA): this is a quick and simple technique for preparing purified, intact total RNA. The system combines the disruptive and protective properties of guanidine thiocyanate and β-mercaptoethanol to inactivate the ribonucleases present in cell extract. The system also incorporates a DNase treatment step, and purification is achieved without any phenol/chloroform extraction.

3. The method elaborated by Liu *et al.* [30] to isolate total RNA from banana fruit tissues: RNA was extracted at room temperature with a high-ionic-strength buffer. Proteins, genomic DNA and secondary metabolites were then removed from the extract by precipitation with pre-cooled potassium acetate and repeated phenol/chloroform/isoamyl alcohol extractions. RNA was recovered by ethanol precipitation without LiCl. This procedure can be completed in less than 4 h.

4. The hot-borate method [31], adapted from that described for cotton [32]: RNA extraction was performed in a basic hot-borate/proteinase K buffer. A RNA-enriched pellet is precipitated from the supernatant with lithium chloride and dissolved in DEPC water. Two phenol/chloroform/isoamyl alcohol (25/24/1) extractions and one chloroform/isoamyl alcohol (24/1) extraction are incorporated into the RNA purification protocol to remove contaminant proteins. Total RNA is precipitated with ethanol.

5. The simple procedure developed by Asif *et al.* [33] for RNA isolation from banana tissue: these authors have modified the CTAB/NaCl method [11] by removing PVP from the extraction buffer and including a simple polysaccharide precipitation step. A RNA-enriched pellet is precipitated from the supernatant with lithium chloride and dissolved in DEPC water before phenol/chloroform extraction. Total RNA is precipitated with ethanol.

On peel and pulp from ripe bananas, in addition to these five protocols, two additional ones among those proposed by Gehrig *et al.* [7] were tested. Their techniques based on GITC (guanidium isothiocyanate) (for technique 6) and GHCL (guanidium hydrochloride) (for technique 7) buffers were selected. Both methods use a high-molecular-weight polyethylene glycol (HMW-PEG). The hot-borate method [31] was also tested on crown tissues. All extractions were performed on a small scale in a 1.5- or 2-mL tube, by using the protocols proportionally.

1.3.3. **Evaluation of RNA quality, quantity and integrity**

After extraction, RNA was recovered by centrifugation for 20 min at 16,000 *g* and 4 °C. The pellet was washed once in 1 mL of 70% cold ethanol, vortexed, and centrifuged for 5 min at 16,000 *g* and 4 °C. The pellet was air-dried at 37 °C, and then resuspended in RNase-free water. The purified RNA was quantified by measuring the absorbance at 260 nm (A_{260}) with a Nanodrop (ND-1000 spectrophotometer, Isogen Life Sciences, Maarssen, Netherlands). An A_{260} of 1 is equivalent to 40 ng RNA•µL^{-1}. The absorbance at 280 nm was also measured, and sample purity was estimated by the [A_{260} / A_{280}] absorbance ratio. RNA integrity was evaluated by the sharpness of the bands corresponding to 28S and 18S ribosomal RNA (rRNA) visualized by ethidium bromide staining on a 1% agarose gel.

1.3.4. **Evaluation of the RNA adequacy for downstream molecular analyses**

RNA extracts were treated for 30 min at 37 °C with 20 units of RNase-free DNase (Roche, Basel, Switzerland) in the presence of 40 units of RNase inhibitor (Roche Diagnostics, Mannheim, Germany). The cDNA-AFLP technique involves three steps: (1) restriction of cDNA and ligation of oligonucleotide adapters; (2) selective amplification of sets of restriction fragments using PCR primers bearing selective nucleotides at the 3’ end; (3) gel analysis of the amplified fragments. Double-stranded cDNA was synthesized from total RNA according to the instructions for use of the Superscript Double-Stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). The double-stranded cDNA was digested with EcoRI and MseI, ligated with EcoRI and MseI adapters, and pre-amplified with the Eco (5’-GACTGCGTACCAATTC-3’) and Mse (5’-GATGAGTCCTGAGTAA-3’) primers, following the instructions of the AFLP Analysis System kit for microorganisms (Invitrogen, Carlsbad, CA, USA). After twenty-fold dilutions of the PCR fragments, specific amplifications were carried out with combinations of an Eco primer and a Mse primer containing two additional bases at their 3’ ends. The Eco primers were labeled with γ^{33}P

dATP. Amplification products were separated by electrophoresis at 50 W on a vertical denaturing polyacrylamide gel (6%) containing 7 M urea. Gels were dried on a Whatman paper before screening with a phosphoimager (BioRad, Richmond, CA, USA). To evaluate the reproducibility of the cDNA-AFLP, a single banana crown was ground in liquid nitrogen to a fine powder and divided into two pools. RNA was extracted according the hot-borate method [31]. After DNase treatment, two different pre-amplifications were performed per sample and two different selective amplifications were performed per pre-amplified sample (Figure 1). Two replicates were performed.

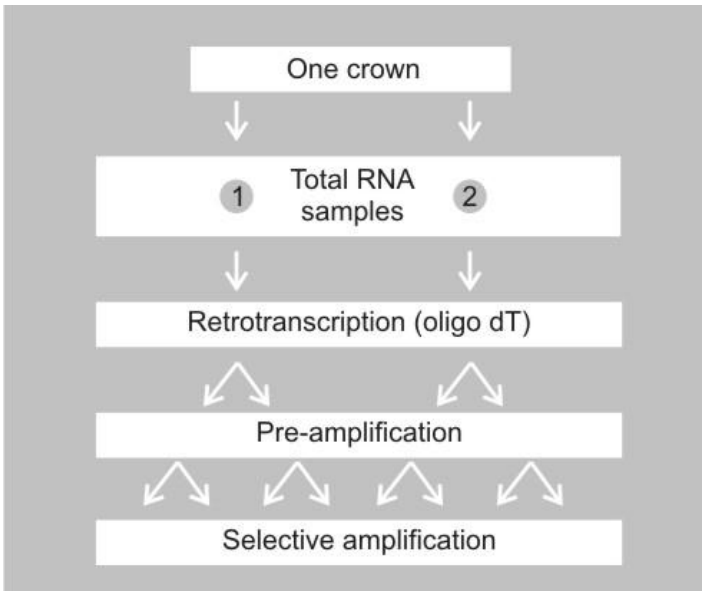

Figure 1. Protocol applied to test the reproducibility of the cDNA-AFLP analyses based on RNA extracted from banana fruit tissues.

1.3.5. *In situ* RNA preservation methods

Four tissue storage methods were compared on crown tissue material. The first two methods involved placing the tissues on RNAlater (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Here, crown tissue was first either sliced into tiny lamellas before storage in RNAlater at 4 °C or ground in liquid nitrogen before storage in RNAlater at 4 °C.

The third conservation method (buffer storage) consisted of storing crown tissues (ground to a fine powder in liquid nitrogen) in the hot-borate buffer [31] (200 mM BORAX, pH 9.0; 30 mM EGTA; 1% SDS; 10 mM DTT; 2% PVP 40; 2% DIECA; 0.5% Igepal) to which 0.5 mg proteinase K•mL^{-1} buffer was added. This was stored at room temperature.

The last method consisted of freeze-drying the crown sample at –80 °C for 24 h (Telstar, Cryodos, Barcelona, Spain) and storing it at room temperature before RNA extraction (freeze-drying storage). The freeze-dried crown samples were stored with silica gel to avoid an aqueous environment.

In each case, RNA extraction was performed 21 d later by the hot-borate method [31]. The extraction efficiency was assessed by analysis of RNA quality, quantity and integrity as described in section 1.3.2..

The adequacy of the RNA, obtained after a buffer or a freeze-drying storage, for downstream molecular application was evaluated by cDNA-AFLP as described in section 1.3.4. Furthermore, this technique allows evaluating the effect of the storage conditions on gene expression by comparing pre- and post-storage differential expression profiles. To do this, three samples were collected from a single crown. One of these served as control and RNA extraction was performed immediately; both of the others were stored either by freeze-drying or in hot-borate storage buffer [31] as described above prior to RNA extraction and cDNA-AFLP. This experiment was repeated twice.

1.4. Results and discussion

1.4.1. Compared performances of the RNA extraction methods

Among the RNA extraction methods tested, the hot-borate method [31] emerges as particularly adequate for banana pulp and peel, whatever the maturity stage, and for the crown, which is particularly fibrous. Total RNA isolated by this method was of high purity and integrity and of sufficient yield, making it suitable for molecular analysis. This was confirmed in several ways.

First, spectrophotometric analysis revealed an [A_{260} / A_{280}] ratio ranging consistently from 2.0 to 2.2, indicating that the RNA isolated by this approach was largely free of contaminating proteins. This was true whatever the tissue type and maturity stage (Table 1). Pure RNA has an [A_{260} / A_{280}] ratio of 2.0, but, for most applications, a lower or higher ratio probably will not affect the results [3]. As a result of variations between individual starting materials and in performing the procedure, the expected range of the [A_{260} / A_{280}] ratio for RNA is 1.7-2.2. Three other methods gave this ratio: Liu *et al.* [30] and both methods described by Gehrig *et al.* [7]. Both commercial kits failed to extract RNA from banana fruit tissues. The TRIZOL reagent was developed for animal tissues, especially those rich in RNase, but it seems unsuitable for recalcitrant plant tissues such as banana. This is in

agreement with the results obtained by other authors [12]. The method based on CTAB [33] was also unsuccessful. The same conclusion was reached with different recalcitrant plant species [13].

Table 1: [A_{260} / A_{280}] ratio values according to different tissue and RNA extraction methods. The best methods give ratios between 1.7 and 2.2. GHCL: guanidium hydrochloride; GITC: guanidium hydrochloride

Methods	Peel		Pulp		Crown
	Green	Mature	Green	Mature	
Trizol® Reagent (Invitrogen)	0.9-1.1	0.9-1.2	0.1-3.4	1.0-1.3	-
SV Total RNA Isolation System (Promega)	0.2-0.8	0.4-1.0	0.06-0.20	0.6-0.9	-
Liu *et al.*[30]	1.7	1.7-2.0	1.7-1.8	1.8-2.1	-
Mbéguié-A-Mbéguié *et al.* [31]	2.0-2.2	2.0-2.2	2.0-2.2	2.0-2.1	2.00-2.14
Asif *et al.* [33]	1.2-1.4	1.2-2.3	1.1-1.5	0.8-1.5	-
Gehrig *et al.* (GITC) [7]	-	1.7-2.0	-	1.0-2.3	-
Gehrig *et al.* (GHCL)[7]	-	1.7-2.0	-	1.7-2.0	-

Secondly, a comparison of RNA yields based on spectrophotometric determinations showed a significantly lower yield (as much as 15 times lower) for the method of Liu *et al.* [30] than for the hot-borate method [31] (Table 2). The yield obtained in this study by this latter method is comparable with those reported for other methods designed for high-phenol fruit tissues [4, 7]. The yield obtained in this study by the hot-borate [31] protocol depended heavily on the tissue type and, to a lesser extent, on the maturity stage. By decreasing yield (Table 2), the different tissues can be ranked as follows: green pulp (67.8–79.5 $\mu g \cdot g^{-1}$ FW) > mature pulp (61.8-65.9 $\mu g \cdot g^{-1}$ FW) > green peel (45.2-60.2 $\mu g \cdot g^{-1}$ FW) > mature peel (43.4-50.5 $\mu g \cdot g^{-1}$ FW) > crown (10.2-17.4 $\mu g \cdot g^{-1}$ FW).

Table 2: Yield values (µg RNA•g^{-1} fresh material), based on A_{260}, according to different tissue and RNA extraction methods. (x) means that the yield could not be calculated because the [A_{260} / A_{280}] ratio was not between 1.7 and 2.2. GHCL: guanidium hydrochloride; GITC: guanidium hydrochloride.

