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Study of the physiological component involved in the development of crown rot in bananas and the role of phenolics in susceptibility variation mechanisms

Étude de la composante physiologique impliquée dans le développement des pourritures de couronne de bananes et rôle des composés phénoliques dans les mécanismes de variation de sensibilité

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Cécile Annie Ewané (2012): Étude de la composante physiologique impliquée dans le développement des pourritures de couronne de bananes et rôle des composés phénoliques dans les mécanismes de variation de sensibilité. **Université de Liège - Gembloux Agro-Bio Tech, Belgique.** 112 pages, 17 tableaux, 16 figures.

La pourriture de couronne est une maladie de conservation qui affecte de façon importante la qualité des bananes d'exportation au Cameroun comme dans la plupart des zones de production. Elle est due à un large complexe parasitaire peu spécifique et opportuniste. L'approche originale de cette étude a été d'abord de mieux comprendre les conditions entourant le développement de la maladie et les facteurs de variation de la sensibilité des fruits, puis de caractériser le contenu phénolique des couronnes de bananes à sensibilité différentielle. Cette thèse a pour objectif de montrer l'influence des facteurs abiotiques et biotiques sur la composante physiologique du fruit à la récolte et l'implication des composés phénoliques dans les mécanismes mis en jeu dans les variations de sensibilité des fruits à cette pathologie.

Au terme de cette étude, il apparait que les facteurs abiotiques et biotiques influencent la composante physiologique du fruit à la récolte, affectant ainsi le niveau de sensibilité des fruits et par conséquent le développement des pourritures de couronne. Au préalable, la fiabilité de la méthode d'estimation des surfaces nécrotiques (INS) a été améliorée. L'influence des facteurs abiotiques (zone de production et date de récolte) sur le niveau de sensibilité des fruits à la pourriture de couronne a été démontrée mais pas celle des variations saisonnières. Les bananes cultivées en zone d'altitude (Ekona, 500 m) sont moins sensibles que celles cultivées en basse altitude (Dia-Dia, Koumba, 80 m). A certaines dates de récolte pendant la saison de pluies, la sensibilité des fruits était élevée en basse altitude. L'influence des facteurs biotiques (parasites foliaires) sur le niveau de sensibilité des fruits aux pourritures de couronne a été démontrée dans deux zones géographiques. Au Cameroun, la maladie des raies noires influence significativement ($P< 0.001$) la sensibilité des fruits à la pourriture de couronnes. En Guadeloupe, la maladie de Sigatoka n'a aucune influence ($P> 0.05$) sur le developpement des pouritures de couronne. L'influence des variations de ratio source-puits (So-Si, facteur abiotique), sur la physiologie du fruit pourrait expliquer cette différence.

L'influence de la modification sévère des ratios source-puits sur la sensibilité des fruits à la pourriture de couronne a été démontrée. Les fruits à faible ratio source-puits étant les plus sensibles. Les bananes de modalités extrêmes (12feuilles/1main, 1feuille/8mains) et à sensibilité différentielle (S^-, S^+) à la pourriture de couronnes ont été utilisées pour la caractérisation biochimique de leur contenu phénolique par les méthodes chromatographiques (GC-MS, HPLC et LC-MS) à deux stades : le jour de récolte avant inoculation (dhbi) et 13 jours après inoculation (13dpi). La dopamine a été identifiée comme étant le métabolite secondaire (alkaloïde phénolique) majoritaire des couronnes de bananes. La norépinéphrine et la normétanéphrine ont été détectées en grande quantité dans les couronnes fraichement récoltés et non inoculées (dhbi), particulièrement dans les couronnes sensibles (S^+). Certains composes phénoliques ont été détectés en quantité plus importante dans les couronnes de bananes les moins sensibles (S^-) comparées aux fruits plus sensibles (S^+). Il s'agit des acides hydroxycinnamiques (acide férulique, acide coumarique et leurs dérivés), ainsi que d'autres composés non identifiés, accumulés le jour de récolte (dhbi) en quantité importante dans les couronnes les moins sensibles (S^-) par rapport aux bananes sensibles (S^+), mais cette quantité diminue 13dpi de manière plus importante chez les fruits sensibles (S^+). Ces résultats suggèrent une possible implication des composés phénoliques dans la défense biochimique des couronnes de banane. Toutefois, le rôle précis de chacun de ces composés phénoliques dans le mécanisme de variations de sensibilité à la pourriture de couronnes n'est pas encore clarifié. Cette étude est le point de départ de la compréhension des fonctions des composés phénoliques dans la défense des couronnes de bananes.

Ce travail constitue une première étude de l'influence des facteurs abiotiques et biotiques sur la composante physiologique des bananes à la récolte au travers de l'évaluation de leur incidence sur le développement des pourritures de couronnes. A notre connaissance, ce travail est le premier qui relie le niveau de sensibilité des fruits au contenu phénolique de leur couronne.

Summary

Cécile Annie Ewané (2012): Study of the physiological component involved in the development of crown rot in bananas and the role of phenolics in susceptibility variation mechanisms. **University of Liège - Gembloux Agro-Bio Tech, Belgium**. 112 pages, 17 tables, 16 figures.

Crown rot is a post-harvest disease caused by a broad unspecific and opportunistic parasitic complex, which affects the quality of export bananas in Cameroon, as well as in most of the production areas around the world. The originality of this research is that it is sets out, not only to investigate on the conditions surrounding the development of the disease and the variable factors of fruit's susceptibility, but equally to characterize the phenolic content of the banana crown with differential susceptibility levels. The study therefore aims at showing the influence of abiotic and biotic factors on the fruit's physiological component at harvest and the involvement of phenolic compounds in the mechanism staked in fruit's susceptibility variations to this pathology.

At the end of this study, it appears that abiotic and biotic factors influence the fruit's physiological component at harvest, affecting thus its susceptibility level and therefore favours the development of crown rot disease. The reliability of internal necrosis surface (INS) assessments method was improved. The influence of abiotic factors (production area and harvest date) on fruit susceptibility was demonstrated without season influence. Fruits grown in high altitudes (Ekona, 500 m) were less susceptible to crown rot than the ones grown in low altitudes (Dia-Dia, Koumba, 80 m). It was noticed that at certain harvest dates within the rainy season, fruit susceptibility was higher in plantations with low altitudes. Concerning biotic factors, *Mycosphaerella* leaf spot disease's influence was shown in two geographical areas. In Cameroon, black leaf streak disease significantly influenced banana's sensitivity to crown rot ($P< 0.001$). In Guadeloupe, Sigatoka disease had no effect ($P> 0.05$) on banana's susceptibility to the development of crown rot disease. The influence of the source-sink ratio variations, an abiotic factor, on fruit physiology could explain these differences.

The influence of severe source-sink ratio modification on fruit susceptibility to crown rot was shown. Fruits with low source-sink ratio were the most susceptible. Bananas of

extreme modalities (12leaves/1hand, 1leaf/8hands) and with differential susceptibility (S^-, S^+) to crown rot were used for the biochemical characterization of their phenolic content at two stages: the day of harvest before inoculation (dhbi) and 13 days post-inoculation (13dpi) by chromatographic methods (GC-MS, HPLC, LC-MS). Dopamine was identified as the major secondary metabolite (phenolic alkaloid) in banana crown. Norepinephrine and normetanephrine levels were high in the dhbi, especially in the S^+ crowns. Hydroxycinnamic acids (ferulic acid, coumaric acid and their derivatives) and other unidentified compounds were accumulated in highly significant quantities (P <0.001) in the dhbi in the less susceptible crown (S^-) as compared to the susceptible ones (S^+), with decreased 13dpi mostly in the susceptible fruits (S^+). These results suggest a possible role of these phenolics in banana crown biochemical defense. However, the main role of each phenolic detected in the susceptibility variations mechanism remains unclear. This study is the starting point to understanding the function(s) of phenolics in banana crown defense.

This is a pioneer study on the influence of abiotic and biotic factors on the banana fruit's physiological component at harvest through the assessment of their incidence on crown rot development. This work appears to be the first to link the level of fruit's susceptibility at two stages (dhbi and13dpi) with their crown phenolic content.

List of abbreviations

13dpi	13 days post-inoculation
AHC	Acid Hydrolysis Conditions
ANOVA	Analysis of Variance
BLSD	Black Leaf Streak Disease
BLSDS	BLSD Severity
$BLSDS_F$	BLSDS at Flowering
$BLSDS_H$	BLSDS at Harvest
CARBAP	Centre Africain de Recherches sur Bananiers et Plantains
CDC	Cameroun Development Corporation
CIRAD	Centre de coopération Internationale en Recherches Agronomique pour le Développement
d.f.	Degree of freedom
dah	Days after harvest
dd	Degree day
dhbi	Day of harvest before inoculation
DOPA	Dihydroxyphenylalanine
EbP	Extraction by-product
EBP	Extracts of the Bound Phenolics
ESI	Electrospray source
ESI-MS	Electrospray source coupled with mass spectrometry
ESP	Extracts of the Soluble Phenolics

FHP	Flowering-to-harvest-period
FW	Fresh Weight
g	Gram
GC-MS	Gas Chromatography coupled with Mass Spectrometry
GL	Greenlife
Gr	Grade of the median fruit
H	Hand
HPLC	High Performance Liquid Chromatography
HR	Hypersensitive Response
i.e.	id est
INS	Internal Necrotic Surface
L	Leaf
l	Liter
L_{350}	Fruit length at 350 dd
L_{900}	Fruit length at 900 dd
LAI	Leaf Area Index
LAR	Localized Acquire Resistance
LC-MS	Liquid Chromatography coupled with Mass Spectrometry
m/z	Mass-to-charge ratio
ME	Methyl Ester
mg	Milligramme
min	Minute
mL	Milliliter

MLSD	*Mycosphaerella* Leaf Spot Diseases
mm	Millimeter
mm^2	Square millimeter
ms	Millisecond
MW	Molecular Weight
NFL	Number of Functional Leaves
NFL_F	Number of Functional Leaves at Flowering
NFL_H	Number of Functional Leaves at Harvest
NH	Number of Hands
NL	Number of Leaves
NMR	Nuclear Magnetic Resonance
NS	Necrosed Surface
P	Probability
PAL	Phenylalanine ammonia-lyase
PCD	Programmed Cell Death
PHP	Plantation du Haut Penja
PIEC	Post-inoculation Environemental Conditions
PPO	Polyphenol oxidase
RP-HPLC	Reverse phase HPLC
R_T	Retention Time
S^-	Less Susceptible
s or sec	Second
S^+	Susceptible

SAR	Systemic Acquired Resistance
SC	Soluble Conditions
SD	Sigatoka Disease
SDS	SD severity
SDS_F	SDS at Flowering
SDS_H	SDS at Harvest
SI	Severity Index
So-Si	Source-Sink
SPM	Sociéte des Plantations du Manengouba
ULg-GxABT	Université de Liège-Gembloux Agro-Bio Tech.
UV	Ultra-violet
YLS	Youngest Leaf Spotted
YLS_F	Youngest Leaf Spotted at Flowering
YLS_H	Youngest Leaf Spotted at Harvest
λ_{max}	Wavelength of maximum absorbance

Introduction

Banana fruits, influenced by some pre-harvest factors, are susceptible to the development of post-harvest diseases mainly anthracnose and crown rot. Crown rot is the main quality defect that affects exported bananas in Cameroon. A broad unspecific and opportunistic fungal parasitic complex among which, *Colletotrichum musae* is the most pathogenic one causes this disease. Fruit contamination can occur within the field, but it mostly occurs during processing at the washing tanks of the packing station. Fungal infection will develop after few days of shipping on banana crowns harvested healthy. The quality of such banana fruits does not enable their sale in the market, and sometimes constitutes a blow on exports and loses for producers.

The use of synthetic fungicides to control post-harvest diseases is systematic. However, this approach is not satisfactory and it is increasingly denounced for the amounts of chemicals used that could be harmful to humans as well as to environment. Consequently, several approaches have been investigated in recent years to find alternatives to applications control.

The influence of environmental conditions on fruit's physiology during growth and the level of contamination determine crown rot development. It is important to understand the relationship between plant's physiology and susceptibility to pathogens; the effects of pathogens on plant growth and on fruits quality. This fruit quality includes the amount of phenolics (defensive compounds) accumulated in fruit tissues from flowering to harvest in order to face further pathogen infection. However, with the current state of knowledge, it is difficult to determine the occurrence of crown rot development and the biochemical mechanisms involved in the fluctuations of the fruit's susceptibility to crown rot.