Methods	Peel		Pulp		Crown
	Green	Mature	Green	Mature	
Trizol® Reagent (Invitrogen)	(x)	(x)	(x)	(x)	-
SV Total RNA Isolation System (Promega)	(x)	(x)	(x)	(x)	-
Liu *et al.*[30]	3.9-5.7	7.6-8.9	3.6-9.9	50.3-56.8	-
Mbéguié-A-Mbéguié *et al.* [31]	45.2-60.2	43.4-50.5	67.8-79.5	61.8-65.9	10.2-17.4
Asif *et al.* [33]	(x)	(x)	(x)	(x)	-
Gehrig *et al.* (GITC) [7]	-	72.0-75.8	-	(x)	-
Gehrig *et al.* (GHCL)[7]	-	102.4-109.7	-	117.5-125.2	-

Thirdly, denaturing agarose gel electrophoresis yielded clear rRNA bands with a 28s rRNA band equal to or more abundant than the 18s rRNA band (Figure 2), indicating that little or no degradation had occurred during extraction with the hot-borate [31] and Liu *et al.*'s [30] methods. In high-quality RNA, the 28s band should be approximately twice as intense as the 18s band [3]. In degraded RNA samples, the 18s band will be enhanced, since the 28s ribosomal RNA is typically degraded to an 18s-like species. No rRNA was observed with any other technique, and notably not with either of those described by Gehrig *et al.* [7]. The latter methods did give, however, a good [A_{260} / A_{280}] ratio (Table 1) and thus a high estimated yield (Table 2). An explanation might be that A_{260} could be falsely boosted by contaminants such as salt, organic solvent or protein, or by one or more of the many chemicals used in nucleic acid purification which absorb at 260 nm [34]. Thus, the [A_{260} / A_{280}] ratio can accurately describe nucleic acid purity, but it can also be misleading [35]. For this reason, it is essential to have both the [A_{260} / A_{280}] ratio and an agarose gel result to evaluate a RNA extraction method.

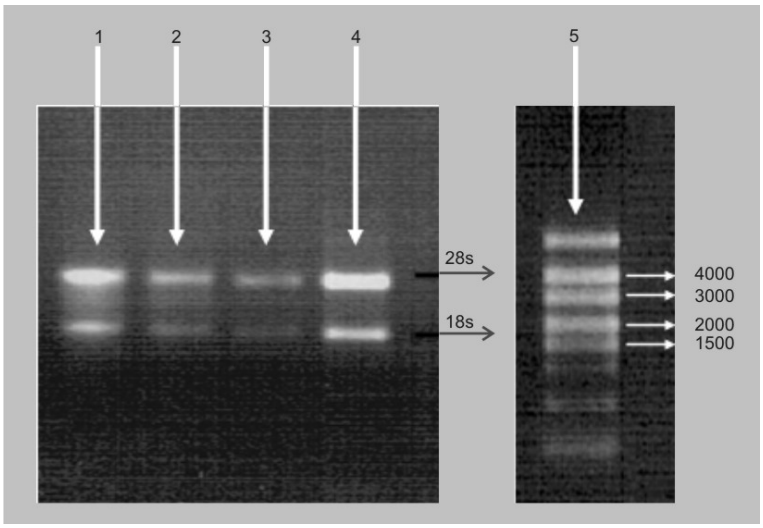

Figure 2. Band patterns obtained by ethidium bromide staining after electrophoresis, through a 1% agarose gel, of total RNA extracted from green banana peel (1 and 3) or pulp (2 and 4). The extraction protocols used were that of Liu *et al.*, [30] (1 and 2) and the hot-borate method [31] (3 and 4). (5) is a RNA ladder and the positions of of the 28S and 18S rRNAs are shown. Similar results were obtained for both protocols with peel and pulp from ripe banana. No rRNAs were observed with any other method tested in this work (resumts not shown).

Finally, the adequacy of the hot-borate method [31] for extracting total RNA from banana tissues in order to generate cDNA for downstream molecular analyses was successfully performed by cDNA-AFLP after DNase treatment (Figures 3 and 4).

For these reasons, although the hot-borate method [31] is labor-intensive and time-consuming, it seems to be the most suitable for extracting RNA from banana tissues in order to carry out genome-wide expression analyses. This RNA extraction technique is applicable to large samples of up to 25 g fresh material [36], but also to small samples of 0.24 g of material in a 2-mL tube, as in this work. It could be more convenient to extract RNA from a small sample, because many samples can be extracted at the same time, and the yield obtained is still sufficient for subsequent molecular applications.

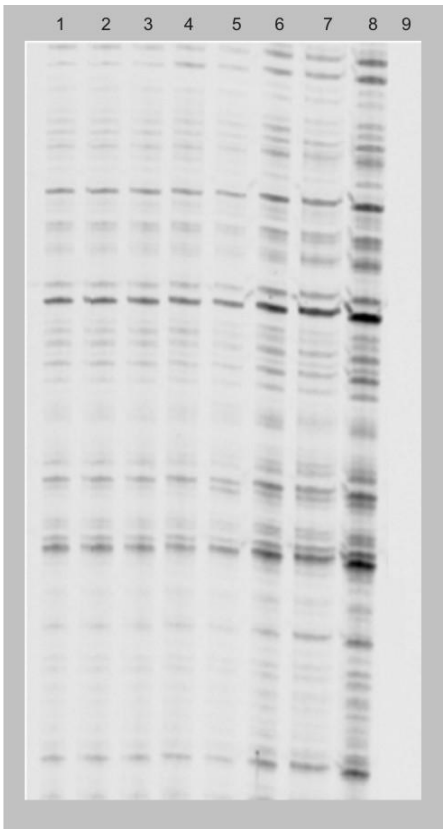

Figure 3. Reproducibility of cDNA-AFLP based on total RNA. cDNA-AFLP was applied to various pools of total RNA from from a single crown according to figure 1. (1) to (8) represent the various cDNA-AFLP pools and (9) is a blank.

1.4.2. *In situ* RNA preservation methods

On the basis of quality, quantity and integrity, it appears that all four methods tested can preserve RNA (Table 3). The choice of a method must thus be based on the experimental constraints and the equipment available. Yet, although all four techniques appeared to preserve RNA quality and integrity, their yields were different. RNAlater is an aqueous tissue storage reagent that stabilizes and protects cellular RNA in intact, unfrozen tissue samples. According to Ambion (Austin, TX, USA), it eliminates the need to process tissue samples immediately or to freeze them in liquid nitrogen for later processing. Tissue pieces can be harvested and submerged in RNAlater for storage without jeopardizing the quality or quantity of RNA obtained by subsequent RNA isolation.

Table 3: [A_{260} / A_{280}] ratio and yield values (based on A_{260}) according to four RNA *in situ* preservation methods. Extractions were performed by the hot-borate method [31].

Methods	[A_{260} / A_{280}] ratio	Yield (µg RNA.g^{-1} fresh material)	28s:18s RNA bands
Crown sliced into tiny lamellas and stored in RNAlater at 4°C for 21 d	1.9-2.0	3.2-5.7	Yes
Crown ground in liquid nitrogen and stored in RNAlater at 4°C for 21 d	1.8	1.5-3.7	Yes
Crown ground in liquid nitrogen and stored in hot-borate buffer [31] + proteinase K at room temperature for 21 d	1.9	10.2-14.0	Yes
Crown freeze-dried and stored for 21 d at room temperature before extraction	2.2	12.0-16.2	Yes

In this study, RNAlater has the advantage of being a simple and fast technique, but its yield is two to five times lower than those obtained by tissue storage in extraction buffer or freeze-drying. For the latter two methods, the yields are similar. These two techniques present the advantages of storage at room temperature and a similar RNA extraction quality and yield to those obtained after immediate extraction of RNA from fresh crowns (Tables 2 and 3). Freeze-drying has not been used extensively in plant tissues [4, 24]. Contradictory results, ranging from good RNA preservation in leaf tea [25] to complete degradation in cotton [26], have been reported. In these previous studies, only the RNA quantity and quality on agarose gel were analyzed [24]. In some cases, authors have tested the expression conservation of a single or two particular genes [19–21, 23, 25]. In this original study, the entire pre- and post-storage genome-wide expression profile were compared, in order to evaluate the eventual global modification of RNA expression that could happen during the freeze-drying process or storage. The results obtained by cDNA-AFLP clearly indicate that the RNA obtained by both buffer storage and freeze-drying was of sufficient quality for molecular application and that cDNA-AFLP pre- and post-storage differential expression profiles were identical (Figure 4). Thus, these two storage methods do not affect either the RNA quantity and quality, or the genome expression. For this study, cDNA-AFLP was selected because it is a non-biased technique based on PCR amplification, offering the advantage of almost no false positives [37]. It provides a straightforward way to check band identity and homogeneity [37] and it is more sensitive than hybridization-based techniques and highly specific. It was also confirmed in this study that cDNA-AFLP is a highly reproducible method for genome-wide expression profile analysis (Figure 3). In both replicates, the steps comprising DNase treatment, amplification and selective amplification led to the same AFLP profile. Reproducibility of

cDNA-AFLP has already been reported by many authors [28, 38–40]. Khun [37] even evaluates the reproducibility of cDNA-AFLP at 100%.

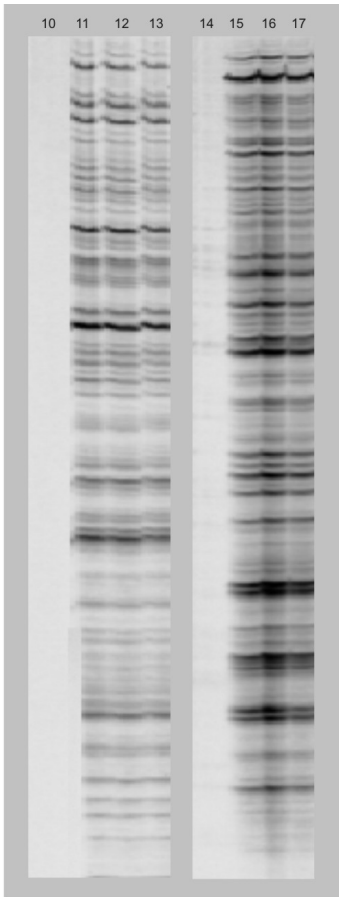

Figure 4. Reproducibility of the cDNA-AFLP gene expression profile whatever the *in situ* preservation method. (10)-(13) = replicate 1; (14)-(17) = replicate 2. (10) and (14) are blanks; (11) and (15) are profiles obtained after immediate RNA extraction from fresh material; (12) and (16) are profiles obtained with RNA from freeze-dried tissues; (13) and (17) are profiles obtained with RNA from tissue samples stored in the hot-borate buffer [31].

Both storage techniques are thus perfectly suitable for cDNA-AFLP applied to the study of differential gene expression in different cell populations. However, freeze-drying, which preserves enzymatic activity, offers further advantages highlighted in this study; they can be summarized: (1) the extraction yield, quality and integrity equal those obtained by fresh tissue RNA extraction or by conventional storage techniques; (2) the freeze-dried RNA can be stored even at room temperature, without any alteration of the expression profile, for further RNA extraction; (3) freeze-drying is an easy way to obtain a fine dry power without using liquid nitrogen, which is not always available or economically feasible in all banana-producing countries; (4) freeze-dried tissues can be easily transported from one laboratory to another and even between countries; (5) a final advantage of freeze-drying is the large amount

of RNA that can be obtained in a single extraction carried out in a 2-mL tube. Such a tube can hold only about 0.25 g fresh sample material, but if the material is freeze-dried, a mere 0.2 g is equivalent to about 2.4 g fresh weight. Assuming the same yield from freeze-dried material as from fresh material, the amount of RNA that can be obtained per tube is ten to twelve times higher when the material is freeze-dried. In applications requiring a lot of RNA, it could be very convenient to meet this requirement while working on a small scale with a 2-mL tube. These advantages make freeze-drying an attractive alternative to conventional storage.

Moreover, the two efficient storage methods proposed in this study to conserve fruit banana RNA *in situ* without altering the quality, quantity or expression profile could probably be applied to other plant tissues in particular fruits which are particularly recalcitrant.

1.5. Conclusion

We thus evaluated and made choices among various techniques spanning all steps required to study differential gene expression in banana fruit. Our results highlight two good, reliable and practical storage methods that preserve RNA *in situ* before extraction without requiring ultra-low temperature. We also identified a RNA extraction method yielding high-quality RNA suitable for gene expression profiling. To illustrate this, we used the extracted RNA successfully to generate cDNA-AFLP differential expression profiles. Thus, it is possible to study gene expression in any banana fruit tissue, whatever the maturity stage, by applying all the procedures selected in our work.

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1.6. References

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2. Identification of genes that may influence quantitatively the banana response to crown rot disease.

To study the molecular mechanisms underlying the susceptibility variation it was important to develop a sampling that allows selecting at harvest two kinds of banana. On one hand fruits with a high susceptibility level to crown rot and on the other hand fruits with a low susceptibility level to compare gene expression between them. The difficulty of such sampling was that crown for molecular analysis should be collected at harvest time whereas the fruit susceptibility can only be assessed once the symptoms appeared on ripe fruit. This corresponds in our essays at 13 days post-harvest. The results presented in chapter 3.2 show that by modifying the source-sink ratio at flowering it could be expected that fruit susceptibility would be highly different. Thus to identify gene expression implied in banana quantitative defence-response to crown rot disease, we have decided to selected our sample on base of their source-sink ratio. cDNA-AFLP method was applied on these samples and results obtain were confirmed by real-time RT-PCR. By this way, genes potentially implied in banana quantitative defence response to crown rot disease were identified.

Results are presented hereafter and will be soon submitted for publication to Physiological and Molecular Plant Pathology.

Lassois Ludivine, Frettinger Patrick, de Lapeyre de Bellaire Luc, Lepoivre Philippe and Jijakli Haissam. *Identification of genes that may influence quantitatively the banana response to crown rot disease*.

Identification of genes that may influence quantitatively the banana response to crown rot disease

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Abstract

Variations in banana fruit susceptibility to the crown rot post-harvest disease have been observed but the molecular mechanisms underlying these quantitative host-pathogen relationships were still unknown. The present study was designed to compare gene expression between crowns of bananas (*Musa acuminata*, AAA, 'Grande-Naine') showing a high susceptibility (S^+) and crowns of bananas showing a low susceptibility (S^-) to *Colletotrichum musae* responsible for crown rot disease. This comparison was performed at two situation time: (i) between crowns (S^+ and S^-) collected one hour before infection and (ii) between crowns (S^+ and S^-) collected 13 days after infection. Gene's expression comparisons were performed with cDNA-AFLP technique and differential expression results obtained were confirmed by real-time RT-PCR for two biological replicate. The limitations of the cDNA-AFLP and the importance of the results validation by an independent method and for two biological replicates are discussed. Among the 3161 transcript-derived fragments (TDFs) screened by cDNA-AFLP, 443 were differently expressed between bananas showing different susceptibility. Only 10% were selected, successfully recovered from the gel, cloned, sequenced and showed non redundant similarities with the data base. Among those, only eleven TDFs differently expressed were finally confirmed for both biological replicates. Two identified genes were involved in signal transduction and three in proteolytic machinery. In addition, two TDFs were similar to pathogenesis-related protein 14, one was similar with a CCR4-associated factor (CAF1) protein, one with a cellulose synthase. Paradoxically, the over-expression of the cellulose synthase gene is associated with banana showing a high susceptibility both in pre- and post-inoculation situation. Finally, the cDNA-AFLP allows identifying that a gene, coding for a dopamine-β-monooxygenase, involved in catecholamine pathway seems to be associated in the quantitative banana responses to crown rot disease. To our knowledge, this work is the first to address both pre- and post-infection gene expression with the same host-pathogen combination and different susceptibility.

2.1. Introduction

Crown rot disease affects export bananas in all producing countries and is considered as one of the main post-harvest diseases of bananas (for a review see Lassois *et al.*, accepted [a]). The symptoms appear on the crown, i.e. the tissue joining the fruit pedicels with each other. The disease develops during shipping, ripening, and storage and has a negative impact on the market value of bananas (Slabaugh and Grove, 1982).

Variations, notably geographic and seasonal, have been reported in the post-harvest development of crown rot disease symptoms (Krauss and Johanson, 2000; Lassois *et al.*, 2008; Lukezic *et al.*, 1967; Shillingford, 1978). It has been suggested that these spatiotemporal fluctuations reflect the variations in the banana fruit quality potential that elaborates during preharvest stages (Lassois *et al.*, accepted [a]). This quality potential depends both on a physiological and a parasitic components, which in turn are influenced by agrotechnical and pedoclimatic factors. The parasitic component reflects the level of crown contamination by the parasitic complex, as well as the pathogenicity of this parasitic complex. Effectively, crown rot results from an unspecific parasitic complex from which *Colletotrichum musae* is the most pathogenic species (Finlay and Brown, 1993). The term "physiological component" refers here to the level of fruit susceptibility to crown rot. To assess the level of fruit susceptibility it is necessary to overcome the influence of the parasitic component. It is then estimated through lesion size after standardized artificial inoculations with *C. musae* (de Lapeyre de Bellaire *et al.*, 2008). Very few studies have linked the spatio-temporal fluctuations of crown rot disease to preharvest factors influencing the level of fruit susceptibility (Lassois *et al.*, 2008). Nevertheless, it has been shown recently that source-sink ratio and the hand position in the bunch affect the banana susceptibility to crown rot disease (Lassois *et al.*, accepted[b]). The genetic and molecular mechanisms underlying these quantitative host-pathogen relationships have not been identified.

Plant defense mechanisms to diseases are mediated by both preformed and inducible responses which lead to significant transcriptional changes in the host (Lucas, 1998). One way to understand the reactions involved in the variation of banana responses to crown rot is then to identify genes involved in these processes via the study of their expression. The cDNA-AFLP technique (Bachem, 1996) has been used to compare genome-wide expression patterns and to identify differently expressed genes potentially related to plant defense mechanisms. This technique does not require prior hypothesis on defense mechanisms and allows the identification of genes without *a priori*. It can thus be used for any biological system,

especially when genomic resources are lacking. In addition, the mechanisms responsible for compatibility interactions and for the quantitative variation of symptoms after infection remain poorly understood, and it is likely that some of the factors involved remain to be discovered. The sensitivity and specificity of cDNA-AFLP analysis are comparable to those of a microarray approach (Reijans *et al.*, 2003). Its reproducibility has also been proved (Kuhn, 2001; Lassois *et al.*, 2009).

The present study was designed to identify genes influencing the susceptibility to crown rot disease in a same banana cultivar. A cDNA-AFLP technique has been used to compare gene expression in banana crowns from plants (*Musa acuminata*, AAA, cv 'Grande-Naine') showing different susceptibility to crown rot disease. In this study, the variation of source-sink ratio previously described (Lassois *et al.*, accepted[b]) has been used to obtain banana plants showing different susceptibility to crown rot disease. Samples were collected before and after infection in order to differentiate constitutive and induced defense responses. The differential expression deduced from cDNA-AFLP data was latter confirmed by an independent technique, the real-time RT-PCR. This latter technique is quick and, thanks to its high sensitivity, requires only a small amount of starting material (Massart and Jijakli, 2006).

2.2. Materials and methods

2.2.1. Fruit sampling

Banana fruits were harvested from plants (*Musa acuminata*, AAA, cv 'Grande-Naine') grown on the Dia-Dia commercial plantation (PHP) in Njombé, Cameroon (altitude: 80 m; annual mean temperature: 26.5°C; annual mean rainfall: 3500 mm). The date of flowering was indicated by tying a colored belt to each bunch (banana inflorescence) at the horizontal finger stage in order to predict the time of harvest. Bunches were also covered with a plastic sleeve at this stage. Bunches were harvested at a constant physiological age (Jullien *et al.*, 2008), i.e. when the mean daily temperature sum accumulated by the fruit at the 14°C threshold between flowering and harvest reached 900 dd. Temperatures were recorded at a weather station on the plantation. The daily average temperature (Td) was estimated from measurements of maximum temperature (Tmax) and minimum temperature (Tmin). To obtain bananas with different levels of susceptibility to crown rot, fruits were harvested from plants characterized by different source/sink ratios (Lassois *et al.*, accepted[b]) established at the flowering stage, leaves being viewed as sources and fruits as sinks:

- Plants showing a low susceptibility to crown rot disease were called "S^-". These plants were obtained through severe trimming of bunches at flowering stage. These plants had 12 leaves and 2 hands (a hand is a group of bananas) left on the bunch. Two plants of this treatment were used, namely, "S^- plant 1" and "S^- plant 2" and constitute the 2 biological replicates.
- Plants showing a high susceptibility to crown rot disease were called "S^+". These plants were reference plants without severe trimming of bunch. At flowering, bunches were not trimmed, so these plants had 12 leaves and 8 hands left on the bunch. Two plants of this treatment were used, namely, "S^+ plant 1" and "S^+ plant 2" and constitute the 2 biological replicates.

Figure 1 outlines the experimental sampling design for fruit susceptibility evaluation and molecular analysis, while table 1 identifies the samples, with their respective sources and processing modalities. Only the second hand of each bunch was collected and each one was divided into 4 clusters of 4 fingers (fruits) without defects. Each sample group consisted of clusters from the same hand, so as to avoid variability due to susceptibility variations among different hands (Lassois *et al.*, accepted[b]). At harvest for each bunch, 1 cluster was used for sampling crown 1 hour before inoculation of *C. musae* (1 hbi - clusters A, C, E and G according to the treatment and the biological replicate) and 3 clusters were inoculated with *C. musae* (figure 1). These 3 clusters were used for fruit susceptibility evaluation in order to confirm the expected S^+ or S^- status of each treatment. One of these 3 was collected for crown sampling 13 days after inoculation when symptoms are developed (13 dpi - clusters B, D, F and H according to the treatment and the biological replicate). The crowns collected at each period (1hbi and 13dpi) for molecular analyses were immediately frozen in liquid nitrogen, freeze-dried at -80°C for 24h (Telstar, Cryodos, Barcelona, Spain), and stored at room temperature before RNA extraction (Lassois *et al.*, 2009) and molecular analysis.

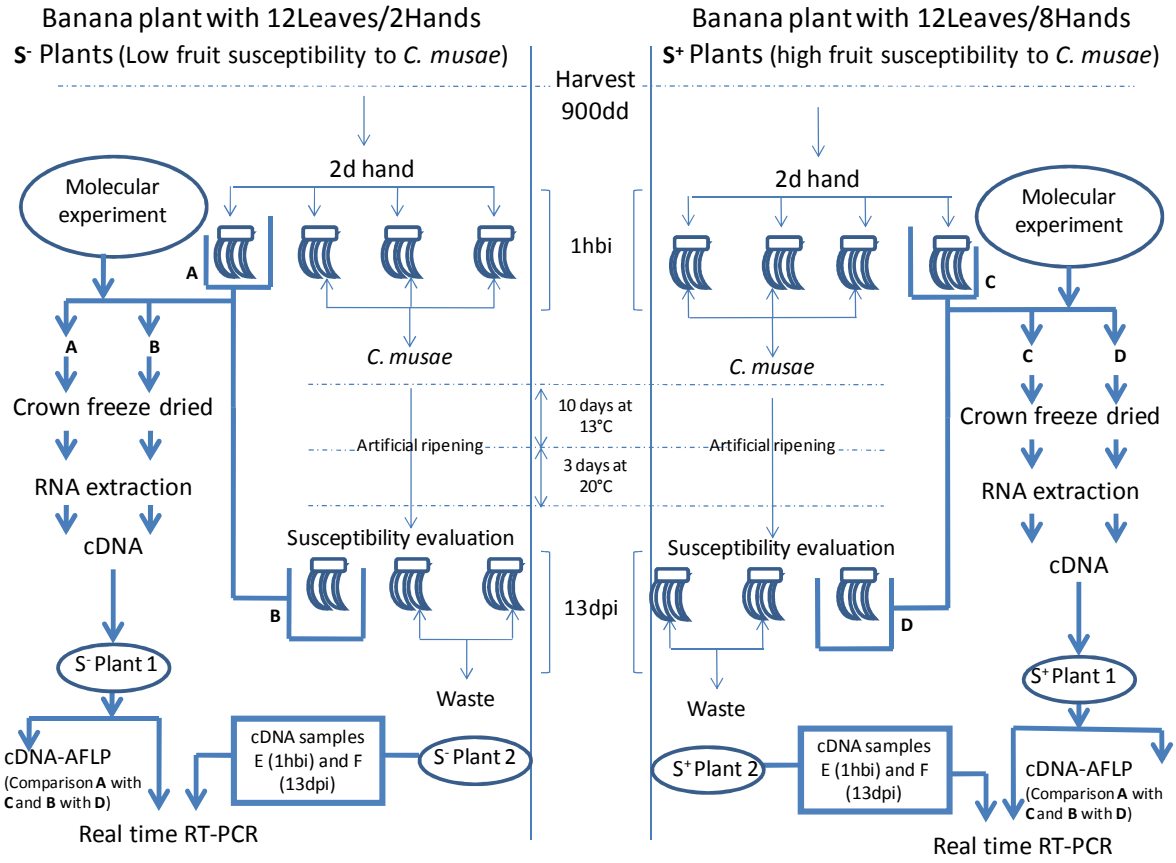
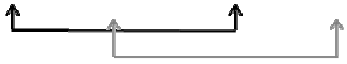