The Plant Pathology Unit of ULg-GxABT has had for many years a research program, in collaboration with CIRAD and CARBAP, which focused on a better understanding of the conditions and the mechanisms that determine the post-harvest crown rot development. This thesis which constitute a part of this research program, aimed to study the abiotic and biotic pre-harvest factor's influence (i) on banana's susceptibility to crown rot, and (ii) on phenolics characterization and their potential involvement in the mechanisms of susceptibility variations.

This research provides new elements of response to the characterization of the banana fruit's physiological component.

List of publications

This thesis contains of the following papers:

1. **Cécile Annie Ewané, Luc de Lapeyre de Bellaire, Philippe Lepoivre, Ludivine Lassois.** Involvement of phenolic compounds in the susceptibility of bananas to crown rot. A review. *Biotechnol. Agron. Soc. Environ.* 2012 **16**(3), 393-404.

2. **Ewané C.A., Lassois L., Brostaux Y., Lepoivre P. and de Lapeyre de Bellaire L., 2012.** The susceptibility of bananas to crown rot disease is influenced by geographical and seasonal effects. *Canadian Journal of Plant Pathology*, DOI:10.1080/07060661.2012.733731.

3. **Ewané C.A., Chillet M., Castelan F., Brostaux Y., Essoh Ngando J., Lassois L., Hubert O., Chilin-Charles Y., Lepoivre P., de Lapeyre de Bellaire L.** Impact of *Mycosphaerella* leaf spot diseases on the banana susceptibility to post-harvest diseases. Submitted to *Fruits*.

4. **Ewané C.A., Nott K., de Lapeyre de Bellaire L., Ngoh Newilah G., Paquot M., Wathelet J.P., Lepoivre P.** Severe modifications in source-sink ratio influence the susceptibility of bananas to crown rot and its phenolic content. Manuscript to be submitted to *Plant Pathology*.

Chapter I. Literature review

I. Crown rot

Crown rot is a post-harvest disease that occurs on tissues joining the bananas finger peduncles. It is the main quality defect that affects exported bananas in Cameroon. In 1998, looses of about 50% have been reported in Port-Vendres (France) on bananas originating from Cameroon (Kameni & Slatter, 1998). A broad unspecific and opportunistic fungal parasitic complex causes this disease. The composition of this parasitic complex differs from one region to another. In Cameroon, the microorganisms commonly isolated from banana washing tanks are *Fusarium spp.* mainly *Fusarium monoliforme, Colletotrichum musae, Verticillium spp., Cephalosporium* spp., *Botrydiploidia* spp., *Gliocladium* spp., *Cladosporium* spp., *Trichoderma* spp., *Penicillium* spp. (Ewané *et al*., Unpublished results).

In banana agricultural practices, chemical control is the most commonly used method against crown rot disease development. The fungicides used are mainly thiabendazol, imazalil and bitertanol. However, the outcome of these chemical applications is unsatisfactory as expected. Shortcomings include the appearance of strains resistant to the active ingredient. The simultaneous use of fungicides having the same mode of action can lead to the development of crossed resistance. It is the case in some *Colletotrichum musae* strains where such resistances have been observed after using benomyl for the black leaf streak disease treatment and thiabendazol for the post-harvest treatment, both having an antimitotic mode of action (Griffee & Burden, 1976; Johanson & Blasquez, 1992; de Lapeyre de Bellaire & Dubois, 1997).

These chemical applications also lead to the presence of fungicides residues (fenarimol, benomyl) in banana peel and pulp (www.fao.org, www.inchem.org), but in an amount below the international maximum residues levels (Ciscato *et al*., 2012).

II. Secondary metabolites in plant defense

1. Introduction

Plants produce a wide range of chemical compounds that are involved in their defense against diseases. Chemical compounds present in plants are grouped into two classes according to the role they play and their omnipresence in plants: these are primary metabolites and secondary metabolites.

Primary metabolites are molecules, which exist in all plant cells and are necessary for plant's survival. Sugars, amino acids, fatty acids, nucleotides and chlorophylls are classified amongst the primary metabolites.

Secondary metabolites have a limited distribution in the plant itself and among the various plant species. These metabolites are important for the propagation and survival of plants, which produce them (Raven *et al.*, 2003). They are substances whose functions are not essential to plant (Judd *et al.*, 2001), but they do have several important functions in plant's life and have a great chemical diversity. This includes their structural role in various supporting and protection tissues; their implication in defense strategies and the particular property of signaling in interactions between plant and its environment.

The three major chemical families representative of secondary metabolites are terpenoids, alkaloids and phenolic compounds (Raven *et al.*, 2003). The limit between primary and secondary metabolites is very thin. Plants hormones such as gibberellins, cytokinins, abscisic acid and brassinosteroids are synthesized by terpenoids pathway but are not secondary metabolites because they are essential to development (they are primary metabolites).

The present study will focus on one group of alkaloids called catecholamines, on phenolic compounds and one particular group of secondary metabolites, which sometimes links the two chemical families called phenolamides.

2. Catecholamines

Catecholamines are a group of biogenic amines with a substituted phenyl 3,4-dihydroxy group and include dopamine, norepinephrine, epinephrine, and their derivatives, and they exist in plants. They are considered as a class of phenolic alkaloids or alkaloids.

Besides the phenolic compounds biosynthesis, phenylalanine oxidation **(Figure 1)** also leads to the formation of tyrosine, the precursor of catecholamines (dopamine and derivatives). Catecholamines are then formed from tyrosine via tyramine or dihydroxyphenylalanine (DOPA). Norepinephrine is the hydroxylated product of dopamine, itself formed by hydroxylation of tyramine. Catecholamines are metabolized in three ways: by methylation, oxidation and conjugation with other phenolic compounds (Szopa *et al*., 2001; Świedrych *et al*., 2004; Kulma & Szopa, 2007).

Catecholamines are also involved in plant's responses to biotic and abiotic stress. These compounds protect plants against pathogens and are involved in nitrogen detoxification (Kulma & Szopa, 2007). A slight increase in catecholamine has been observed in potato leaves after mechanical wounding (Szopa *et al*., 2001). This shows the catecholamines as biochemical stress markers in plants (Świedrych *et al*., 2004). In banana tissues, catecholamines mainly dopamine have been identified and suggested to probably play an important role in banana defense mechanism (Mulvena *et al*., 1969; Muirhead & Deverall, 1984; Valette *et al*., 1998; Kanazawa & Sakakibara, 2000; Wuyts *et al*., 2007; Lassois *et al*., 2011).

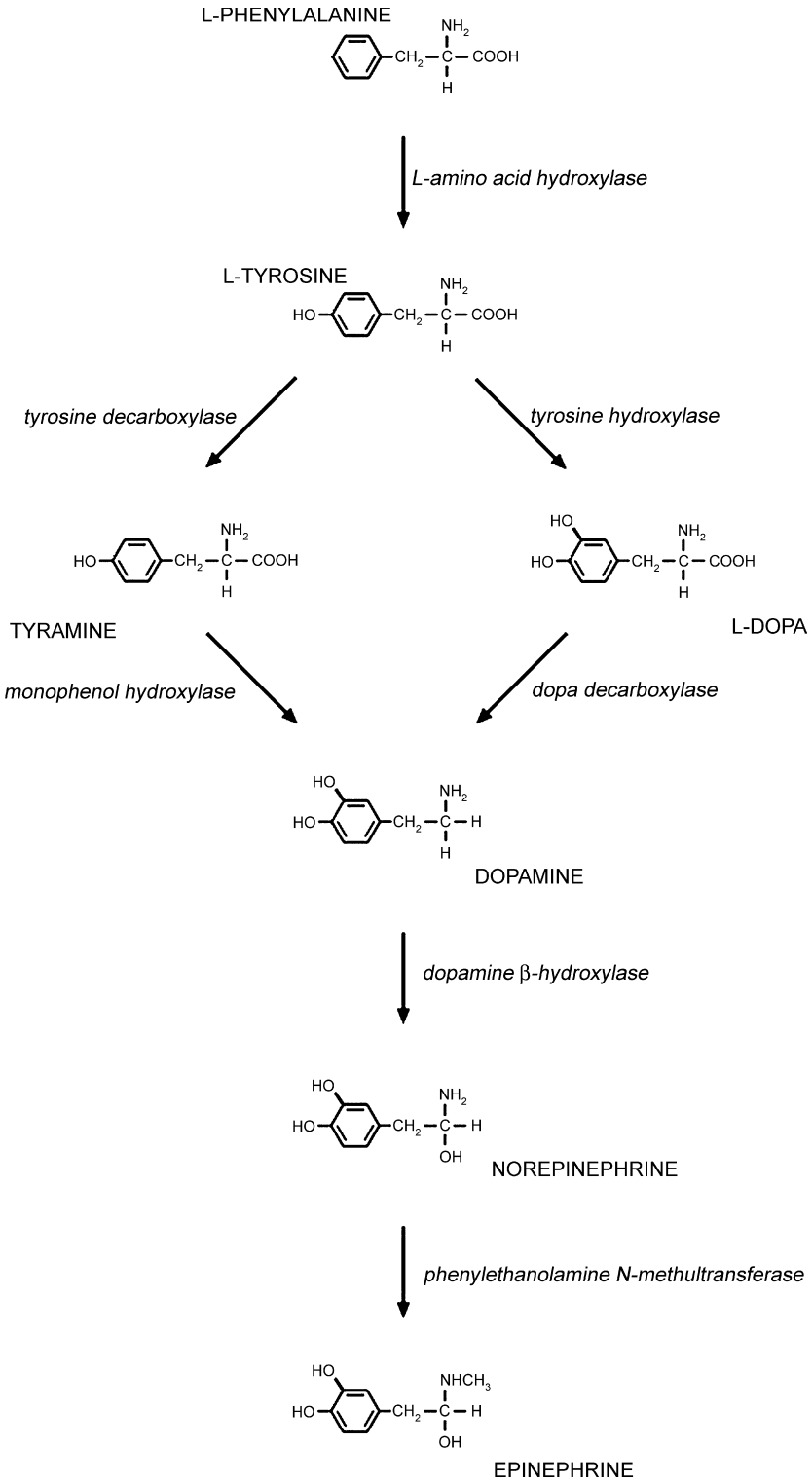

Figure 1: Plant catecholamine synthesis pathway (Kulma & Szopa, 2007).

3. Phenolic compounds

Phenolic compounds are the most studied secondary metabolites because of their close involvement in interactions between plants and their environment. Phenolic compounds, their glycosides, and their esters are widespread and abundant in plants where they intervene in both plant's physiology and defense. They constitute one of the means by which the plant adapts its physiology and its metabolism to defend itself against various biotic or environmental aggressions (Macheix *et al*., 2005). The involvement of phenolic compounds in plant resistance mechanisms varies according to the species, but generally their role is related to their antibiotic, antinutritional, or unpalatable properties. They can intervene in cell wall reinforcement and antimicrobial activity, in scavenging reactive oxygen species, or in modulating stress-related phytohormones taking part in signaling (Lattanzio *et al*., 2006). However, the function of many phenolic products remains unknown.

4. Phenolamides

Phenolamines are frequently referred to as hydroxycinnamic acid amides (HCAA) or phenylamides. They constitute a diverse and quantitatively major group of secondary metabolites resulting from the conjugation of a phenolic moiety with a polyamine or a deaminated aromatic amino acid. In a wide range of plants, hydroxycinnamic acids such as p-coumaric or ferulic acid can occur conjugated to the β-phenylethylamine-alkaloid tyramine, methoxytyramine, tyramine, dopamine or octopamine, forming the corresponding N-hydroxycinnamic acid amides (Facchini *et al*., 2002; Kulma & Szopa, 2007; Bassard *et al*., 2010). Synthesis of these amides is activated by fungal elicitors, attempted infection by fungi, viruses, bacteria, and in some cases by wounding (Kulma & Szopa, 2007). They seem to have specific functions in plant development and defense as metabolic intermediates and final products (Bassard *et al*., 2010).

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III. Involvement of phenolic compounds in the susceptibility of bananas to crown rot. A review

The level of susceptibility of banana fruit to crown rot disease is fluctuating. However, mechanisms behind these fluctuations are not well understood. Since phenolic compounds are involved in defense mechanism of banana plant against fungi and nematodes, they can also be involved in these variations of fruit susceptibility. In this paper, we present crown rot disease as well as the different factors that can influence the disease incidence and the level of fruit susceptibility. We then reviewed the phenolic compounds, their biosynthesis and the factors (biotic and abiotic) influencing their biosynthesis, with a particular focus on phenolic compounds involvement in plant defense mechanism, notably in banana tree defense.