Figure 1: Experimental procedure. As bananas from plants with 12 leaves and 2 hands are waiting to be less susceptible than bananas from plants with 12 leaves and 8 hands (Lassois *et al.*, accepted[b]), two plants with each configuration were used as source material (S^- plant 1, S^- plant 2, S^+ plant 1 and S^+ plant 2). Only the second hand of each bunch was collected and each one was divided into 4 clusters of 4 fingers without defects. The "S^- plant 1" (S^- for low susceptibility) and "S^+ plant 1" (S^+ for high susceptibility) were used as sources of material for cDNA-AFLP analysis and for one replicate of the real time RT-PCR. Samples A and B were collected from "S- plant 1" respectively 1 hour before inoculation (1hbi) and 13 days post-inoculation (13dpi). Likewise, samples C and D were collected from the "S+ plant 1" respectively 1 hour before inoculation (1hbi) and 13 days post-inoculation. Following the same procedure, samples E (1 hbi) and F (13 dpi) were collected from "S- plant 2" and samples G (1 hbi) and H (13 dpi) from "S+ plant 2". These samples provided the second biological replicate used for result confirmation by real time RT-PCR. Furthermore, 3 clusters of each selected hand were used to evaluate fruit susceptibility to *Colletotrichum musae* at 13 dpi to confirm the expected S^+ or S^- status of each source.

Table 1: Presentation of the various collected samples used for molecular analysis in order to identify genes differently expressed in banana crowns showing high and low susceptibility to crown rot disease. S^- stands for low susceptibility and S^+ for high susceptibility. 1 hbi: Crowns collected 1 hour before inoculation of clusters from the second hand; 13 dpi: Crowns collected 13 days after inoculation of clusters from the second hand. The cDNA pools obtained after RNA extraction and reverse transcription are labeled A to H. In the cDNA-AFLP analysis, sample A was compared with C (two susceptibility levels, 1 hbi) and sample B with D (two susceptibility levels, 13 dpi). In the real-time RT-PCR assays, A and E yielded biological replicates, as did B and F, C and G, D and H.

	Biological replicate 1				**Biological replicate 2**			
Susceptibility level	S^- plant 1		S^+ plant 1		S^- plant 2		S^+ plant 2	
Source/sink ratio	12leaves/2hands		12leaves/8hands		12leaves/2hands		12leaves/8hands	
Collecting time	1hbi	13dpi	1hbi	13dpi	1hbi	13dpi	1hbi	13dpi
Crown identification (cfr Figure 1)	A	B	C	D	E	F	G	H
cDNA-AFLP comparison								

2.2.2. **Inoculation of fruits for the evaluation of susceptibility to crown rot**

The *C. musae* strain was isolated in Njombé, Cameroon. It is sensitive to thiabendazole and was stored at –20°C in a glycerol solution (30%). Before use, it was grown at 25°C in Mathur medium ($MgSO_4.7H_2O$: 2.5g/l; KH_2PO_4: 2.7g/l; peptone: 1 g/l; yeast extract: 1g/l; saccharose: 10 g/l; agar: 15 g/l) for 10 days. Conidia were removed by flooding the plates with sterile distilled water and filtration through a 45-µm sieve. The concentrations of the conidial suspension was determined with a Mallassez cell.

Smoothly and similarly cut crowns were obtained with a sharp knife, leaving as much crown tissue as possible. The latex from the crown tissue was eliminated with absorbent paper and the crowns were surface-sterilized by immersion in 50% ethanol. Fifty microliters of *C. musae* conidial suspension containing 10^4 conidia/ml was applied to the centre of the freshly exposed crown tissue and covered with a small paper filter. Two hours after application of the conidial suspension, the clusters were packed in punched polyfilms normally used in the industry, placed in commercial boxes, and stored at 13°C for 10 days to simulate shipment. Artificial ripening was then initiated by dipping the bananas for 5 seconds in an ethrel

solution (480 g/l), after which the clusters remained at 20°C for another 3 days before crown rot assessment. The internal progression of the rot was determined by cutting the cluster crown longitudinally in two and measuring the surface of rot spread into the crown, from the original inoculation point. This "internal necrotic surface" (INS), calculated by assuming a rectangular shape, was expressed in mm^2 (figure 2). The average value of the 3 replicates was taken as a measure of fruit susceptibility to crown rot.

Figure 2: Evaluation of crown susceptibility. The internal necrotic surface (INS) was calculated assuming a rectangular shape and is expressed in mm^2.

2.2.3. **RNA isolation and reverse transcription**

Total RNA was extracted from the eight lyophilized crown samples (A to H, table 1) by the hot borate method (Wan and Wilkins, 1994) modified by Mbéguié-A-Mbéguié *et al.* (2008) and as described by Lassois *et al.* (2009). RNA extracts were treated for 30 min at 37°C with 20 units of RNase-free DNase (Roche, Basel, Switzerland) in the presence of 40 units of RNase inhibitor (Roche Diagnostics, Mannheim, Germany). The RNA yield was determined by measuring the absorbance at 260 nm with an ND-1000 nanodrop spectrophotometer (Isogen Life Sciences, Maarssen, the Netherlands), and sample purity was estimated by the A_{260}/A_{280} absorbance ratio. RNA integrity was evaluated by the sharpness of the bands corresponding to 28S and 18S ribosomal RNA (rRNA) visualized by ethidium bromide staining on a 1% agarose gel. Double-stranded cDNA was synthesized from total RNA according to the instructions for use of the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA).

2.2.4. **Identification of genes differently expressed between crowns of high and low susceptibility to crown rot disease**

The cDNA pools derived from S^+ plant 1 and S^- plant 1 (samples A to D in table 1 and figure 1) were subjected to cDNA-AFLP. For each collection time (1 hbi or 13dpi) the S^+ and S^- samples were compared. A was thus compared with C (1 hbi) and B with D (13 dpi).

2.2.4.1. **cDNA-AFLP**

Double-stranded cDNA was digested with EcoRI and MseI, ligated with EcoRI and MseI adapters, and pre-amplified with Eco (5'-GACTGCGTACCAATTC-3') and Mse (5'-GATGAGTCCTGAGTAA-3') primers according to the instructions of the AFLP Analysis System kit for microorganisms (Invitrogen, Carlsbad, CA, USA). After 20-fold dilution of the PCR fragments, specific amplifications were carried out with 8 primer combinations of an Eco primer and an Mse primer containing two additional bases at their 3' ends. The Eco primers were labeled with γ^{33}P dATP. Amplification products were separated by electrophoresis at 50 W on a vertical denaturing polyacrylamide gel (6%) containing 7 M urea. Gels were dried on Whatman paper before autoradiography.

2.2.4.2. **Isolation and reamplification of differently expressed transcription-derived fragments (TDFs)**

The film and gel were aligned and the fragments of interest were excised from the gel with a sterile razor blade and immersed overnight in 100 µl distilled water. Of this solution 5µl was used in a standard PCR with the same EcoRI and MseI primers as used in the preamplification step described above. After separation of the amplified products in a 1.2% agarose gel, the bands were excised and purified as recommended with the *QiaEx II gel extraction Kit* (Quiagen, Venlo, Pays-Bas).

2.2.4.3. **Cloning and sequencing of reamplified differently expressed TDFs**

The DNA fragments were ligated to the pJET1.2 vector according to the instructions of the *Clonejet PCR Cloning Kit* (Fermentas, Vilnius, Lithuania) and cloned into *Escherichia coli* (Ultracomp™ INVαF', Invitrogen, Carlsbad, CA, USA). After transformation, bacterial cells were spread onto NZY medium (2.1% NZY, 1.5% agar; pH 7.0) containing 100 µg/ml ampicillin. After incubation for at least 30 h at 37°C, 5 colonies per fragment were chosen and cultured overnight at 37°C in liquid NZY medium containing ampicillin. Plasmids containing

the PCR product were extracted with the *GeneJet Plasmid miniprep Kit* (Fermentas, Vilnius, Lithuania), according to the instructions. The presence of inserts in plasmids was checked by standard PCR amplification with *pJET Forward* and *Reverse* primers (Fermenta,Vilnius, Lithuania).

2.2.4.4. **Sequence analysis**

Three colonies were selected for sequence analysis for each TDF. Sequencing was carried out with an automated sequencer (AbiPrism3730XL, Applied Biosystems, Foster City, CA, USA) by Macrogen Inc. (Seoul, South Corea) with the *pJET Forward* primer of the Clonjet^{TM} PCR Cloning Kit (Fermentas, Vilnius, Lithuania). Sequence alignments were performed and the sequence of each fragment corrected by means of the *Bioedit sequence alignment editor* software developed by the Microbiology Department of North Carolina University (USA). Sequence homology was determined with the Basic Local Alignment and Search Tool (BlastX program) from the National Center for Biotechnology Information (NCBI). The identified putative protein sequences were used in queries against the UniProt database. Homologies with *E-value* scores below 10^{-8} were considered significant.

2.2.5. **cDNA-AFLP fragment validation by real-time RT-PCR**

2.2.5.1. **Experimental design**

The results obtained by cDNA-AFLP were confirmed for selected TDFs by real-time RT-PCR (with two independent biological replicates, table 1). In these confirmation tests, expression-level differences between identically processed S^- and S^+ samples were determined for both collection times (1 hbi and 13 dpi) and expressed as described in the legend of table 5. Hence, samples A and E yielded biological replicate, as did B and F, C and G, D and H. Template-free controls (water instead of cDNA) were included. Three technical replicates were done for each biological replicate.

2.2.5.2. **Primer design**

For each TDF, the forward and reverse primers were designed on the basis of the isolated sequence, with the online *Primer3* software under default settings. Newly designed primers were ordered from and synthesized at Eurogentec (Seraing, Belgium).

2.2.5.3. **Real time RT-PCR**

Real-time RT-PCR was carried out with StepOne^{+} Real-Time PCR systems (Applied Biosystems, Foster City, CA, USA). Each PCR amplification was performed in 20 µl reaction mixture consisting of 10 µl Maxima Sybr Green qPCR Master Mix 2X (Fermentas), 2 µl each of the forward and reverse primers (10 µM), 1 µl cDNA template (1ng/µl), and 5µl PCR-grade water. The cycling conditions were: pre-incubation for 10 min at 95°C, followed by 40 cycles, each consisting of 30s denaturing at 95°C, 40s annealing at 52°C, and 45s elongation at 72°C, the last cycle ending with a final 10-min extension at 72°C. Melting curve analysis (60 to 95°C) was performed to validate the gene specificity of the primers. Only genes with clear melting curves were taken for further data analysis. Samples showing irregular melting peaks were excluded from the quantification procedure. Differences in expression were calculated according to the "Delta-delta method" (Pfaffl, 2001), elongation factor 1-alpha (Ef1a) being used as an internal control for normalization (Czechowski *et al.*, 2005; Eungwanichayapant and Popluechai, 2009). RT-PCR analyses and subsequent calculations were performed with the StepOne^{TM} software (Applied Biosystems, Foster City, CA, USA).

2.2.5.4. **Functional annotation of differently expressed genes**

Genes identified by cDNA-AFLP analysis and confirmed in both real time RT-PCR biological replicates to be differently expressed to a significant degree in S^{+} and S^{-} banana crown tissues were grouped into gene ontology (GO) categories according to biological process and molecular function. To obtain the GO annotations of our proteins, we downloaded those of *Arabidopsis* proteins from the TAIR website as reference annotations. The *Arabidopsis* protein GO annotations were extended to the proteins found in this study.