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Focus on:

Involvement of phenolic compounds in the susceptibility of bananas to crown rot. A review

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Crown rot of bananas, caused by a fungal parasitic complex, is one of the main quality defects of exported bananas. Major variations in the susceptibility of bananas to crown rot have been observed in different production zones. The physiological state of the banana fruit at harvest is said to influence its response to pathogenic attack and thus to modulate its susceptibility to crown rot. The susceptibility of bananas to this disease, however, appears to be influenced by many pre-harvest factors, although the underlying defense mechanisms have not been clearly identified. A recent report based on molecular analyses suggests that phenolic compounds might be involved in the different variations in the susceptibility of bananas to crown rot. Results of other earlier studies point to an involvement of phenolic compounds in the defensive reactions of banana plants against various pathogens. The present paper reviews the current state of knowledge on the variations in the susceptibility of bananas to crown rot and takes stock of what is known about phenolic compounds in relation to their potential involvement in the defense mechanisms of the banana plant.
Keywords. Bananas, fruit susceptibility, crown, rots, defense mechanisms, phenolic compounds.

Implication des composés phénoliques dans la sensibilité des bananes à la pourriture de couronne (synthèse bibliographique). La pourriture de couronne des bananes provoquée par un complexe parasitaire fongique est l'un des principaux défauts de qualité des bananes d'exportation. Des variations de sensibilité importantes des bananes à la pourriture de couronne ont été observées dans différentes zones de production. Il a été suggéré que l'état physiologique du fruit à la récolte influence la réponse des bananes à l'attaque des micro-organismes pathogènes et module ainsi sa sensibilité à la pourriture de couronne. La sensibilité des bananes à la pourriture de couronne est influencée par plusieurs facteurs pré-récolte, bien que les mécanismes de défense sous-jacents n'aient pas été clairement identifiés. Récemment, une étude basée sur des analyses moléculaires a suggéré que les composés phénoliques pouvaient être potentiellement impliqués dans les variations de sensibilités des bananes aux pourritures de couronne. D'autres études antérieures avaient déjà suggéré l'implication de composés phénoliques dans les réactions de défense du bananier contre diverses attaques de pathogènes. Cette étude se propose de faire l'état de l'art sur les variations de sensibilité des bananes à la pourriture de couronne, et de faire le point sur les composés phénoliques et leur implication potentielle dans les mécanismes de défense du bananier.
Mots-clés. Banane, sensibilité du fruit, pourriture de couronne, mécanisme de défense, composés phénoliques.

1. INTRODUCTION

Banana is cultivated in more than 120 countries over about 10 million hectares. It ranks first in global fruit production with just over 106 million tons being produced annually worldwide. Cropping systems around the world are diverse and production targets include subsistence, sale in local or national markets, and international export. Some bananas, such as plantains, can be cooked, while others are classified

as "dessert" bananas. Dessert bananas for export belong exclusively to the Cavendish subgroup and are subjected to an important international trade (Loeillet, 2005). Banana is currently, in terms of volume, the first exported fruit and ranks second after citrus fruits in terms of value. Total world exports of banana in 2006 accounted for 16.8 million tons (FAOSTAT data, 2006). Thus the banana industry is of vital importance to banana-producing countries.

Banana trees and fruits are susceptible to attack by several diseases, including two main post-harvest diseases, namely banana anthracnose and crown rot. In Cameroon, the main quality defect that affects exported banana is crown rot. This disease is caused by a broad unspecific and opportunistic fungal parasitic complex. Fruit contamination can occur within the field but it mostly occurs in the washing tanks at the packing station where processing favors the penetration of pathogens into the crown tissues. The banana crowns, which were healthy at harvest, will then develop a fungal infection after a few days of shipping. Upon arrival, the quality of the banana fruits does not allow them to have a good position in the European market, and this sometimes constitutes a blow to exports. The use of synthetic fungicides to control banana tree diseases and post-harvest diseases is systematic. However, this approach is not satisfactory and is increasingly being criticized for the amounts of chemicals used, which could be harmful to humans as well as to the environment. Consequently, several approaches have been developed in recent years to find alternatives to chemical control.

Variations have been noticed in the development of crown rot disease partly related to environmental conditions during growth. The occurrence of crown rot therefore depends firstly, on the level of susceptibility of the banana fruit to being influenced physiologically by environmental conditions during growth and secondly, on the level of contamination. Occurrence of crown rot also depends on the amount of defensive compounds (phenolics) accumulated in fruit tissues from flowering to harvest to fight pathogenic infections. Indeed, plant defense strategies involve the combination of several structural and biochemical mechanisms, including accumulated phenolic compounds and lignin-like polymers. However, with the current state of knowledge, it is extremely difficult to determine the mechanism responsible for the variability in the susceptibility of the fruit to crown rot.

Up-to-date information will be presented below both on pre-harvest factors that influence the susceptibility of bananas to crown rot (especially biotic and abiotic factors) and on the involvement of phenolic compounds in plant defense mechanisms in relation to environmental conditions that support the plant's growth.

2. BANANA CROWN ROT

Exported bananas are subjected to various phytosanitary constraints throughout the production chain and these alter fruit marketability. Crown rot disease in particular causes major damage and economic losses in most banana-producing countries (Krauss et al., 2000). Losses of more than 86% have been reported for non-chemically-treated bananas exported from the Philippines (Alvindia et al., 2000).

2.1. Crown rot disease

Crown rot is a disease of the tissue uniting the peduncles. It occurs when banana hands are detached from their central axis, called the stalk. Banana hands are cut in clusters of 5-7 fruits, causing the exposure of wounded crown tissues to fungal infection (Muirhead et al., 2000). Fungal colonization of the crown starts afterwards, and the degeneration of the fruits does not require highly specialized parasites. Indeed, crown rot is a complex infection, which results from the activity of several microorganisms, of which *Colletotrichum musae* is the most pathogenic species (Finlay et al., 1993; Krauss et al., 2000; Muirhead et al., 2000; Lassois et al., 2010a). The infection is, however, not visible when the bananas are placed in commercial boxes and the first symptoms do not appear until after a few days of shipping. The disease evolves quickly during ripening. The rot is initially superficial and later progresses into the tissues. In some cases, it can invade the peduncles and later the fruits (**Figure 1**). The main effects on fruit quality are seen in rottening and in the induction of early ripening during shipping because of the ethylene that is released, either by stressed tissues and necrosed fruits or by fungal mycelium. When crown rot is frequently observed in bananas of a specific origin, production of bananas from this origin may be excluded from the market.

Crown rot disease is currently controlled by the systematic use of post-harvest fungicidal treatments. This approach, however, has several drawbacks:

– the effectiveness of fungicides depends on the time of year that they are applied and on the production area;
– the frequent appearance of fungicide-resistant strains prevents complete disease control;
– the fungicidal slurries rejected around packing stations after post-harvest treatment of banana crowns may cause environmental pollution.

In addition, these post-harvest chemical sanitation treatments may have wider-reaching effects, with chemical exposure affecting workers at the packing stations, and even consumers. Evidence of the possible effect on consumers can be seen in the fungicidal

Figure 1. Banana crown rot — *La pourriture de couronne des bananes*.

a: necrosed fruit clusters — *bouquets de fruits nécrosés*; **b**: internal necrosis — *nécrose interne*; **c**: necrosis spread to peduncles and fingers — *progression de la nécrose sur les pédoncules et les doigts*.

residues regularly detected on banana peel and also in the pulp. These drawbacks have led to research focusing on alternative control methods.

2.2. Susceptibility of bananas to crown rot

Variations in disease incidence. Geographical and seasonal variations have been observed in the incidence of crown rot disease (Lukezic et al., 1967; Shillingford, 1978; Krauss et al., 2000; Lassois et al., 2008). In a study conducted in Honduras, Lukezic et al. (1967) showed that the incidence of the disease varied in the course of the year, being higher in summer and lower during the colder period. They also found these variations to be unrelated to variations in the parasitic complex isolated from banana crowns. Similarly, in Jamaica, high levels of disease incidence were found to correlate with periods of high temperature (Shillingford, 1978). It has been hypothesized that such spatiotemporal fluctuations in disease incidence could reflect the variations in the quality potential of the fruit, acquired in the field (Lassois et al., 2010a). This quality potential would include two components, which would themselves depend on the agrotechnical and pedoclimatic factors prevailing during the growth phase of the banana plant. The first of these components is parasitic and the second is physiological. The parasitic component reflects the level of crown contamination by the fungal complex and the pathogenicity of this complex. The physiological component, for its part, reflects the fruit's response to pathogenic attack, which is related to the physiological state of the fruit at harvest, and this state modulates the fruit's susceptibility to crown rot.

Variations in the level of fruit susceptibility. Recently, it has been shown that several pre-harvest factors influence the susceptibility of bananas to crown rot. These factors are: geographical and seasonal variations; bunch age; source-sink ratio; and biotic stress factors.

Geographical variation. A multilocal study carried out in Cameroon over a year showed lesser crown rot development at higher altitude (500 m) than at lower altitude (80 m) (Ewané, personal communication).

Seasonal variation. In their study, Lassois et al. (2008) showed that the level of fruit susceptibility to crown rot disease was variable over a 10-week period in the same banana plot in Guadeloupe. The multilocal study carried out in Cameroon suggested that fruits harvested during the rainy season might be more susceptible to crown rot than those harvested during the dry season (Ewané, personal communication).

Bunch age and source-sink (So-Si) ratio. A within-bunch gradient of fruit is susceptible to crown rot. The first hands to initiate are more susceptible than those that initiate last (Lassois et al., 2010a). On the other hand, the So-Si ratio of the banana tree during its growth phase also has a significant influence on fruit susceptibility (hands being regarded as the sinks and leaves as the sources). When the total sink is decreased by the removal of banana hands from the bunch, the fruit's susceptibility to crown rot also decreases. Moreover, a linear relationship between fruit age and fruit susceptibility to crown rot has also been demonstrated in Guadeloupe, the oldest fruits being the most susceptible (Forret, 2008).

Biotic stress factors. The incidence of *Mycosphaerella* leaf spot disease also has an influence on crown rot disease, which is generated by host-pathogen interaction (Ewané, personal communication).

Among the great diversity of reactions and products involved in plant defense responses, phenolic compounds have been repeatedly proposed as potential participants in banana tree defense mechanisms (Beverragi et al., 1995; El Hadrami, 1997; Valette et al., 1998; Collingborn et al., 2000; Kanazawa et al., 2000; Someya et al., 2002; de Ascensao et al., 2003; Wuyts et al., 2007; Kavino et al., 2009). A recent report

suggested that these compounds might contribute to determining the strength of the response of bananas to crown rot (Lassois et al., 2011).

3. PHENOLIC COMPOUNDS

Phenolic compounds are the most studied secondary metabolites because of their considerable involvement in plant-environment interactions. They are molecules belonging to very diverse chemical families having in common an aromatic ring bearing at least one phenol hydroxyl substituent. Some phenolic compounds have several hydroxyl group substituents, which can undergo esterification, methylation, etherification or glycosylation reactions (Raven et al., 2003; Macheix et al., 2005; Lattanzio et al., 2006). The molecular weight of phenolic compounds is variable, being lower in simple compounds, higher in those with complex structures, and higher still in polymerized tannins.

3.1. Classes of phenolic compounds

Phenolic compounds are classified according to:
- the nature and complexity of the carbonaceous skeleton;
- the degree of skeletal modification (degree of oxidation, hydroxylation, methylation, etc.);
- the link between the base unit and other molecules such as carbohydrates, lipids, proteins, or the link to other secondary metabolites, possibly polyphenols (Macheix et al., 2005).

The phenolic compounds of plants include the simplest forms (hydroxybenzoic and hydroxycinnamic acids), condensed forms (tannins), and forms related to non-phenolic macromolecules (certain glucidic components of the pecto-cellulosic wall, cutin and suberin). Only two groups of phenolic compounds are mainly related to the plant walls: firstly, low-molecular-weight hydroxycinnamic acids related to various cell wall compounds, and secondly, lignins, which are polymers of monolignol units bound by oxidative coupling (**Figure 2**).

Simple phenols (C6). These are compounds with one (monophenol-like catechin) or several phenolic groups (di, tri- and oligophenols): phenol, benzoquinone, pyrogallol, pyrocathecol, etc. (Lattanzio et al., 2006).