2.3. Results

2.3.1. **Banana samples**

As expected, by modification of the source-sink ratio at flowering we were able to obtain banana plants with different susceptibility levels. As shown in figure 3, crowns obtained from both 12-leaf/2-hand banana plants (average INS values: 123 mm^2 and 168 mm^2) were less susceptible to crown rot disease than the crowns obtained from both 12-leaf/8-hand banana plants (INS values: 369mm^2 and 395mm^2). RNA was successfully extracted from all 8 freeze-dried crown samples (results not shown).

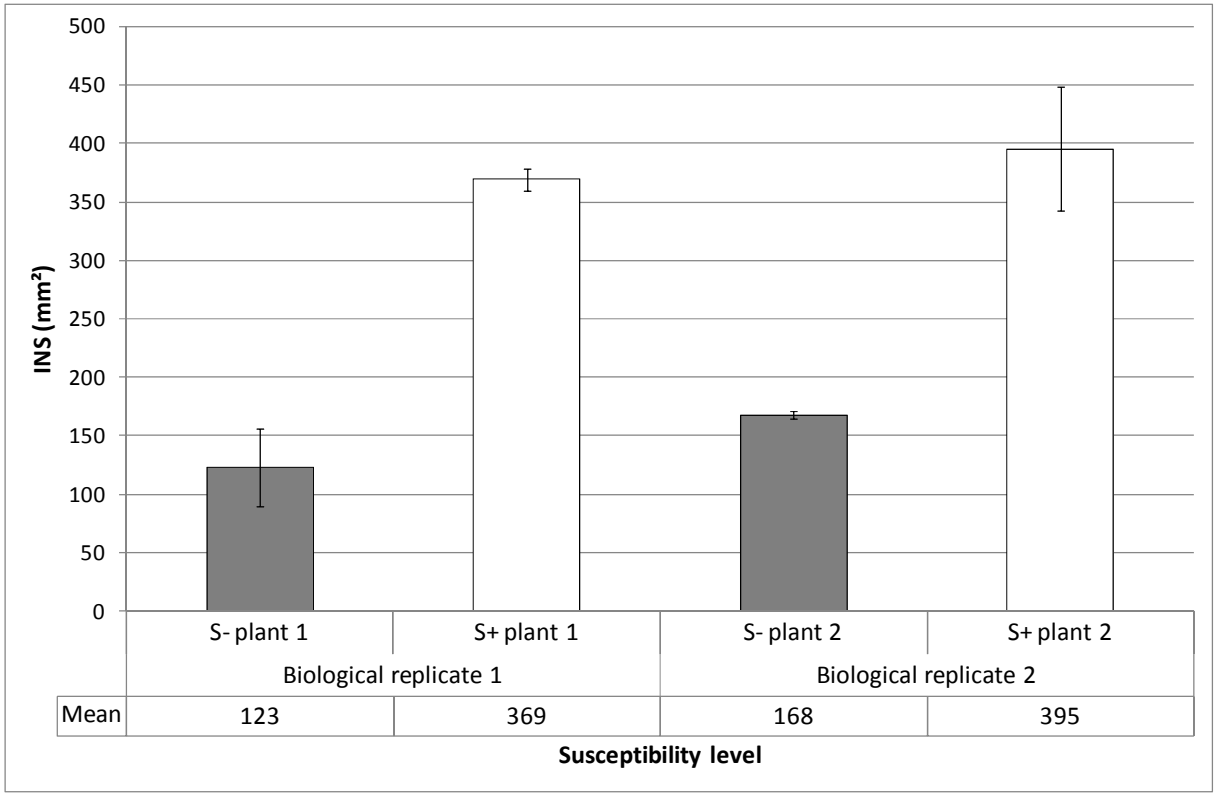


Figure 3: Susceptibility of the banana crowns used for molecular analyses, as determined by measuring the internal necrotic surface (INS, expressed in mm^2). The INS average and standard deviation of the 3 clusters of the same second hand are given for the 2 independent biological replicates and for the 2 different susceptibility level banana plants.

2.3.2. **Isolation of differently expressed genes**

The cDNA pools from S^- plant 1 (less susceptible to crown rot) and S^+ plant 1 (more susceptible) at both collection times were subjected to cDNA-AFLP screening. A total of 8 combinations of EcoRI+2/MseI+2 primers were used and more than 3100 bands were amplified from the cDNA pools. The cDNA-AFLP technique revealed (table 2, a and b) a total of 157 amplicons differentially expressed between the high- (S+) and low- (S-) susceptibility samples collected before inoculation (1 hbi) (i.e. about 10% of the total amplicons on the corresponding profile); and 286 amplicons differentially expressed between the high- and low-susceptibility samples collected 13 days after inoculation (13 dpi) (about 14% of the total amplicons). Only bands showing a clear intensity difference between S^+ and S^- upon visual inspection, without other bands nearby and with a size superior to 100 bp, were excised from the polyacrylamide gels (figure 4), cloned and sequenced.

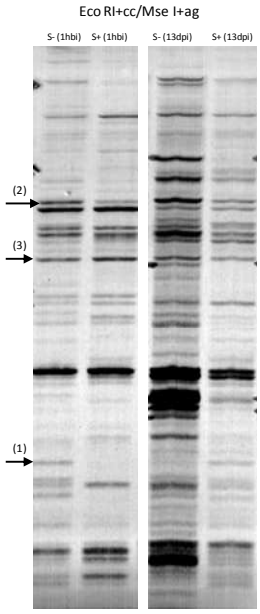


Figure 4: Autoradiography showing a typical result for one selective Eco RI/MseI primer combination for comparing high-susceptibility (S^+) banana tissues with low-susceptibility banana tissues (S^-) at two times: 1 hour before inoculation (1 hbi) and 13 days post-inoculation (13 dpi). Arrows indicate examples of: (1) a transcripts of an induced gene; (2) transcripts of an upregulated gene; and (3) transcripts of an unaffected gene. Differentially expressed transcript-derived fragments (TDFs) were excised from the gel, reamplified, cloned and sequenced.

A total of 32 and 99 bands (TDF) were selected respectively from samples collected before inoculation (1hbi) and 13 days after inoculation (13 dpi). Among them, 16 (for 1 hbi) and 62 (for 13 hpi) were successfully recovered from the gels, reamplified, cloned, and sequenced (table 2, a and b). These 78 distinct nucleotide sequences were then subjected to a BlastX homology search. This analysis revealed non-redundant significant homology with plant gene sequences in the public database for 15 of the 16 selected TDFs from the " 1 hbi " profile (table 3a); and 31 of the 62 TDFs selected from the "13 dpi" profile (table 3b). The remaining 31 TDFs from "13 dpi" profile showed no significant homology to any known plant sequence.

Table 2a: Overall results of cDNA-AFLP analysis (with 8 primer combinations) and real-time RT-PCR confirmation for material harvested 1 hour before inoculation (1hbi).

Expression profile (1hbi)	Number
cDNA fragments displayed (100-450bp) in S^- and S^+	822 and 683
Differently expressed fragments	157
Upregulated in S^-	62
Downregulated in S^-	95
TDFs selected and excised	32
TDFs successfully recovered from the gels, reamplified, cloned and sequenced	16
Non redundant significant similarities with the database after analysis	15
Selected TDFs for real time RT-PCR confirmation	7
cDNA-AFLP result confirmation by real time RT-PCR/ Biological replicate 1	7
cDNA-AFLP result confirmation by real time RT-PCR/ Biological replicate 2	6

Table 2b: Overall results of cDNA-AFLP analysis (with 8 primer combinations) and real-time RT-PCR confirmation for material harvested 13 days post-inoculation (13dpi).

Expression profile (13dpi)	Number
cDNA fragments displayed (100-450bp) in S^- and S^+	811 and 845
Differently expressed fragments	286
Upregulated in S^-	166
Downregulated in S^-	120
TDFs selected and excised	99
TDFs successfully recovered from the gels, reamplified, cloned and sequenced	62
Non redundant significant similarities with the database after analysis	31
Selected TDFs for real time RT-PCR confirmation	21
cDNA-AFLP result confirmation by real time RT-PCR/ Biological replicate 1	17
cDNA-AFLP result confirmation by real time RT-PCR/ Biological replicate 2	5

Table 3a: BlastX identities of non-redundant clones identified by comparing the cDNA-AFLP profiles of highly susceptible and less susceptible banana crown tissues collected 1 hour before inoculation. The table also shows the number of cases in which differential expression was confirmed by real-time RT-PCR (two independent biological replicates). BR1: biological replicate 1; BR2: biological replicate 2.

cDNA-AFLP Fragment	Size (bp)	cDNA-AFLP regulation S^-	Homology	Annotation	Organism origin	E-value (%similarity)	Real Time RT-PCR confirmation	
							BR1	BR2
48b.1	447	+	ABF71990	Putative protein kinase	*Musa acuminata*	8e-29 (82%)	yes	yes
44b.2	620	-	ABF70116	dual specificity protein phosphatase family protein	*Musa balbisiana*	2e-18 (57%)	yes	yes
31.1	411	-	NP_195309	RING-type ubiquitin ligase	*Arabidopsis thaliana*	8e-21 (79%)	yes	yes
33.2	348	+	EEF29330	dopamine beta-monooxygenase	*Ricinus communis*	5e-34 (74%)	yes	yes
44.1	607	-	NP_001053150	Hypothetical protein	*Oriza sativa*	5e-65 (87%)	yes	yes
47.1	490	-	NP_565766	Glycolip transfer protein	*Arabidopsis thaliana*	7e-39 (87%)	yes	yes
48.1	454	-	CAN81047	Hypothetical protein	*Vitis vinifera*	6e-65 (91%)	yes	no
73.1	245	-	CAO60899	Hypothetical protein	*Vitis vinifera*	3e-06 (61%)	Not done	
73.2	248	-	CAN81194	Hypothetical protein	*Vitis vinifera*	7e-33 (98%)	Not done	
32.1	402	+	NP_187791	Unknown protein	*Arabidopsis thaliana*	1e-20 (68%)	Not done	
36.1	193	-	NP_001060105	Hypothetical protein	*Oriza sativa*	6e-19 (85%)	Not done	
37.1	186	-	EEC73391	Hypothetical protein	*Oriza sativa*	2e-16 (80%)	Not done	
51b.1	366	-	NP_001055242	Hypothetical protein	*Oriza sativa*	9e-15 (56%)	Not done	
70.2	426	-	CAN66568.1|	Hypothetical protein	*Vitis vinifera*	3e-14 (65%)	Not done	
5.2	282	+	EEE54897	Hypothetical protein	*Oriza sativa*	7e-07 (54%)	Not done	

Table 3b: BlastX identities of non-redundant clones identified by comparing the cDNA-AFLP profiles of highly susceptible and less susceptible banana crown tissues collected 13 days post-inoculation. The table also shows the number of cases in which differential expression was confirmed by real-time RT-PCR (two independent biological replicates). BR1: biological replicate 1; BR2: biological replicate 2.

cDNA-AFLP Fragment	Size (bp)	cDNA-AFLP regulation S^-	Homology	Annotation	Organism origin	E-value (%similarity)	Real Time RT-PCR confirmation	
							BR1	BR2
294.2	298	+	NP_001104954	Cellulose synthase	*Zea mays*	3e-43 (96%)	yes	yes
283.1	444	+	EEF32539	Putative glycolipid transfer protein	*Ricinus communis*	4e-20 (92%)	yes	yes
232.2	119	+	ACG29071	Serine carboxypeptidase II-3	*Zea mays*	1e-12 (89%)	yes	yes
317.1	425	-	EEF47930	Putative ubiquitin carboxyl-terminal hydrolase	*Ricinus communis*	3e-52 (78%)	yes	yes
190.2	618	+	ABG66307	CCR4 associated factor 1-related protein	*Capsicum annuum*	1e-58 (77%)	yes	yes
284.1	434	-	EEF44770	Putative lactoylglutathione lyase	*Ricinus communis*	5e-28 (84%)	yes	no
197.2	418	+	AAF23074	Heat shock protein 70	*Triticum aestivum*	6e-53 (99%)	yes	no
145.2	298	-	EEF32493	Putative ribose-5-phosphate isomerase	*Ricinus communis*	1e-10 (81%)	yes	no
220.2	390	+	EEF37576	Putative sulfate transporter	*Ricinus communis*	1e-27 (86%)	yes	no
220.3	395	+	BAE99290	β-N-acetylhexosaminidase -like protein	*Arabidopsis thaliana*	2e-59 (90%)	yes	no

cDNA-AFLP Fragment	Size (bp)	cDNA-AFLP regulation S$^-$	Homology	Annotation	Organism origin	E-value (%similarity)	Real Time RT-PCR confirmation	
							BR1	BR2
178.1	270	+	A2XDD6	Homeobox-leucine zipper protein HOX13	*Oryza sativa*	9e-12 (64%)	yes	no
233.2	129	+	ABC72694	Granule-bound starch synthase	*Cephalostachyum mannii*	3e-12 (97%)	yes	no
231.3	147	+	NP_195906	Pentatricopeptide (PPR) repeat-containing protein	*Arabidopsis thaliana*	3e-09 (82%)	yes	no
241.1	458	+	AAL59042	Putative tetratricopeptide repeat protein	*Oryza sativa*	5e-45 (86%)	yes	no
243.2	343	+	BAD30585	Putative 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase	*Oryza sativa*	3e-31 (83%)	yes	no
291.2	348	+	EEC70392	Hypothetical protein	*Oryza sativa*	6e-27 (73%)	yes	no
216.1	466	+	AAX07420	Actin 2	*Musa acuminata*	1e-74 (98%)	yes	no
198.1	291	+	ACG32616	expp1 protein precursor	*Zea mays*	1e-25 (84%)	no	
170.2	405	+	ABF98518	Putative protein kinase	*Oryza sativa*	3e-59 (89%)	no	
182.2	169	-	AAO43609	Caffeic acid O-methyltransferase	*Sorghum bicolor*	2e-09 (76%)	no	
288.1	357	+	Q9XF47	Cytosolic fructose 1,6 biphosphatase	*Musa acuminata*	4e-42 (97%)	no	

cDNA-AFLP Fragment	Size (bp)	cDNA-AFLP regulation S$^-$	Homology	Annotation	Organism origin	E-value (%similarity)	Real Time RT-PCR confirmation	
							BR1	BR2
285.1	417	+	Q0JDM0	Zinc finger CCCH domain-containing protein 27	*Oryza sativa*	9e-41 (81%)	Not done	
283.2	448	-	ACG28752	Serine/threonine-protein kinase NAK	*Zea mays*	1e-46 (81%)	Not done	
183.1	170	+	CAN60313	Putative serine/threonine protein kinase	*Vitis vinifera*	5e-13 (79%)	Not done	
182.1	167	-	EEF50185	Putative serine/threonine protein kinase	*Ricinus communis*	4e-14 (80%)	Not done	
302.1	205	-	CAO46913	Unnamed protein product	*Vitis vinifera*	2e-09 (89%)	Not done	
143.1	334	+	CAO45614	Unnamed protein product	*Vitis vinifera*	1e-31 (88%)	Not done	
318.2	419	+	CAO66159	Unnamed protein product	*Vitis vinifera*	3e-38 (82%)	Not done	
312.2	120	-	CAN71825	Hypothetical protein	*Vitis vinifera*	7e-10 (88%)	Not done	
290.1	361	-	EEE60281	Hypothetical protein	*Oryza sativa*	8e-32 (74%)	Not done	
171.1	373	+	CAN72176	Hypothetical protein	*Vitis vinifera*	5e-37 (82%)	Not done	

2.3.3. **Confirmation of differently expressed fragments by real-time RT-PCR**

All TDFs from tables 3a and 3b, for which specific primer pairs were successfully designed, were selected for confirmation of their differential expression by real time RT-PCR: Here, cDNA pools from all four plants were used, providing two independent biological replicates.

2.3.3.1. **TDFs isolated 1 hbi**

Primer pairs were successfully designed, amplifying the expected products for only 7 of the 15 TDFs (table 4).

Table 4: Nucleotide sequences of the specific primers used in real-time RT-PCR analyses. *collection time of the sample from which the fragment was isolated.

Target cDNA	Isolation*	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product size (Bp)
44.1	1hbi	GTGAATAAGCCCCGAGTTGA	GAATCCCTGCAGTTGGATGT	208
44B.2	1hbi	ATGGTGATGAGGAGGCTTTG	GCTGGTAAAAGGGGGAAAAC	192
48B.1	1hbi	TTTACTCGGGGAAGGAGGTT	TCTGTCAGCCATTCCTTGTG	150
31.1	1hbi	CAACAGGGGAGAAGAATTGC	CGCCTACACAGTGGACAAGA	151
47.1	1hbi	TGTTCACTCCCTCATTGGAAG	CCAGCCTCGTTATGTTACCC	168
48.1	1hbi	GCTATGATGCTTTCACTCTTG	GTTCTTCACGGGGATTCG	119
33.2	1hbi	GACGCAACAGGGGAGAAG	CGAAAGAGAGGAAAGCAAGC	218
317.1	13dpi	CCATCAGGAAGCATTCCATT	GTGCCCTCAACAACAGTTCC	203
170.2	13dpi	TGATGGATTCCAAGAAAAACG	AAGACTTGGAGCCAATGGTG	160
197.2	13dpi	AACCCTGATGAGGTTGTTGC	TGTTGTGTTCCTTGGGATGA	156
216.1	13dpi	GTTTGGATCTTGCTGGTCGT	GATCAAGGATGGCTGGAAGA	272
190.2	13dpi	TCCGCTACATCTGGGAGTTC	ACCAGTCCCGACGACATTAG	169
220.3	13dpi	AGGTACACGGTTGAGGATGC	GCTCACATCTAGGGGCTCAG	162
291.2	13dpi	TCGAGGAAGACTGCCAAGAT	GTCAGATGCCCGATCGTTAT	169
288.1	13dpi	TGGCAAATGCTGTCAAATGT	GCGATGGATCCACAAAAGTT	242
294.2	13dpi	GAATGAACCACATTCCAGCA	CTTTTGGAGAGGGTGGCATA	151
241.1	13dpi	CGAGGCTATTGCTGACATGA	ACCAATTCCCCATCTCCTTC	155
243.2	13dpi	AAAGCTTGGCTGCTTCACAT	CATTGCATCACAGACCTTCG	203
283.1	13dpi	ACCAATTCCCCGTATCCTTC	AACCTCCTTCCCCGAGTAAA	256
178.1	13dpi	GCTGCTCCTAGCCGAGATAA	CGCCGTCCTTGTAGATCAAT	125
284.1	13dpi	TGCTGAGGAAGAAGGCTCAT	CCTGAGTAACCCCAAGGACA	258
198.1	13dpi	TTGTGTGTCTCAGGGAGCAG	CAGCTTGGAAACCTCGTACC	155

231.3	13dpi	GCAGGCCAAATGAGAATACC	GCGTACCAATTCCACGATCT	120
232.2	13dpi	TAATGGAGGTCCCGGGTGT	CCAATTCCAAGCATAAGGATTC	115
182.2	13dpi	CTCACAGCGATGGAGGTAGG	GCGGCGAAGAGATAAGTGAC	100
145.2	13dpi	TGGTTGCTTTTGTGTTGGTT	CCTCAAATTTTCTCCGTCCA	113
220.2	13dpi	AGGTACACGGTTGAGGATGC	GCTCACATCTAGGGGCTCAG	162
233.2	13dpi	ATGCCCTGAAGCTGGATAAG	TCCTGAGTAATGCACCTTTGG	103

The real-time RT-PCR results for biological replicate 1 (material from S^+ plant 1 and S^- plant 1) confirmed the changes in expression of all 7 tested TDFs (table 3a). Only one TDF (48.1) coding for a hypothetical protein did not show the same regulation in the biological replicate 2 (table 3a). The 6 TDFs which had the same pattern of expression for the two biological replicates were found to encode proteins showing similarity to 1 putative protein kinase (48b.1), 1 protein of the dual specificity protein phosphatase family (44b.2), 1 protein of the RING-type ubiquitin ligase family (31.1), 1 dopamine-β-monooxygenase (33.2), 1 hypothetical protein (44.1), and 1 glycolipid transfer protein (47.1) (table 3a). The 6 corresponding primer combinations were also used in real-time RT-PCR experiments on the cDNA pools obtained from the samples collected 13 dpi from all four plants (table 5). The putative protein kinase and the dopamine-β-monooxygenase appeared upregulated in the S^- tissues collected 1 hbi, becoming highly upregulated in the tissues collected 13 dpi. The member of the dual specificity phosphatase family, the RING-type ubiquitin ligase family, and the glycolipid transfer protein appeared downregulated in the S^- tissues collected 1 hbi, becoming highly upregulated in the S^- tissues collected 13 dpi. The last protein, a hypothetical one, showed high downregulation in the tissues collected at both times (table 5).

Table 5: Genes differently expressed to a significant degree in S^+ and S^- banana crown tissues and results of real-time RT-PCR confirmations of differential transcription (two biological replicates). Genes identified by cDNA-AFLP were grouped into gene ontology categories according to biological process and molecular function. The leftmost column shows the time of collection (1 hbi: 1 hour before pathogen inoculation; 13 dpi: 13 days post-inoculation) of the tissues in which the genes were identified by cDNA-AFLP analysis. The rightmost column provides, on a semi-quantitative scale, an estimate of the extent of differential transcription of the selected genes, as measured in confirmatory real-time RT-PCR assays performed on material collected at both times. A "+" sign means upregulation in S^- as compared to S^+ crown tissue, and a "-"sign means downregulation in S^- as compared to S^+ crown tissue. The "regulation levels" defined for S^- tissue are ±1: less than 2-fold up- or downregulation; ±2: 2- to 5-fold up- or downregulation; ±3: 5- to 10-fold up- or downregulation; ±4: more than 10-fold up- or downregulation. When only one level appears, it is the mean of the results of the two biological replicates. When two levels appear, the two replicates showed different regulation trends and the values obtained for both replicates are given separately."

Isolation	TDF	Annotation	GO biological process		GO molecular function		Regulation level	
							1 hbi	13 dpi
1hbi	48b1	Putative protein kinase	GO: 0006468	Protein amino acid phosphorylation	GO: 0004672	Protein kinase activity	+2	+4
1hbi	44b2	Dual specificity phosphatase family protein	GO: 0006470	Protein amino acid dephosphorylation	GO: 0016791	Phosphatase activity	-4	+4
1hbi	31.1	RING-type ubiquitin ligase (C3H4 RING zinc finger family protein)	GO: 0006511	Ubiquitin-dependent protein catabolic process	GO: 0005515	Protein binding	-2	+3
13dpi	317.1	Putative ubiquitin carboxyl-terminal hydrolase	GO: 0006511	Ubiquitin-dependent protein catabolic process	GO: 0004221	Ubiquitin thiolesterase activity	-1; +1	+2
13dpi	232.2	Serine carboxypeptidase	GO: 0006508	Proteolysis	GO: 0004185	Serine-type carboxypeptidase activity	+2	+3
1hbi	47.1	Glycolipid transfer protein	GO: 0046836	Glycolipid transport	GO: 0017089	Glycolipid transporter activity	-1	+3
13dpi	283.1	Glycolipid transfer protein	GO: 0046836	Glycolipid transport	GO: 0017089	Glycolipid transporter activity	-1	+3
1hbi	33.2	Dopamine beta-monooxygenase	GO: 0042420	Dopamine catabolic process	GO: 0004500	Dopamine monooxygenase activity	+1	+4
13dpi	294.2	Cellulose synthase	GO: 0030244	Cellulose biosynthetic process	GO: 0016760	Cellulose synthase activity	-2	-2

Isolation	TDF	Annotation	GO biological process		GO molecular function		Regulation level 1 hbi	Regulation level 13 dpi
13dpi	190.2	CCR4 associated factor 1-related protein	GO: 0009451	RNA modification	GO: 0004540	Ribonuclease activity	-1; +1	+2
1hbi	44.1	Hypothetical protein					-4	-4

2.3.3.2. **TDFs isolated 13 dpi**

Twenty-one primer pairs were successfully designed and they amplified the expected products (table 4). Among the corresponding 21 TDFs, only 5 had the same pattern of expression in both real-time RT-PCR biological replicates (table 3b). They were found to encode proteins showing similarity to 1 cellulose synthase (294.2), 1 putative glycolipid transfer protein (283.1), 1 serine carboxypeptidase (232.2), 1 putative ubiquitin carboxyl-terminal hydrolase (317.1), and 1 protein related to the CCR4-associated factor 1 (190.2) (table 3b). The 5 corresponding primer combinations were also used in real-time RT-PCR experiments performed on the cDNA pools derived from samples collected 1 hbi from all four plants (table 5). Serine caroboxypeptidase appeared upregulated in S^- tissues collected 1 hbi and slightly more so in tissues collected 13 dpi. Cellulose synthase showed similar downregulation in S^- tissues collected 1 hbi and 13 dpi. The putative glycolipid transfer protein appeared downregulated in S^- tissues collected 1 hbi, becoming highly upregulated 13 dpi. For both the putative ubiquitin carboxyterminal hydrolase and the protein related to CCR4-associated factor 1, the biological duplicates showed similar results for 13 dpi (upregulation in S^- tissues) but different results for 1 hbi: slight downregulation in tissues from S^- plant 1 but slight upregulation in tissues from S^- plant 2.