Phenolic acids (C_6-C_1 or C_6-C_3). These are benzoic or hydroxybenzoic acids (gallic acid, ellagic acid), and cinnamic or hydroxycinnamic acids such as caffeic, coumaric, ferulic, and chlorogenic acid (Manach et al., 2004; Macheix et al., 2005; Lattanzio et al., 2006). A group of small phenolic molecules is derived from the subclass of hydroxycinnamic acids and is called phenylpropenes.

Flavonoids (C_6-C_3-C_6). These are present in plant vacuoles, where they are sometimes water-soluble or sometimes act as pigments (Raven et al., 2003). Flavonoids are the most abundant phenolic compounds in nature and are classified according to the degree of oxidation and unsaturation of their heterocyclic ring (Scalbert et al., 2000). Two classes of flavonoids can be distinguished: 4-oxoflavonoids and anthocyanidins (Manach et al., 2004).

Lignins (C_6-C_3)n. These are extremely complex phenolic polymers. Of the biopolymers, lignins rank second in abundance after cellulose. The synthesis of these compounds results from a three-dimensional polymerization of three basic phenolic molecules (called monolignols): coumarylic, coniferylic and sinapylic alcohol, corresponding respectively to p-coumaric, ferulic and sinapic acid (Macheix et al., 2005). The complexity of lignins results from the potential association of these units *via* various chemical bonds, in a manner that is neither ordered nor repetitive, so as to generate an amorphous, hydrophobic polymer.

Tannins (C_6-C_3-C_6)n. These are found in several forms with different types of chemical reactivity and composition: water-soluble tannins, condensed tannins, catechic tannins and proanthocyanidins (Macheix et al., 2005). Proanthocyanidins have a high molecular weight and are a group of condensed (chain dimers or oligomers) flavan-3-ols often related to cell walls.

The variability of phenolic compound classes in plants is far more complex than presented above. This is only intended to familiarize the reader with the classes of compounds relevant to the research topic.

3.2. Biosynthesis of phenolic compounds

The biosynthesis pathway of phenolic compounds is well characterized (**Figure 2**). Phenolic compounds are formed *via* the well-known shikimate pathway from simple sugars resulting from primary metabolism. Synthesis of phenylalanine begins with carbohydrates transforming into erythrose-4-phosphate (pentose phosphate pathway) and phosphoenolpyruvate (glycolysis), which enter into the shikimic acid metabolic pathway. Phenylalanine deamination by phenylalanine ammonia-lyase (PAL) is the first crucial stage in the biosynthesis of the large majority of phenolic compounds (Macheix et al., 2005). In addition to its involvement in the production of phenolic compounds, phenylalanine oxidation also leads to the formation of tyrosine, the precursor of catecholamines

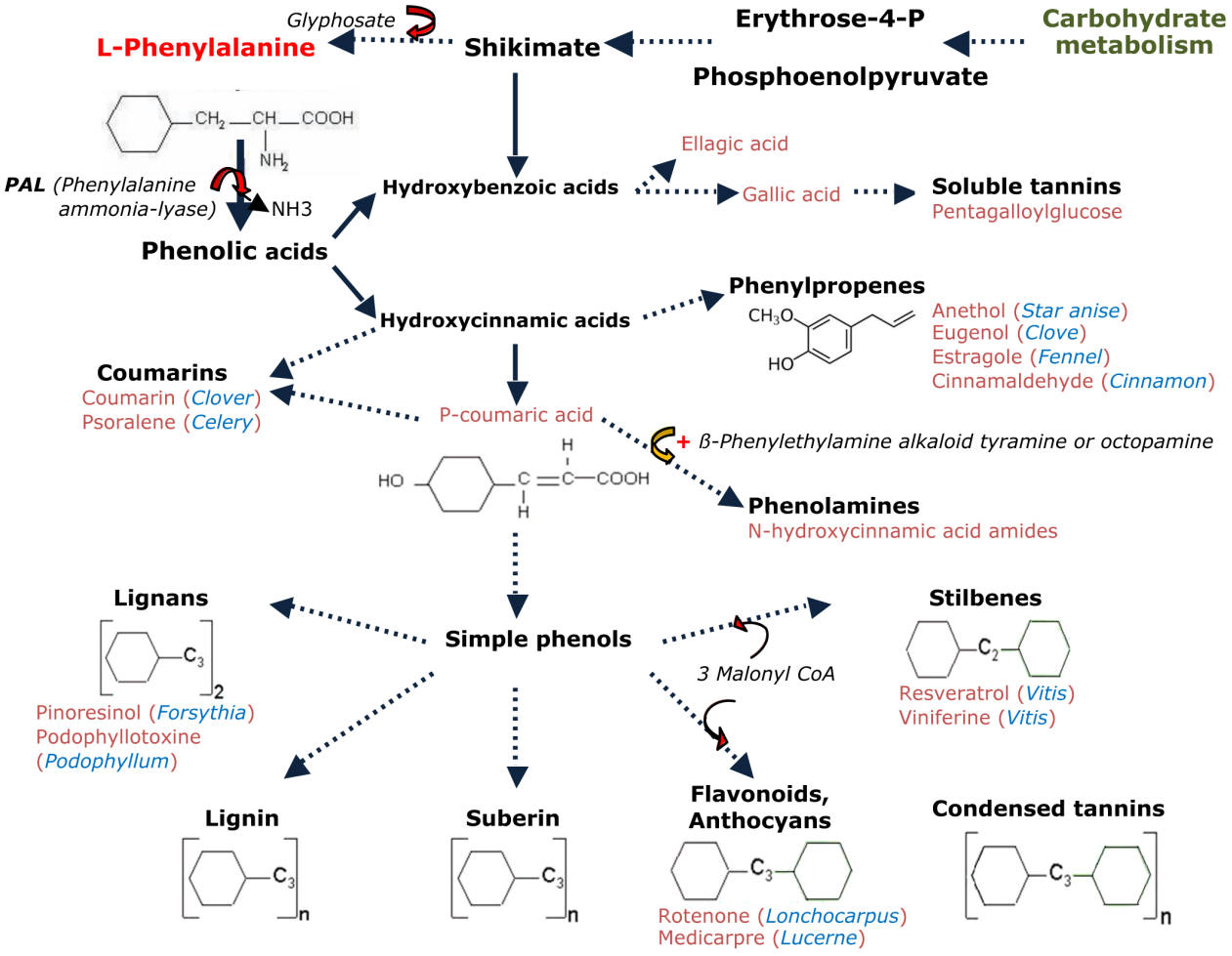

Figure 2. Simplified pathway of phenolic compound synthesis (adapted from Roland Douce, 2005) — *Voie de synthèse simplifiée des composés phénoliques (d'après Roland Douce, 2005).*

(dopamine and derivatives) (Kulma et al., 2007), which are abundant compounds in the banana tree and fruit (Muirhead et al., 1984; Kanazawa et al., 2000; Wuyts et al., 2007).

In plants, the phenolic composition varies considerably both qualitatively and quantitatively between species and between individuals of the same species. Some phenolic compounds are ubiquitous in plants (*e.g.* hydroxycinnamic acids), some are common (*e.g.* anthocyanins) and others are specific to certain families or species (*e.g.* isoflavonoids and stilbenes). Besides plant variety, a wide range of biotic and abiotic stresses (physical, chemical and biological factors) both external and endogenous can influence the level of plant phenolic content both before and after harvest. Changes in the level of plant phenolic content are effected through modulation of the phenolics metabolism: light (visible and UV), temperature, osmotic potential, plant nutrition, growth regulators, biotic elicitors, the fruit maturation state at harvest, the photoperiod, moisture, processing, and storage (Manach et al., 2004; Kalt, 2005; Macheix et al., 2005; Lattanzio et al., 2006; Ksouri et al., 2008; Xu et al., 2008; He et al., 2010; Treutter, 2010). This leads to an alteration of a very broad palette of functions, including the release or synthesis of stress metabolites involving polyphenols. For example, environmental stresses can affect photosynthesis and the assimilation of carbon required for growth, development and defense, leading to an influence on the final phenolics concentration in plant tissues. In the following sections, the influence of biotic and abiotic factors on phenolics synthesis is fully discussed.

Influence of biotic factors. Plants face daily parasitic attacks by pathogenic microorganisms, herbivores and insects. Parasites have developed different strategies for colonizing plants, since they need regulation mechanisms to effectively adapt to changes in their environment. Development of a pathogen within

a host plant occurs only under certain conditions. These conditions relate to the environmental factors influencing the host plant's physiological state, the parasite's capacity to attack and colonize its host, and the host's capacity to establish mechanisms capable of stopping colonization by the parasite. Plants have, however, developed preformed and induced defense mechanisms effective against pathogenic infections.

Following penetration by pathogens of the physical and chemical constitutive barriers of a susceptible or a resistant plant, induced structural and chemical barriers are activated in order to prevent the pathogen's progression. This results in a multitude of defense responses. For instance, the defense response of the tomato tissue is seen in an increase in soluble phenol (Zhu et al., 2004), and/or the deposit of lignin and lignin polymers (Mandal et al., 2007; Panina et al., 2007). When a plant cell encounters a pathogen, this also provokes other reactions such as the hypersensitive response (HR), which may confine the pathogen at the penetration point (Lepoivre, 2003; Lattanzio et al., 2006; Van Loon et al., 2006; Ferreira et al., 2007). However, phenolic compounds have different rates of accumulation depending on whether plant-pathogen interaction is compatible or incompatible.

The HR triggers a general resistance mechanism, rendering uninfected parts of the plant less sensitive to further attack by pathogens (Lattanzio et al., 2006). Resistant reactions involving phenolic compounds are induced in the adjacent cells surrounding the infected zone, forming a localized acquired resistance (LAR). In distal tissue cells, a systemic acquired resistance (SAR) is established (Lepoivre, 2003; Macheix et al., 2005; Lattanzio et al., 2006; Van Loon et al., 2006; Ferreira et al., 2007). Stilbene phytoalexines (resveratrol), for example, are accumulated in grapewine berries following fungal infection by *Botrytis cinerea*, but also in healthy grapes on the same cluster or near infected grape clusters (Adrian et al., 2000). In the case of the banana tree, the presence of *Mycosphaerella* leaf spot diseases during growth influences the susceptibility of bananas to post-harvest diseases (Ewané, personal communication), but the possible involvement of phenolic compounds has not been investigated.

Influence of abiotic factors. Abiotic factors – *e.g.* pedoclimatic, agronomic and agrotechnical – influence the phenolic biosynthetic process in plant tissue cells before and after harvest. The geographical location of the production zone can influence the biosynthesis of polyphenols in plant tissues. Tropical and high altitude plants contain higher proportions of flavonoids than do temperate plants (Lattanzio et al., 2006), probably because of differences in climatic regimes.

Seasonal variability in plant phenolic content may be due to the effects of climatic factors such as solar radiation, temperature, rainfall (water availability) and the hydrothermal coefficient between seasons. Spatiotemporal variations have been found to influence the susceptibility of bananas to crown rot, but further investigations are needed to provide evidence for the influence of these variations on the fruit's phenolic content. A wide array of abiotic stimuli, such as salinity and drought, are capable of triggering changes in the plant's metabolism and these changes enhance the production of plant secondary products (Khan et al., 2011).

High levels of solar radiation coupled with high temperatures lead in some cases to modifications in the accumulation of polyphenols in the plant. The resulting modifications make it difficult to determine whether physiological changes related to phenolic synthesis in the plant are caused by the effects of either solar radiation or high temperature. The literature has reported that temperature can be positively or negatively correlated to the accumulation of polyphenols during growth or processing for several plant species (Kalt, 2005; He et al., 2010; Treutter, 2010). A prominent example of the effect of temperature and light can be seen in the significantly high level of phenolics found in the seed and skin of winter berries compared to summer berries (Xu et al., 2011).

Some information on the effect of light exposure, UV and solar radiation on PAL and phenolics synthesis has been reported in the literature for several plant species. The influence of other factors has also been cited, such as soil fertility, fertilization mode (nutrient supplies), irrigation (water availability), rootstocks, elevated atmospheric CO_2, and pre-harvest treatment (Kalt, 2005; Lattanzio et al., 2006; He et al., 2010; Treutter, 2010; Pombo et al., 2011). Hence, it is not only the energy provided for carbon assimilation (carbon resources for biosynthesis) but also the quality, namely UV fractions (light intensity), which stimulates the formation and accumulation of certain phenolic compounds in plants (Ghasemzadeh et al., 2010; Treutter, 2010). The influence of soil with high levels of aluminum chloride in grape leaves has been shown to stimulate the production of resveratrol (Adrian et al., 2000).