2.4. Discussion

Transcriptome analysis is a common way of discovering differences in gene expression between two samples because regulation of gene activity occurs primarily on transcription level. cDNA-AFLP presents the advantage to be an open-end methods requiring only standart instrumentation and incurring low costs. But its drawback is a high fraction of cDNA molecules escaping detection. Indeed, although in theory the cDNA-AFLP technique allows analysis of the whole transcriptome, in practice the estimated transcriptome coverage ranges from less than 20% to 73% (Venkatesh *et al.*, 2005). Means reasons are because (i) of the lack of suitable restriction sites (Breyne *et al.*, 2003) ; (ii) gene discovery is based on subjective band selection ; and (iii) because this is a laborious and time-consuming technique requiring some selection at various steps. Furthermore, in the present study, we have retained only fragments showing homology with sequences in a database, amounting to only 60% of our fragments. Similar or lower percentages are reported in the literature for cDNA-AFLP (Botton *et al.*, 2008; Chen *et al.*, 2003; Dong *et al.*, 2004; Dilger *et al.*, 2003; Guo *et al.*, 2006; Trindade *et al.*, 2004). Results obtained in this study provide a supplementary proof of the escaping detection. Indeed, different genes were put in evidence between 1hbi and 13dpi

detection while the real-time RT-PCR shows that these genes were differently expressed in both stages. It is thus essential to bear in mind that some interesting genes involved in banana response to crown rot disease might not have been identified in our study and may remain to be discovered.

This study also highlights the importance of confirming results with an independent technique. In the runs performed on material derived from S^+ plant 1 and S^- plant 1 (the same material as used for cDNA-AFLP analysis), the real-time RT-PCR confirmed the initially differential expression for 86% of the cDNA-AFLP fragments. Non-confirmation is likely due to the fact that a band in a gel can contain a mixture of several fragments (Chen *et al.*, 2006; Hsu *et al.*, 2008; Zhang, 2003). This introduces artefactual cloning (Bachem *et al.*, 1998) and can also possibly lead to misinterpretation (overestimation) of cDNA-AFLP fragment expression levels in the gel. In the literature, investigators report confirmation rates of 30% (Campalans *et al.*, 2001; Fukuda *et al.*, 1999) to 100% (Ditt *et al.*, 2001) for genes identified by cDNA-AFLP analysis and tested with an independent technique. Moreover, our results also demonstrate the necessity of confirming results with biological replicates before concluding that a gene is involved in a molecular process, since the initial differential expression was confirmed in both biological replicates for only 46% of our selected genes.

Despite these limitations, the cDNA-AFLP technique has provided useful information allowing a step towards understanding the mechanisms involved in the variations of banana susceptibility to crown rot disease and this is the first work carried on this pathosystem. Because of the compatibility of the interaction, we expected significant transcriptional changes to occur among genes associated with cellular changes involved in general defense responses. It was no surprise to find genes involved in signal transduction or proteolytic pathways among those potentially involved in banana defense responses. It is well known that these pathways are complex but required to regulate defense responses, even though the exact regulatory mechanisms are not yet known.

The sequences encoded by fragments 48b.1 and 44b.2 respectively show homology with a putative protein kinase and a protein of the dual specificity phosphatase (DSP) family. Proteins of both types catalyze reversible protein phosphorylations involved in signaling pathways. It is known that synthesis of such proteins is triggered by an array of stimuli and that they target a broad range of downstream effectors, thereby regulating various processes such as defense responses (Agrawal *et al.*, 2002; Agrawal *et al.*, 2003; Lecourieux-Quaked *et al.*, 2000; Nurnberger *et al.*, 2004; Rakwal *et al.*, 2001).

The proteins corresponding to fragments 31.1 (a RING-type ubiquitin ligase) and 317.1 (a putative ubiquitin carboxyl-terminal hydrolase) belong the proteolytic machinery that plays key roles in the regulation of biological processes in plants (Edelmann and Kessler, 2008), including ones mediating responses to pathogens (Devoto *et al.*, 2003; Ellis *et al.*, 2002; van der Hoorn and Jones, 2004; Zeng *et al.*, 2006). More specifically, proteins of this type are involved in the 26S proteasome pathway, considered to be the main proteolytic pathway in eukaryotes. Over the past years, approximately a dozen ubiquitinylation-related components have been identified as being involved in plant-pathogen interactions (Zeng *et al.*, 2006), but in most cases, the exact nature of the regulation is not known. Most proteins of this type are induced in response to pathogen or elicitor challenges (Boyes *et al.*, 1998; Ramonell *et al.*, 2005; Salinas-Mondragón *et al.*, 1999; Serrano and Guzman, 2004; Takizawa *et al.*, 2005; Zeng *et al.*, 2006).

In addition to the 26S proteasome pathway, there are other proteases that may be involved in plant defense (Avrova *et al.*, 1999; Kruger *et al.*, 2002; Pautot *et al.*, 1993; Tornero *et al.*, 1996; Xia *et al.*, 2004). Serine carboxypeptidase (232.2) is a protease with a well-established function in protein turnover for the mobilization of N-resources, notably during seed germination (Dal Degan *et al.*, 1994; Granat et al, 2003) and wound stress (Moura *et al.*, 2001). Our results suggest that serine carboxypeptidase could be also implied in plant-pathogen response. This protein appears to be upregulated in S^- crown both pre- and post-infection, but to a lesser extent 1hbi.

The proteins corresponding to fragments 47.1 and 283.1 show high similarity to glycolipid transfer protein type 1 of *Arabidopsis thaliana (*AtGLTP1). They belong to the lipid-transfer protein 1 (LTP1) family, classified as pathogenesis-related family 14 (PR-14). Such proteins can be activated by elicitation and accumulate in plants, mounting a defensive response against pathogens, including fungi (Van Loon *et al.*, 2006; Buhot *et al.*, 2004). Yet the precise role of LTPs in defense responses remains to be discovered. AtGLTP1 has been shown in vitro to enhance the intervesicular trafficking of glycosphingolipids (GSLs), but the precise in vivo biological function is still unknown (Brown and Mattjus, 2007). Both banana genes showed the same regulation profile: slightly lower expression in S^- than in S^+ crown tissue before pathogen inoculation and much higher expression in the former than in the latter 13 days post-inoculation.

On the other hand, cDNA-AFLP providing clues that may contribute to understanding the mechanisms involved in banana responses to crown rot disease. One is CCR4-associated

factor 1 (CAF1). CAF1 proteins are required for regulated deadenylation of a broad spectrum of stress-responsive mRNAs (Liang *et al.*, 2009), but the biochemical and physiological functions of CAF1 proteins are not clearly established (Liang *et al.*, 2009). They have been implicated in the regulation of plant growth (Sarowar *et al.*, 2007) and defense responses to biotic (Lee *et al.*, 2004; Liang *et al.*, 2009; Sarowar *et al.*, 2007) and abiotic stress (Liang *et al.*, 2009; Walley *et al.*, 2007). In our study, as in all known cases, CAF1 overexpression appears to result in enhanced resistance. No constitutive differential expression was noted prior to inoculation. Investigators have linked up-regulation of multiple pathogenesis-related genes to over-expression of CAF1 genes (Liang *et al.*, 2009; Sarowar *et al.*, 2007). It will be interesting to study in our model the expression levels of some pathogenesis-related proteins in order to corroborate previous results and to attempt to better understand the role of CAF1 in resistance mechanisms.

Expression of the gene encoding a protein similar to cellulose synthase (294.2) is down-regulated in S^- banana crowns. This is somewhat surprising, as cellulose is a cell-wall component expected to play a role in forming a physical barrier against fungal penetration. Nevertheless there is evidence that when cellulose synthesis is reduced by mutation, lignin synthesis (Cano-Delgado *et al.*, 2003) and defense responses are activated through various signaling pathways (Cano-Delgado *et al.*, 2003; Ellis *et al.*, 2002). Here we provide no information on lignin synthesis, but we do show a correlation between reduced expression of the cellulose synthase gene and enhanced resistance. This downregulation seems constitituve, as S^- crowns showed the same level of downregulation both pre- and post-infection. Perhaps a plant more exposed to pathogen attack because of a low cellulose synthase level produces compensating defense mechanisms that are more efficient.

Finally, cDNA-AFLP analysis has enabled us to identify a gene showing homology with a dopamine-β-monooxygenase (DoH) combined with a cytochrome B561 (CB) (33.2). DoH-CB proteins form a recently identified group of proteins, likely to play a key role in catecholamine biosynthesis pathway in plants (Tsubaki *et al.*, 2005; Verelst and Asard, 2004). Cathecholamines are biogenic amines possessing a 3,4-dihydroxy-substituted phenyl ring. Among them are phenylethylamine, tyramine, dopamine, norepinephrine, and epinephrine (Kuklin and Conger, 1995). In banana fruits, dopamine is formed only through hydroxylation of tyramine (Smith, 1980). Dopamine hydroxylation by dopamine-β-monooxygenase leads to synthesis of norepinephrine, which is subsequently methylated to epinephrine. From a broad range of plants, bananas, and particularly Cavendish bananas, have the richest content in cathecolamines and particularly in dopamine : 100 µg/g FW in Cavendish banana peel as

compared with 7 µg/g FW for potato, the second richest plant of this list (Kulma and Szopa, 2007). This observation suggests that cathecolamines might play an important role in banana physiology.

Various functions, including plant pathogen resistance, have been proposed for catecholamines (Swiedrych *et al.*, 2004; reviewed in Kulma and Szopa, 2007). Effects are probably due to the antioxidant properties of catecholamines themselves and also to cell-wall deposition and to the antioxidant properties of derivatives (Kulma and Szopa, 2007). Whether catecholamines can stimulate some defense responses more directly remains to be seen (Kulma and Szopa, 2007). However, catecholamine, dopamine, or oxidation products are suggested to be involved in banana resistance mechanisms (Mace, 1963; Muirhead and Deverall, 1984; Valette *et al.*, 1998; Wuyts *et al.*, 2006; Wuyts *et al.*, 2007). Particularly, it has been shown that dopamine oxidation products have a brown-red color and show a strong antifungal activity against *C. musae* (Muirhead and Deverall, 1984). These authors suggested that four of the five antifungal products reported (Brown and Swinburne, 1980; Brown and Swinburne, 1981; Swinburne, 1978) subsequently to the infection of green bananas by hyaline appressoria of *Colletotrichum musae* were dopamine oxidation products (contrarily to melanized appressoria that do not germinate on green bananas, these infections from hyaline appressoria are rapidly blocked and a brown-red coloration of neighboring tissues is observed). It is noteworthy that the peripheral necrotic tissues observed here in the S- banana crowns showed a specific red-brown color (data not shown). Then, our results suggest that dopamine oxidation products might play an important role in susceptibility to crown rot disease.

The factors involved in overexpression of dopamine oxidation in S- plants might be complex. Effectively, catecholamine biosynthesis and content depend on various factors: plant development stage (Lyte, 1997; Swiedrych *et al.*, 2004b; Szopa *et al.*, 2001); growth conditions (Swiedrych *et al.*, 2004b); environmental factors (Kulma and Szopa, 2007) such as the pH (Homeyer and Roberts, 1984), brightness (Endress *et al.*, 1984; Protacio *et al.*, 1992), temperature (Swiedrych *et al.*, 2004); and stress conditions including (i) biotic factors such as pathogen infection (Facchini *et al.*, 1996), and (ii) abiotic stresses such as drought, ABA treatment, UV light, and wounding (Swiedrych *et al.*, 2004b; Swiedrych *et al.*, 2004; Szopa et al., 2001). In the case of cactus (Steelink *et al.*, 1967) and potato (Szopa et al., 2001) wounding is accompanied by a rise in the dopamine concentration, and wounded tissues appear to stimulate dopamine production in the surrounding healthy tissue.

Similarly, in the case of crown rot of bananas we can hypothetize that wounding at harvest could also influence cathecolamine biosynthetic pathway.

It would be interesting to learn more about the role of dopamine-β-monooxygenase and other catecholamines in the response of bananas to crown rot. We have shown that the dopamine-β-monooxygenase gene is overexpressed in bananas less susceptible to the disease. One should make sure, however, that this enzyme and some others are active and that their substrates are present in the crowns. By determining the levels of various catecholamines in crowns with different levels of susceptibility it might be possible to gain interesting information about their respective roles. Furthermore, by measuring the potential antifungal activities of catecholamines one could learn whether they are active by themselves in defense mechanisms or whether they are precursors of other active compounds.

Finally, it is necessary to evaluate the expression levels of all these genes in other situations of susceptibility level variation. To be sure that they are really involved in quantitative regulation of banana defense responses and not in other pathways that might be triggered by a change in the source-sink ratio quite independently of any plant defense response. Furthermore, because plant defense responses invariably are by nature multicomponent processes, it is not easy to determine which components are both necessary and sufficient to confer protection.

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CHAPTER V

Conclusions and Perspectives

We propose a new way to view the post-harvest development of banana crown rot disease. The idea is that that crown rot development depends notably on fruit physiology at harvest, which in turn depends on pre-harvest factors. Although it has been suggested previously that fruit physiology may affect disease incidence, this has never been investigated in detail. Two aspects of the fruit physiology were considered in this study. In the first part (i), the importance of the fruit physiological component at harvest in post-harvest crown rot disease was highlighted (a) and the influence of some environmental pre-harvest factors on this component was studied (b). The second part of the study (ii) focused on the genetic determinant implied in the observed variation of susceptibility.

(i) a. The conducted assays have shown that the fruit physiological component at harvest has a significant effect on post-harvest crown rot development. In two production areas (Cameroon and Guadeloupe), fruit susceptibility was found to fluctuate significantly over time. Under Guadeloupian growth conditions, bananas harvested over an 11-week period interval showed a trend towards increasing susceptibility by 4-fold. However, the pre-harvest factors affecting fruit physiology and determining the susceptibility remained unknown. In both cases we suggested that climate conditions during banana growth were probably the most important pre-harvest parameter influencing the post-harvest susceptibility to crown rot. Indeed, climate conditions can vary from week to week whereas other parameters can be assumed to be reasonably constant in our assays (although they were not actively monitored). It quickly became apparent, however, that the environment-plant relationship determining fruit susceptibility is very complex. It was notably difficult to correlate fruit susceptibility with any particular environmental parameter, as different parameters tended to vary simultaneously in the field (results not shown).

b. Because of the apparent complexity of plant-environment interactions in determining susceptibility, we chose to focus on two pre-harvest factors: the source-sink ratio at flowering and the hand position on the bunch. Both were found to influence significantly the susceptibility of banana to crown rot. Within a bunch, there appeared a gradient of susceptibility to crown rot ($r=-0.95$), the hands initiated first (the upper ones) being more susceptible than those initiated last (the lower ones). Furthermore, when the sink was decreased by removal of many hands, the fruit susceptibility decreased. These observations suggest that susceptibility is influenced by the stage of fruit development and by fruit filling characteristics. These two parameters are in close interaction with – and dependent on – the soil-climate

conditions and agro-technical factors of the production area. We propose that plant susceptibility is regulated according to the nutritional balance established during plant growth. This balance varies between plants and also between fruits of the same bunch. It should result from all physiological interactions between the whole plant and environmental factors, however slight. Macro-environmental stress factors such as cloudiness, drought, fertilizer imbalance, poor soil water-holding capacity, the presence of pathogens, etc. probably have a greater impact on this balance than micro-environmental factors. Yet because plants growing close to one another can show very different susceptibility, we hypothesize that the nutritional balance of each plant, and even of each fruit, is affected by soil and leaf micro-environmental factors and by plant competition. The nutritional balance might affect the plant-pathogen interaction in two ways: by influencing the ability of the plant to establish defence mechanisms (notably through changes in secondary metabolism) and by altering the bioavailability of nutrients necessary for pathogen development.

Prospects: The ubiquitous nature of the fungi encountered in the disease complex, their diversity, and their highly variable occurrence and pathogenicity complicate the study of the parasitic component of the fruit quality potential. To reduce the disease, a better understanding of the pre-harvest factors that predispose bananas to crown rot would be helpful, rather than focusing on the fungi involved. Physiological studies should be designed under standardized and controlled conditions throughout the banana growth phase. These conditions should allow to examine separatly the effect of each parameter of the complex environment-host-pathogen interactions. On the basis of such knowledge, it should be possible to reduce crown rot disease by controlling environmental factors, rather than through systematic chemical control with its known drawbacks.

Practical applications: As a first measure liable both to reduce fruit susceptibility and to increase fruit size, we recommend early hand removal on the field. This practice should be easy to apply on a large scale on industrial plantations. It is already applied in some areas to increase fruit size.

On the basis of our results, we further propose advices when evaluating fruit susceptibility to crown rot disease. It is important to standardize the hand sampling method. The hands to be used in an experiment should be collected from the same position on each bunch, with a preference for the third one. If more than one hand

per bunch is needed, it is essential to work with successive hands. Furthermore, samples should be collected over the shortest possible time period, to avoid the high week-to-week variability described in this study.

(ii) The second part of this work focused on genes whose expression-level variations may be linked to the fruit susceptibility variation observed. cDNA-AFLP analysis was used, so as to keep our approach as broad and as unbiased as possible. We obtained fundamental information about genes that may quantitatively influence banana defence mechanisms. To our knowledge, this work is the first to address both pre- and post-infection gene expression with the same host-pathogen combination and different susceptibility. Some of the genes identified as potentially influencing the defence-response participate in signalling pathways or are part of the plant's proteolytic machinery. These mechanisms are nonspecific regulatory processes known to respond to environmental modifications generally. Their identification thus sheds no light on the mechanisms involved in susceptibility variation. The products of other identified genes show homology with glycolipid transfer proteins, a CAF1 protein, and a cellulose synthase. These proteins have already been linked to responses to pathogens in other models, but their precise role remains to be discovered. Expression of the CAF1 gene seems to be induced by the pathogen, as no constitutive differential expression was noted prior to inoculation. Overexpression of the CAF1 gene is associated with enhanced resistance, and we found this overexpression to persist 13 days post-inoculation. Paradoxically, overexpression of the cellulose synthase gene is associated with high banana susceptibility both pre- and post-inoculation. cDNA-AFLP analysis, furthermore, highlighted a gene whose predicted product shows homology with a dopamine-β-monooxygenase (DoH) combined with a cytochrome B561. Such genes play a key role in catecholamine action in plants. This seems particularly interesting, because other investigators have previously suggested that catecholamines, dopamine, or their oxidation products play a key role in banana resistance mechanisms. More detailed research is necessary to determine whether catecholamines contribute to determining the strength of the banana defence-response to crown rot disease.

Our results confirm the reproducibility of the cDNA-AFLP method. It is a sensitive method allowing the discovery of new genes (40% of the identified fragments showed no similarity to any database gene) and of genes whose expression is low. Our work also shows the limitations of cDNA-AFLP analysis.

Although in theory this technique allows visualizing and analysing the whole transcriptome, in practice it allows only partial coverage, because it is laborious and time-consuming and requires selection at various steps. In our case, selection resulted from using only one restriction enzyme pair on the cDNA pool (genes lacking the corresponding restriction site could not be detected), from using only 8 primer combinations for the amplifications, and from selecting only some of the differently expressed fragments for further analyses. We retained only fragments that were successfully recovered from the gels, re-amplified, cloned, and sequenced, for which specific primers could be designed and which showed homology to database genes. It is worth stressing that only 60% of the selected fragments showed homology to database genes. All these selection steps were necessary and they are frequently applied (Costa Mondego *et al.*, 2003), but it is essential to bear in mind that interesting genes involved in plant stress responses may have escaped identification in our study and would thus remain to be discovered.

This study also illustrates the importance of validating cDNA-AFLP results by an independent method and on two biological replicates before concluding that a gene is involved in a biological process. The expression-level differences observed by cDNA-AFLP analysis were confirmed by an independent technique for 86% of the identified fragments, and only 46% of them were validated on the second biological replicate.

Prospects: It is necessary to confirm the involvement of the identified genes in banana defence responses to be sure that the observed expression differences are not merely a side effect of the change in the source-sink ratio and of the mechanical injury of the bunch. This could be done by studying the expression of these genes under other conditions of susceptibility variation. Furthermore, it would be interesting to evaluate the expression level of these genes between varieties showing different degrees of resistance to crown rot. This would make it possible to assess the potential utility of these genes as selection markers for varietal improvement. Then it would be interesting to measure the catecholamine level and associated enzyme activities in fruits showing different susceptibility levels so as to gain interesting information about their respective roles in defence responses. Furthermore, by testing catecholamines for antifungal activity, one could learn whether they are active by themselves in defence mechanisms or whether they are precursors of other active compounds.

The present results give fundamental informations about mechanisms which govern the fruit susceptibility to crown rot disease. However, no one could be use, in the current state of knowledge, to direct the crop management. Nevertheless, further characterization and functional analyses of the genes that are identified in this study should lead to a more comprehensive understanding of plant-pathogen interactions.

Another important contribution may come from the fragments (40% of the total) that showed no similarity to any database gene. Although we excluded them from the present study, their isolation constitutes a first step to a better understanding of the plant-pathogen interactions. However, further caracterization is needed and could allow to identify original and/or specific mechanisms that govern the banana-crown rot interactions.

Practical applications: To perform our gene expression study we had to solve two major problems. Firstly, we wanted to study banana gene expression at the harvest stage and 13 days post-harvest on samples collected in Cameroon, where the necessary materials for extracting RNA are not available. We demonstrated that by freeze-drying our samples it was possible to preserve RNA without affecting its quality, and hence without compromising the results of our expression studies. This technique offers several advantages and can probably be transposed to other plant models.

The second difficulty was to extract efficiently, from bananas containing high levels of polyphenols and polysaccharides, RNA of sufficient quality to allow differential gene expression analysis. The proposed methods in this work, from *in vivo* sample RNA conservation to differentially expressed gene analysis by passing throught the RNA extraction step, allow studying differently expressed genes in any banana fruit tissue. These methods should prove useful wathever the transcriptome analysis method applied.

The results of this original study constitute the first demonstration of the importance of the fruit physiological component at harvest in post-harvest crown rot development. This observation is probably transferable to other plant-pathogen systems. Indeed plant stress responses depend on the plant-environment interactions that determine the physiological state of the plant. This should encourage researchers to view disease development more broadly, as a process that depends not only of a parasitic component but also on the plant physiological

status. Characterizing the banana fruit physiological component is complex, but there is evidence that integrated control management must take into account the pre-harvest factors that influence fruit susceptibility. We hope our pioneering research will make possible more detailed studies leading to practical agronomic transfer and to developing a banana crop management system that is more sustainable in terms of human and environmental constraints and needs.