Cultural practice is another factor that influences the phenolic content of plants and allows the control of plant growing conditions. Since carbohydrate availability is a prerequisite for phenylpropanoid accumulation (Treutter, 2010), the degree of ripeness or physiological age of the fruit at harvest (maturity) has a considerable effect on the concentration and proportions of the various polyphenols (Macheix et al., 1990; Macheix et al., 2005). Moreover, So-Si ratio modification has been shown to improve the chemical composition of *Vitis vinifera* cultivars through an impact on anthocyanin accumulation (Mota et al.,

2010). So-Si ratio modification also influences the susceptibility of the banana fruit to crown rot (Lassois et al., 2010b). An evaluation of the level of phenolic compound synthesis has not yet been undertaken. Furthermore, cultural conditions such as organic or sustainable agriculture provide higher amounts of polyphenols in strawberries, blackberries, and corn than do conventional or hydroponic conditions without stress (Asami et al., 2003).

Several studies have reported the influence of processing and storage on the synthesis of phenolic compounds or on degradation in fruits, vegetables and plants (Kalt, 2005; Lattanzio et al., 2006; He et al., 2010; Treutter, 2010). At the packing station, during the preparation of banana clusters, physical injury to the fruit through cutting of the crown tissues may lead to oxidative degradation of polyphenols. This oxidative degradation of polyphenols causes their transformation into brown pigments that are polymerized to different degrees or else it causes polyphenol synthesis in the banana tissues. In addition, certain water-soluble phenolics present in the wounded crown may leach from fruit tissues into washing tanks. Finally, the number of phenolic compounds known to be synthesized and stored by higher plants is in the thousands and rising; however, the functions of many phenolics remain unknown.

3.3. Localization and function of phenolic compounds in plants

Biosynthesis of phenolic compounds occurs at various sites in plant cells, such as the chloroplasts, the cytoplasm and the endoplasmic reticulum membrane. Polyphenols (relatively hydrophilic) usually accumulate in the central vacuoles of guard cells, epidermal cells and the subepidermal cells of leaves and shoots. Some polyphenols are found covalently linked to the plant cell wall (lignin); others are found in waxes (related to lipidic structures) or on the external surfaces (cuticle) of plant organs (Lattanzio et al., 2006). The localization of a phenolic compound within a tissue reflects its physiological function or its participation in interactions of the plant with its environment (Macheix et al., 2005). For example, polyphenols with a role in signaling or defense are often stored at strategically important sites (Lattanzio et al., 2006).

Phenolic compounds, both preformed and induced, play an essential role in the balance of the plant within its environment and its capacity to adapt to environmental changes (Macheix et al., 2005) through biochemical, physiological and molecular responses. Phenolic compounds intervene in major physiological mechanisms of the plant, such as growth, reproduction, pigmentation, rhizogenesis, vitrification and resistance to pathogens, by performing many functions (Cheynier, 2005; Lattanzio et al., 2006). Polyphenols play a key role as the major red, blue, and purple pigments of plants, as antioxidants and metal chelators, and as agents acting both above and below ground in signaling between the plant and other organisms and as UV light screens (Cheynier, 2005; Lattanzio et al., 2006). Polyphenols have multiple effects on tissue maturation processes and on the sensory qualities of plant-derived food products, including astringency, bitterness and aroma. The compounds are also responsible for the browning caused by wounds to fruit or by conservation accidents and notably for the effect of low temperatures on pineapple and banana. In banana, dopamine has been identified as a substrate for polyphenol oxidase, resulting in the browning reaction, and the chemical is also responsible for tissue discoloration (Kanazawa et al., 2000; Someya et al., 2002; Maneenuam et al., 2007). As polyphenols play a role in regulating plant-environment interactions, some researchers attribute an ecological function to these compounds. Polyphenols are, for instance, reported to modulate litter decomposition processes and the mineralization rate in plants (Macheix et al., 2005; Lattanzio et al., 2006). Plants thus contain several classes of polyphenols with different localizations and roles.

3.4. Involvement of phenolic compounds in plant defense mechanisms

Phenolic compounds involved in plant defense are either preformed (constitutive) or synthesized *de novo* (postinfectional). Preexisting phenols are antibiotic (antifungal) compounds such as simple phenols, phenolic acids, flavonols, and dihydrochalcones. These preexisting phenols are called phytoanticipins in order to distinguish them from phytoalexins, which are synthesized from remote precursors in response to pathogenic attack (Lattanzio et al., 2006). Phenolic compounds synthesized *de novo* accumulate in response to plant infection by a pathogen. This defensive response involves the rapid increase of specific phenolic compounds at the infected site, particularly phytoalexins, which can inhibit a broad range of microorganisms (Lepoivre, 2003; Macheix et al., 2005; Lattanzio et al., 2006). The result is the development of plant resistance to disease. An example of the involvement of polyphenols in plant defense is their action in the programmed cell death of one part of the plant. The rate at which programmed cell death occurs depends on whether the host-pathogen interaction is compatible or incompatible. During the establishment of a pathogen in host tissues, there is an increase in the activity of specific enzymes such as PAL, peroxidase and polyphenol oxidase.

These enzymes, which consume oxygen and produce fungitoxic quinones, make the medium unfavorable for the further development of pathogens (Lattanzio et al., 2006). PAL is the key enzyme involved in phenolic compound metabolism through the phenylpropanoid pathway (Dixon et al., 2002). Peroxidase catalyzes the condensation of phenol into lignin and is also involved in phenol metabolism (Passardi et al., 2004). Polyphenol oxidase oxidizes constitutive plant phenols into quinones, which have bactericidal and fungicidal properties, and is also involved in the oxidative detoxification of pathogen phytotoxins (Macheix et al., 1990; Yoruk et al., 2003).

Another example of a phenolic compound that contributes to the plant's defense mechanisms is lignin. Lignin is a phenolic polymer, which plays a fundamental role in solute conductance, mechanical support and disease resistance. In response to abiotic stress, to wounding or to pathogenic infection, the deposition of lignins, lignin polymers and other phenolic substances related to the cell wall are observed. This contributes to both a thickening of the cell wall (conferring greater rigidity and mechanical resistance) and to an increase in cell hydrophobicity. Lignin thus acts as a physical barrier against pathogenic invasion. In addition, lignin deposits reduce the diffusion of enzymes and toxins that the pathogen releases in order to facilitate host tissue colonization. Lignin also deprives the pathogen of the plant water and nutrients necessary to its proliferation (Macheix et al., 2005; Lattanzio et al., 2006).

3.5. Involvement of phenolic compounds in banana tree defense

Since phenolic compounds are potential targets for the improvement of plant resistance against pathogenic attack, it is possible that they are involved in the susceptibility of bananas to crown rot disease and a better understanding of their involvement in banana tree defense is required. The phenolic composition and the role of several banana tree tissues (leaves, fruits, roots) have been described in the literature review, but their potential role in the variation of susceptibility of banana crowns to disease is not yet known (**Table 1**).

Preformed defense mechanisms. The leaf surface wax, cuticle, suberin and cell wall are the first preformed physical barriers in banana. These preformed physical barriers are also involved in banana tree defense mechanisms, and contain phenolic compounds (lignin, cross-linking hydroxycinnamic acids) in their structures. The cuticle forms an envelope that protects the aerial parts of plants from the majority of attacks

Table 1. Some phenolic compounds identified in banana tree tissues — *Quelques composés phénoliques identifiés dans les tissus du bananier.*

Phenolic alkaloids	**Tissues**	**Class (*Subclass*)**	**References**
Dopamine Norepinephrine	Fruits Roots	Catecholamines	Valette et al., 1998; Kanazawa et al., 2000; Wuyts et al., 2007
Phenolic compounds	**Tissues**	**Class (*Subclass*)**	**References**
		Phenolic acids	
Gallic acid, vanillin	Fruit	*Hydroxybenzoic acids*	Mendoza et al., 1992; Mendez et al., 2003
Chlorogenic, caffeic, *P*-coumaric, ferulic, vanillic and synapic acids	Fruits, roots	*Hydroxycinnamic acids and derivatives*	Wade et al., 1993; Valette et al., 1998; de Ascensao et al., 2003; Wuyts et al., 2007
Catechin Gallocatechin	Fruits	Simple phenols *Flavan-3-ol*	Mendoza et al., 1992; Wade et al., 1993; Someya et al., 2002; Mendez et al., 2003
		Flavonoids	
Rutin, naringin	Fruits Leaves, roots	*Flavonols* *Flavanones*	Valette et al., 1998; Kanazawa et al., 2000; Wuyts et al., 2007
Anthocyanidins		*Flavonoids derivatives*	Collingborn et al., 2000
		Tannins	
Tannins catechic, Proanthocyanidins	Fruits Roots	*Hydrolysable tannins* *Condensed tannins*	Mendoza et al., 1992; El hadrami, 1997; Valette et al., 1998
Lignin	Roots	Lignin	Valette et al., 1998; de Ascensao et al., 2003; Wuyts et al., 2007

by pathogenic fungi (Pollard et al., 2008). Suberin, localized in the underground parts of the plants such as the bark and scar tissues, contains high quantities of ω-dicarboxylic acid, hydroxycinnamic acids (mainly ferulate) and fatty alcohols (Pollard et al., 2008; Schreiber, 2010). The cuticle and suberin control the diffusion of gases, of water, and of solutes in and out of the plant. They also play a protective role towards biotic and abiotic stress (Pollard et al., 2008; Schreiber, 2010). The pecto-cellulosic wall plays the role of "skeleton" while maintaining a certain plasticity/elasticity in the plant, allowing growth and cellular divisions (Macheix et al., 2005), but also being involved in physiological resistance.

The preformed chemical barriers of plants are a plethora of peptides, proteins and secondary metabolites (phenolics, sulfur compounds, etc.), which act as antimicrobial compounds during defense responses against microorganisms (Lattanzio et al., 2006). Some of these compounds are present in their biologically active form, and are therefore directly toxic; others are present in the form of inactive precursors (combined forms). In studies comparing varieties of banana susceptible to black leaf streak disease with partially resistant varieties, certain mesophyll cells specialized in the storage of phenolic compounds (proanthocyanidins) were found to be lower in number in the susceptible varieties than in the partially resistant ones (Beveraggi et al., 1995; El Hadrami, 1997).

Induced defense mechanisms. After infection by different banana pathogens, a rapid increase in both phenolic compounds and phenylpropanoid pathway enzymes has been observed in banana tree tissues. Induced structural barriers are activated following the presence of pathogens in susceptible or resistant plants, and this results in a multitude of defense responses. When a plant recognizes a pathogenic invader, its defense mechanisms are induced, and chemical barriers are produced as a result. These chemical barriers are generally in the form of "*de novo*" synthesized molecules and once produced, these molecules become accumulated.

Several studies on the interaction between banana tree roots and their various pathogens have suggested an involvement of phenolic compounds in the defense responses of the banana tree. These studies focused on cell wall lignification, on histochemical detection of phenolic compounds and on the quantification of flavonoids, proanthocyanidins and hydroxycinnamic acids after infection of banana roots by phytophagous nematodes (Valette et al., 1998; Collingborn et al., 2000; Kanazawa et al., 2000; de Ascenceo et al., 2003; Wuyts et al., 2007). In resistant banana cultivars, a higher content of several phenolic compounds such as dopamine and cyanidin-related compounds, lignin, and ferulic acid (esters and hydrolyzation products of ferulic acid) was observed (Wuyts et al., 2007). Moreover, the role of these compounds in defense mechanisms such as physical barriers, toxic agent and protection against pathogenic attack was suggested.

In other studies, several phytoalexins (phenylphenalenones, irenolone, and emenolone types) have been induced in green banana fruits after wounding or inoculation with *Colletotrichum musae* (Luis et al., 1993; Kamo et al., 1998; Kamo et al., 2001). Recently, in the interaction between *Musa* spp. and *Fusarium oxysporum* f. sp. *cubense* (Panama disease), resistant hybrid plantlets (leaves and roots) were found to contain higher concentrations of phenol and pathogenesis-related proteins and higher activity levels of PAL and oxidative enzymes (peroxidase, polyphenol oxidase, superoxide dismutase, catalase) than sensitive hybrid plantlets (Kavino et al., 2009). During penetration of host tissues, phenolic substances are released around the pathogen hyphae, causing considerable morphological changes such as cytoplasmic disorganization and loss of protoplasmic contents (Kavino et al., 2009) and thus forming a chemical barrier that blocks or at least slows down microorganism progression.

As mentioned above, many phenolic compounds, particularly dopamine, have been identified in various banana tree tissues. Among a broad range of plants, bananas have the richest dopamine content: 100 $\mu g \cdot g^{-1}$ FW in Cavendish banana peel as compared with 7 $\mu g \cdot g^{-1}$ FW for potato, the second richest in this list of dopamine-rich plants (Kulma et al., 2007). This observation suggests that dopamine might play an important role in banana physiology. Dopamine results from the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine, and is responsible for the browning of the fruit peel and pulp. Products arising from the decomposition of banana dopamine seem to have a fungitoxic activity against *C. musae* (Mulvena et al., 1969; Muirhead et al., 1984). Moreover, a recent study on the molecular mechanisms involved in the variations in the susceptibility of banana fruit has highlighted overexpression of the dopamine-β-monooxygenase gene in bananas less susceptible to crown rot (Lassois et al., 2011).

4. CONCLUSION

The occurrence of crown rot disease seems to correlate with the physiological state of the fruits at harvest. Plant polyphenols are secondary metabolites involved in the defense mechanisms of plants against fungal pathogens. These phenolic compounds are also involved in the defense responses of the banana tree, although their mechanism(s) of action and their

antagonistic properties are not known. Thus, the possible involvement of phenolic compounds in variations found in the susceptibility of bananas to crown rot means that these compounds represent a potential target for developing alternatives to the chemical control of the disease. It is thus essential to measure pools of phenolic compounds, particularly dopamine, in fruits displaying differential susceptibility, so as to identify key "defense" compounds and to gain a better knowledge of the defense mechanisms of the banana crown tissues.

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Chapter II. Aims and proposed research

The relation between the fruit quality potential and the physiological component as well as factors influencing this fruit's quality potential in the field and in the banana commodity channel (Figure 1) were well presented and developed by Lassois *et al*. (2010a).

Recent advances have been done in the understanding of crown rot post-harvest development through the characterization of the banana fruit's physiological component. The fruit quality potential at harvest was thus considered as a key factor in crown rot development. Some pre-harvest factors influencing banana's susceptibility to crown rot have been determined (Lassois *et al*., 2010b), and some genes potentially involved in banana susceptibility variation have been identified (Lassois *et al*., 2011). Nevertheless, due to the complexity of this research topic, the results obtained laid the basis for the comprehension of the post-harvest development of crown rot.

The research proposes a new, interesting and original approach to the characterization of the banana fruit's physiological component. It consists of a physiological study in order to determine the abiotic and biotic pre-harvest factors influencing banana's susceptibility to crown rot, followed by a biochemical study to analyze the phenolic content of banana crown tissues with differential susceptibility level to crown rot. The different steps of the scientific strategy of this thesis are presented in Figure 1.

The aim of this study is to characterize the fruit physiological component at harvest in relation with banana crown rot post-harvest development through an evaluation of fruit's susceptibility after standardized artificial inoculations (de Lapeyre de Bellaire *et al*., 2008) and banana crown phenolics content analysis.

Specific aims are to study the influence of abiotic pre-harvest factors (Chapter 3.1.) and biotic pre-harvest factors (Chapter 3.2.) on crown rot post-harvest development. An essential step of this part is the improvement on the methodology for the assessment of banana's susceptibility to crown rot by artificial inoculations in order to determine the small variations in the banana fruit's susceptibility to the disease and to increase the reliability of necrotic surface assessments. This part was essentially realized in Cameroon with the collaboration of CARBAP and CIRAD. In the Second part of the work, the role of phenolics (catecholamines and polyphenols) in susceptibility variation mechanisms was analyzed through chromatographic methods (Chapter 4). In this part, chromatographic techniques (GC-MS, HPLC, LC-MS) were applied in order to identify and quantify the phenolic content of banana

crown different susceptibilities to crown rot. Field work was carried out in CARBAP (Cameroon) for plant material while the analytical part was done in ULg-GxABT.

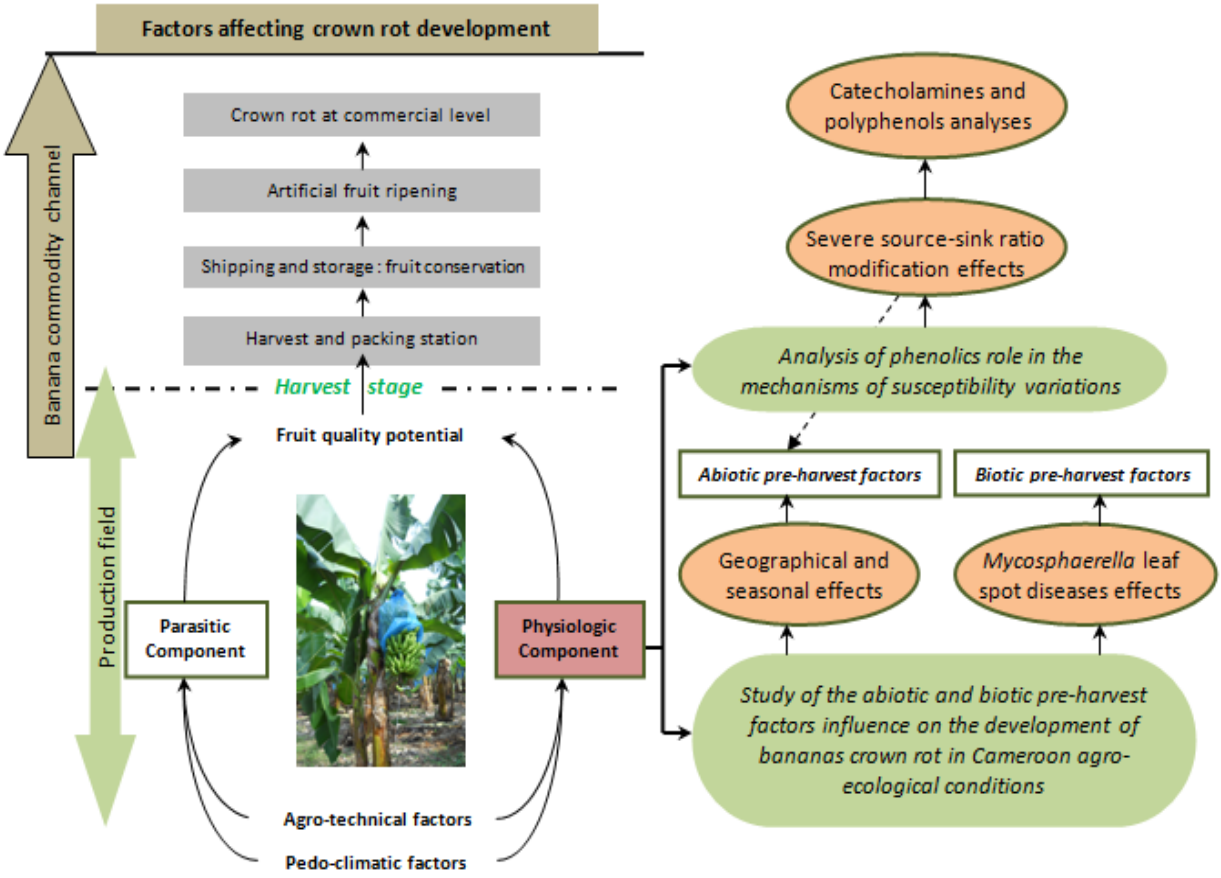

Figure 1: Diagram representing different steps of banana commodity channel and the scientific strategy of this thesis.

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Chapter III. Study of the influence of abiotic and biotic pre-harvest factors on crown rot development of bananas in Cameroon agro-ecological conditions

I. The susceptibility of bananas to the crown rot disease is influenced by geographical and seasonal effects

Some pre-harvest factors such as the fruit's position on the bunch and source-sink ratio at flowering stage have been recently identified as influential to the fruit's susceptibility to crown rot. Since these pre-harvest factors are keys in the post-harvest development of crown rot disease and its control, a better understanding of these pre-harvest factors could limit the risk of development of the post-harvest diseases. Because of the complexity of the research topic, a continuous assessment of the pre-harvest factors has been made through the identification of two abiotic pre-harvest factors affecting the level of the fruit susceptibility to crown rot in field conditions. Prior to the evaluation of the level of susceptibility to crown rot, it was essential to increase the reliability of necrotic surface assessments so that small variations in banana fruit's susceptibility to crown rot could be determined. An improvement on the methodology was effected in order to assess the banana's susceptibility to crown rot by artificial inoculations and therefore stabilize the post-inoculation environmental conditions.

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The susceptibility of bananas to crown rot disease is influenced by geographical and seasonal effects

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Abstract: Crown rot of banana fruits is caused by a complex of fungal pathogens, the most common of which is *Colletotrichum musae*, and is one of the main quality defects of exported bananas. Susceptibility of banana fruits to crown rot is influenced by many pre-harvest factors. The aim of this study was to improve on the methodology for the evaluation of fruit susceptibility and to verify whether cultivation areas in Cameroon as well as seasonal variations have an influence on the susceptibility to crown rot. Fruit susceptibility was evaluated on a monthly basis throughout a year (including the dry and rainy seasons) in three banana plantations located in very different agro-ecological conditions (two in a lowland area and one in a highland area). Fruit susceptibility was determined through an internal necrotic surface (INS) assessment after artificial inoculation with *C. musae*. The standardization of post-inoculation environmental conditions enabled more reliable INS assessments. Fruit susceptibility was found to be significantly influenced by cultivation area ($P < 0.001$) since fruits grown in low altitude (Dia-dia, Koumba, 80 m) were more susceptible than fruits grown in high altitude (Ekona, 500 m). Although no seasonal effect was observed ($P = 0.075$), there was a highly significant date effect ($P < 0.001$). This was specifically the case in low-altitude plantations where fruit susceptibility was higher for some harvest dates within the rainy season. In Ekona, fruit grade and number of leaves on the banana plant were found to be significantly higher than in the two other locations, while black leaf streak disease severity was significantly lower. The potential relationship with fruit susceptibility is fully discussed.

Keywords: banana, crown rot, fruit susceptibility, geographical and seasonal effects, *Musa* spp, post-harvest diseases

Résumé: La pourriture du collet de la banane est causée par un complexe d'agents pathogènes fongiques, dont le plus courant est *Colletotrichum musae*, et est un des principaux défauts de qualité des bananes exportées. La réceptivité de la banane à l'égard de la pourriture du collet est influencée par plusieurs facteurs relatifs à la période avant-récolte. Le but de cette étude était d'améliorer la méthodologie permettant d'évaluer la susceptibilité des fruits et de vérifier si les régions de culture au Cameroun de même que les variations saisonnières avaient une influence sur la réceptivité à l'égard de la pourriture du collet. La sensibilité des fruits a été évaluée mensuellement dans le cours d'une année (y compris durant la saison sèche et la saison des pluies) dans trois plantations localisées dans des régions différentes sur le plan des conditions agroécologiques (deux dans les basses terres et une dans les montagnes). La susceptibilité des fruits a été déterminée par l'évaluation de la nécrose interne de la surface (NIS) après inoculation artificielle avec *C. musae*. La normalisation des conditions environnementales post-inoculation a permis d'obtenir des évaluations de la NIS plus fiables. La susceptibilité des fruits s'est avérée significativement influencée par la région de culture ($P < 0.001$) étant donné que les fruits cultivés dans les basses terres (Dia-Dia, Koumba, 80 m) étaient plus susceptibles que ceux cultivés en altitude (Ekona, 500 m). Bien qu'aucun effet saisonnier n'ait été observé ($P = 0.075$), il y avait un effet significatif relatif à la date ($P < 0.001$). C'était le cas particulièrement dans les plantations localisées dans les basses terres où la

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susceptibilité des fruits était plus grande à certaines dates de récolte durant la saison des pluies. À Ekona, le calibre des fruits et le nombre de feuilles sur les bananiers étaient significativement supérieurs aux deux autres régions, tandis que la gravité de la maladie des raies noires était significativement moindre. La relation potentielle quant à la susceptibilité des fruits y est discutée en détail.

Mots clés: banane, effets géographiques et saisonniers, maladies de conservation, *Musa* spp, pourriture du collet, sensibilité des fruits

Introduction

In Cameroon, the main quality defect of banana fruit exported to Europe is related to a post-harvest disease known as crown rot. This post-harvest disease is caused by a broad unspecific and opportunistic fungal complex. Within this complex, *Colletotrichum musae* (Berk. & Curt.) Arx is the most commonly described pathogenic species (Finlay & Brown, 1993; Krauss & Johanson, 2000; Lassois *et al*., 2010a). *Colletotrichum musae* is also responsible for banana anthracnose and wound anthracnose. At harvest, crowns of fruit appear to be healthy but after a few days of shipping, fungal infection develops superficially and internally, affecting the crowns, the pedicels and ultimately the banana pulp. The formation of clusters at the packing station involves cut sections that are penetration tracts for pathogens. Fruit contamination can occur within the field, but it mostly occurs in the washing tanks at the packing station (Shillingford, 1977; Finlay *et al*., 1992).

The control of post-harvest diseases of bananas intended for export mainly relies on post-harvest fungicide treatments (de Lapeyre de Bellaire & Nolin, 1994). Unfortunately, the chemical control method currently used is not satisfactory and has many other shortcomings, including: (i) resistance build-up in pathogen populations (Johanson & Blazquez, 1992; de Lapeyre de Bellaire & Dubois 1997); (ii) unacceptable fungicide residues in fruit; and (iii) potential human and environmental hazards with the fungicide mixtures used for post-harvest treatment of banana crowns rejected in packing stations after packing. Therefore, interest in non-chemical alternatives for the management of crown rot and other post-harvest diseases of bananas is increasing.

Geographical and seasonal variations (temperature and rainfall) have been shown to play a role in the incidence of post-harvest diseases in bananas (Lukezic *et al*., 1967; Shillingford, 1978; Chillet & de Lapeyre de Bellaire, 1996; Chillet *et al*., 2000; Krauss & Johanson, 2000). It has been suggested that these spatiotemporal fluctuations could reflect the variations in the quality potential of the fruit, acquired in the field. This quality potential would include two components: the fruit susceptibility to the post-harvest diseases (physiological component) and the level of fruit contamination by fungal pathogens (pathological component). Both components depend on pre-harvest factors including agronomic practices and soil–climate factors (Chillet & de Lapeyre de Bellaire, 1996; Chillet *et al*., 2000; Lassois *et al*., 2010*b*). Alternative control methods as well as any control measures should rely on a better understanding of these pre-harvest factors as previously shown for wound anthracnose, another post-harvest disease of bananas (de Lapeyre de Bellaire *et al*., 2000; Chillet *et al*., 2006, 2007). However, little is known about pre-harvest factors which influence the post-harvest development of crown rot disease. Although pedo-climatic conditions and agrotechnical factors are known to influence the development of this disease (Lukezic *et al*. 1967; Shillingford, 1978; Krauss & Johanson, 2000), only few studies link these fluctuations to either fruit susceptibility (Lassois *et al*., 2008, 2010*a*) or to fruit contamination.

The aim of this work was therefore to examine the effects of geographical and seasonal variations on banana fruit susceptibility to crown rot. Preliminary studies were conducted in order to show how the standardization of the post-inoculation environmental conditions (PIEC) during the assessment of banana susceptibility to the disease by artificial inoculations could improve accuracy of assessment. In the second part, cultivation areas and seasonal variations were evaluated to understand their effects on the susceptibility of bananas to crown rot disease.

Materials and methods

Plant material

Banana fruits (*Musa acuminata* Colla [AAA group, Cavendish subgroup] 'Grande Naine') were harvested from commercial banana farms in Cameroon. Bunches were tagged at flowering. They were also covered with a plastic sleeve at this stage. Bunches were harvested at a constant physiological age (Jullien *et al*., 2008), determined when the sum of the mean daily temperature accumulated by the fruit between flowering and harvest reached 900 degree days (dd) according to the procedure described earlier (Ganry & Chillet, 2008). Temperatures were recorded at a weather station in the plantation so as to predict the time due for harvest. An electronic probe (Tinytag Plus, TGP-4017; Gemini Data Loggers,

Chichester, UK) with regular recording intervals (every 15 min) was installed in each of the experimental plots. Average daily temperature was calculated from these data.

Evaluation of susceptibility to crown rot through artificial infection

The susceptibility of banana fruits to crown rot was evaluated through artificial inoculation of banana clusters with *C. musae*. The second hand (occasionally the third hand was also harvested if needed) of each bunch was harvested in the field the day before experimentation (15 bunches per harvest date) and stored at 13 °C. In the laboratory, for each banana plant, the hands were divided into three to five clusters of four healthy banana fingers according to the experiments. These clusters were rid of any defects and the fingers located at the ends of the hands were systematically eliminated. The cuttings of the crowns were square, with regular and clear-cut sections in order to obtain similar crowns between the clusters. As commonly done in previous research on crown rot (Lassois *et al*., 2008; Bastiaanse *et al*., 2010), once latex exuded, crown tissues were dried with absorbent paper and sterilized by dipping in 50% alcohol. Fruits were then laid out at room temperature for 2 h so as to allow the crowns to dry. The *C. musae* isolate Co-CMR-65 is a standard monosporic strain, that has been previously isolated from a banana fruit harvested in Njombé (Cameroon), tested for its pathogenicity and preserved at −20 °C in a glycerol solution (30%) in order to avoid fungal variations. New cultures of the *C. musae* strain were initiated, 15 days before each inoculation, starting from freezed cryotubes of mycelium. Thereafter, on the fifth day, the strain was transferred to Mathur's medium ($MgSO_4.7H_2O$, 2.5 g L^{-1}; KH_2PO_4, 2.7 g L^{-1}; peptone, 1 g L^{-1}; yeast extract, 1 g L^{-1}; saccharose, 10 g L^{-1}; agar, 15 g L^{-1}) and incubated at 25 °C for 10 days. Conidia were harvested in sterile distilled water and passed through a 45 μm sterile sieve and the concentration of conidia was maintained at 10^4 conidia mL^{-1} using a Malassez cell (Marienfeld, Germany). A 50 μL droplet of the *C. musae* suspension was then deposited midway through the exposed surface of the crown. A sterilized filter paper was placed on the droplet in order to maintain the inoculum in place. Three hours after inoculation, the fruit clusters were packed in perforated plastic bags in commercial boxes. They were stored under different conditions according to the protocol assigned to each experiment as described below. At the end of the storage period, an evaluation of the internal progression of the crown rot was carried out. The clusters were divided into two parts and the transverse cutting of the crown allowed a visualization of the surface of rot spread into the crown (Fig. 1). This necrotic surface (INS) was adjusted to a rectangular shape and the length (L) and the width (W) of this surface were measured. The 'Internal Necrosis Surface' expressed in mm^2 was calculated according to the rectangle surface formula: $INS = L \times W$. Its average value was taken as a measure of fruit susceptibility to crown rot.

Fig. 1. Clusters of bananas divided into two parts through the transverse cutting of the crown for the visualization of internal crown rot symptoms that was adjusted to a rectangular shape for measurements of the necrotic surface.

Experimental sites

Experiments were carried out in three commercial banana farms located in representative pedo-climatic conditions, as follows: (1) Dia-dia: this plot was located in a plantation belonging to Plantation du Haut Penja (PHP), at 80 m altitude near Njombé in the Littoral region with an average temperature of 27 °C and a mean annual rainfall of 2500 mm; (2) Koumba: this plot was located in a plantation belonging to Société des Plantations du Manengouba (SPM), at 80 m altitude near Mbanga in the Littoral region with an average temperature of 26 °C and a mean annual rainfall of 2500 mm; (3) Ekona: this plot was located in a plantation belonging to Cameroun Development Corporation/Delmonte (CDC/Del Monte), at 500 m altitude on the slopes of Mount Cameroon, Moliko near Buea in the South-west region with an average temperature of 22 °C and a mean annual rainfall of 2300 mm.

The climate in the banana-producing areas of Cameroon is equatorial and it is characterized by two main and contrasting seasons: a rainy season with heavy rains from April to October and a dry season from November to March. The average temperature and rainfall during the dry season are 27 °C and 63 mm $month^{-1}$ in Dia-dia and Koumba; 23 °C and 45 mm $month^{-1}$ in Ekona, respectively. During the rainy season, the average temperature and rainfall are 26 °C and 309 mm $month^{-1}$ in Dia-dia and Koumba; 22 °C and 301 mm $month^{-1}$ in

Ekona, respectively. The rainy season was considered, in this study, as a period with rainfall higher than 150 mm month^{-1}.

Experiment 1: evaluation of fruit susceptibility through artificial infection and the standardization of post-infection environmental conditions

This experiment was conducted in September 2007, in the packing house of two commercial banana plantations: Koumba and Dia-dia. For each repetition of the experiment, 15 banana bunches were selected. Five clusters (A, B, C, D, E) were cut off from the second and third hands. The susceptibility of each cluster to crown rot was assessed as described previously. Clusters of the same treatment (A, B, C, D, E) were packed in the same commercial box and stored under stable conditions at 13 °C for different periods before necrotic assessment: A: 7 days; B: 9 days; C: 11 days; D: 13 days; E: 15 days. Fruits were allowed to ripen naturally without the use of applied ethylene. Each experiment was repeated three times for three consecutive weeks.

Experiment 2: Evaluation of geographical and seasonal effects on fruit susceptibility through artificial infection and post-infection environmental conditions similar to commercial export conditions

This experiment was conducted between April 2006 and May 2007 in Dia-dia, Koumba and Ekona. At each location, 15 banana plants were selected and labelled at the flowering stage, and three clusters were cut off from the second hand of each bunch at harvest time. The susceptibility of bananas to crown rot was evaluated as described previously. After inoculation, banana fruits were placed in commercial boxes and stored in conditions which simulate commercial exportation. Boxes were stored at 13 °C for 10 days to simulate shipping. Artificial ripening was then initiated by dipping the bananas for 5 s in an ethrel solution (2-chloroethyl phosphonic acid, 480 g L^{-1}), and keeping the bananas at 20 °C for another three days before crown rot assessment. This experiment was repeated every month during a year.

Experiment 3: Evaluation of geographical and seasonal effects on fruit susceptibility through artificial infection and standardization of post-infection environmental conditions

This experiment was conducted between November 2007 and October 2008 in Dia-dia, Koumba and Ekona. At each location, 15 banana plants were selected and labelled at the flowering stage. Three clusters were cut off from each second hand harvested and the susceptibility of bananas to crown rot was evaluated as described previously. After inoculation, fruits were stored in stable conditions at 13 °C for 13 days in order to evaluate their susceptibility. In addition to the assessment of fruit susceptibility, some fruits and banana plant characteristics were measured at harvest stage (900 dd) in the three locations:

- the number of leaves (NL) on the plant,
- the number of hands (NH) per bunch,
- the grade of the median fruit (Gr) on the second hand.

Black leaf streak disease (BLSD) of bananas caused by *Mycosphaerella fijiensis* (Morelet) can lead to substantial reduction in the leaf area with direct effects on fruit quality and fruit growth. The severity index of BLSD was also assessed, according to the method described by Gauhl *et al*. (1993). This method involves a visual estimation of the necrotic area per plant, scored according to a six-disease grade scale. This was done on all the leaves of each banana plant harvested at 900 dd. The assessments were conducted on a monthly basis during a year in order to evaluate seasonal effects.

Statistical analyses

The effects of geographical and seasonal variations on banana susceptibility to crown rot were analysed. This was done by subjection of the average INS values calculated for the three clusters to partially nested mixed four-way ANOVA performed with Minitab software v.15.1, each cluster being taken as an experimental unit and location, season, date and bunch as factors. The different variables were analysed by subjection of the value (INS, Gr, NH, NL and BLSD severity) to a mixed three-way ANOVA performed with Minitab software v.15.1 with location, date and bunch as factors. Multiple comparisons of the means were done by applying Tukey test at a 5% probability level.

Results

Importance of post-infection environmental conditions on necrotic assessments

In order to evaluate the effect of the standardization of post-infection environmental conditions (PIEC) on INS assessment, we compared the data of experiment 2 (PIEC

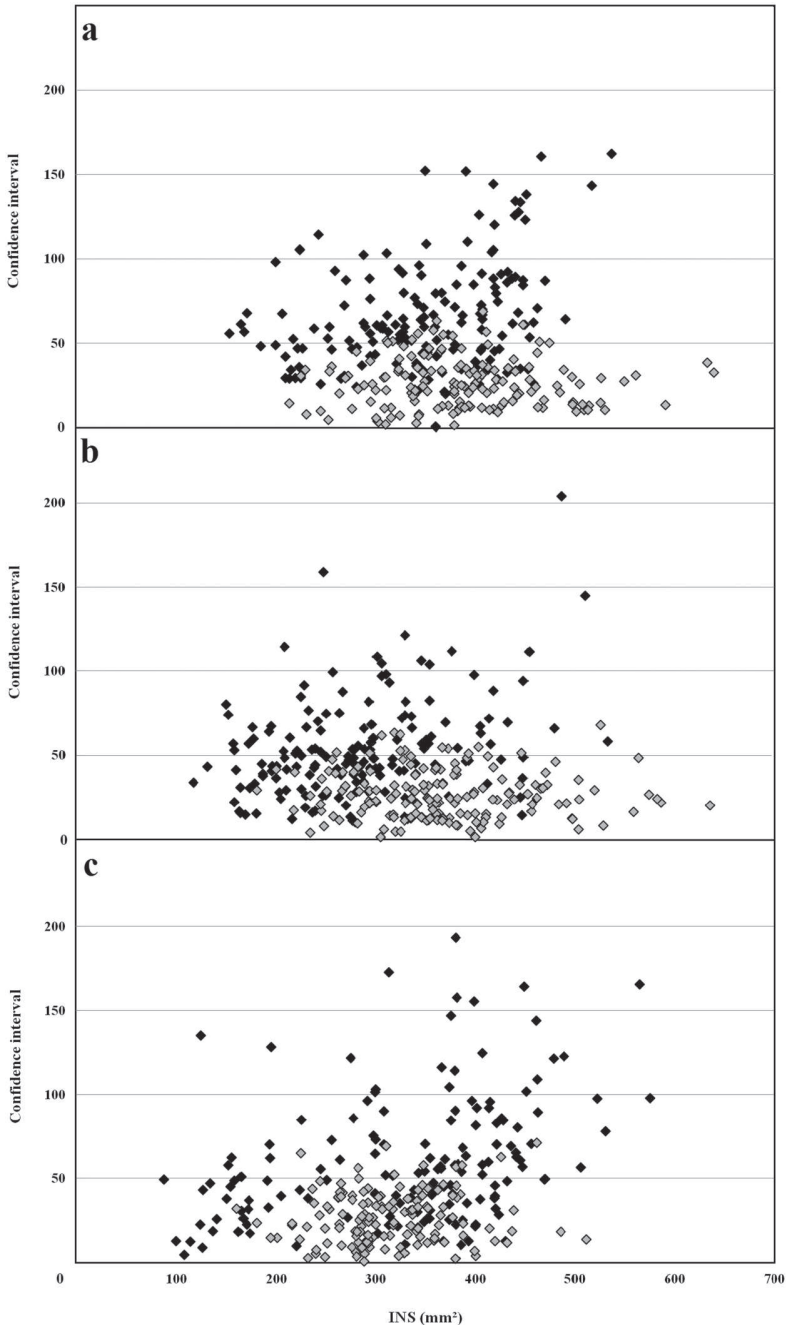

Fig. 2. Representation of different means of INS versus their confidence intervals at 95% assessed in two experiments differing by their post-inoculation environmental conditions in (**a**) Dia-dia, (**b**) Koumba and (**c**) Ekona. In experiment 1 (Black ◆) conducted from June 2006 to May 2007, bananas were stored at 13 °C for 10 days to simulate shipment; then artificial ripening was initiated with ethylene and bananas remained at 20 °C for another 3 days before crown rot assessment. In experiment 2 (Grey ◇) conducted from November 2007 to October 2008, bananas were stored at 13 °C for 13 days. Each point represents the INS of three clusters per harvested bunch. Fifteen bunches were harvested per month in the three locations (Dia-dia, Koumba and Ekona) during 12 months of experimentation.

similar to commercial conditions) and data of experiment 3 (stable PIEC). This comparison showed that the confidence intervals of the means for INS were reduced at all three locations where PIEC was standardized at 13 °C and where fruit were allowed to ripen naturally (Fig. 2*a*, *b*, *c*). For further analysis of banana susceptibility to crown rot disease, only data obtained with these new post-harvest environmental conditions was considered.

Time-course of necrotic assessments under stable conditions

The time-course of INS at 13° C (experiment 1) is typically a sigmoid curve (Fig. 3). From harvest day (day 0) to day 7, necrotic surface extension was low. From the days 7 to 13, necrotic extension was linear. After day 13, the necrotic surface increased slowly and stabilization was at day 15. This time course was the same for Dia-dia and Koumba locations. As linear extension of necrotic surface ended at day 13, this date was chosen for all further estimation of the level of fruit susceptibility to crown rot.

Geographical and seasonal variation effects on banana susceptibility to crown rot

Here, the data from experiment 3 where PIEC have been stabilized were the only ones considered. The cultivation area was found to significantly influence ($P < 0.001$) the susceptibility of fruit to crown rot (Table 1). Fruit susceptibility to crown rot was consistently higher in the two low-altitude plantations (average value of INS: 385.8 mm^2 in Dia-dia; 366.4 mm^2 in Koumba) compared with the high-altitude plantation (average value of INS: 313.4 mm^2 in Ekona). As shown in Fig. 4, the level of fruit susceptibility to crown rot in Dia-dia (Fig. 4*a*) and Koumba (Fig. 4*b*) seems to be higher during the rainy season compared with during the dry season. However, seasonal variation over the year had no significant effect ($P = 0.075$) on fruit susceptibility to crown rot (Table 1). It should also be noted (Table 1) that during the same season, there was a significant date effect on fruit susceptibility ($P < 0.001$). This date effect was especially marked in low-altitude zones (Fig. 4*a*, *b*), as confirmed by the significant interaction ($P = 0.011$) between location and date within a season (Table 1), although no significant interaction between location and season was observed ($P = 0.111$). Moreover, the susceptibility level was particularly higher for both low altitude locations for harvests in the months of July, August and September (Fig. 4*a*, *b*). A highly significant bunch effect was also observed ($P < 0.001$), as expected, from previous studies (Lassois *et al.*, 2010*a*). Hence, two statistically different groups were distinguished between fruits harvested in the low-altitude plantations of Dia-dia and Koumba and those harvested at Ekona (Table 2).

Table 1. Variance analysis of geographical and seasonal effects on banana susceptibility to crown rot.

	Internal necrosis surface (INS)		
Source	d.f.	*F*	P
Location	2	18.70	< 0.001
Season	1	3.93	0.075
Date (Season)	10	8.23	< 0.001
Location * Season	2	2.40	0.111
Location * Date (Season)	20	1.90	0.011
Bunch (Location Season Date)	425	3.97	< 0.001

d.f. is the degree of freedom, *F* is the value of F test and P is the probability.

Banana fruit and banana plant characteristics at harvest

The grade of the median fruit at harvest was consistently higher ($P < 0.001$) in the high-altitude plantation (average value of Grade: 37.4 mm in Ekona) than in the

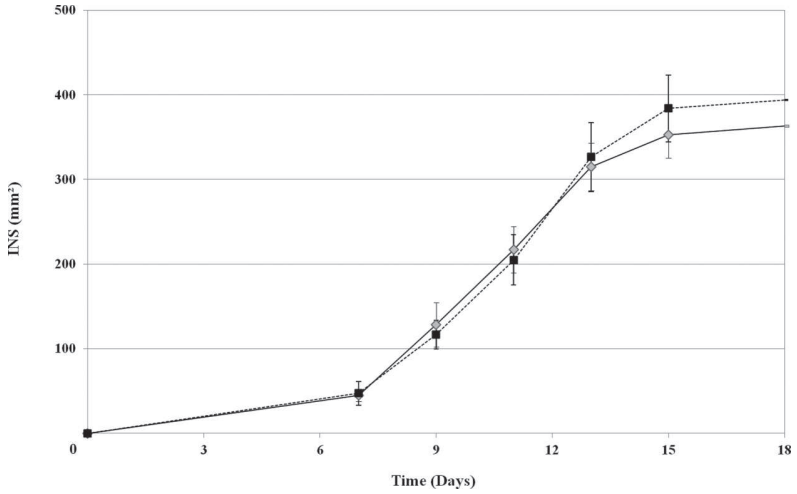

Fig. 3. Time course of the variation of internal necrotic surface (INS) in Dia-dia (——) and Koumba (– – – –). Each point represents the average mean of three replicates for each location (for each repetition INS was assessed over 15 fruits). Error bars represent standard deviation.

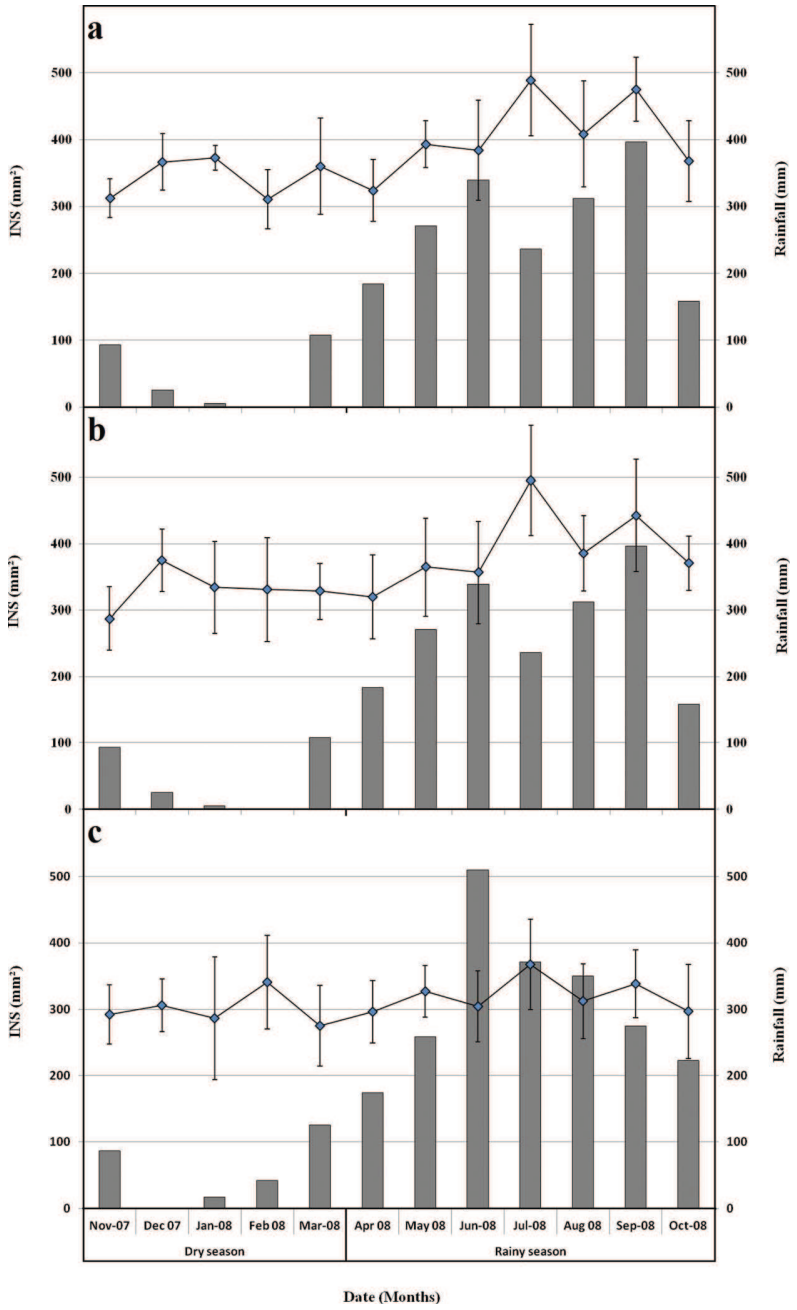

Fig. 4. Seasonal variation of the internal necrotic surface (INS — ◆—) on banana clusters inoculated with a conidial suspension of *C. musae* (10^4 conidia mL^{-1}) during one year assessment in (**a**) Dia-dia, (**b**) Koumba and (**c**) Ekona. For each date of harvest, INS is the mean of 15 replicates (bunches) and standard errors are represented by vertical bars. The shaded columns represent the average monthly rainfall.

low-altitude plantations (average value of Grade: 32.7 mm in Dia-dia; 32.5 mm in Koumba). Moreover, the number of leaves at harvest (NL) was also significantly higher ($P < 0.001$) in the high-altitude plantation (average value of NL: 9.6 in Ekona) as compared with the low-altitude plantations. Hence, two statistically different groups were distinguished between fruits harvested at Ekona and those harvested at Dia-dia and Koumba (Table 2). In addition, BLSD severity was significantly lower in Ekona than in the two other locations. Although the number of hands of the bunches harvested (NH) differed significantly amongst the three locations ($P = 0.006$), the average number of hands in Ekona did not differ from the two other locations (Table 2). As such, bananas harvested in Ekona

Table 2. Mean values and standard deviations of internal necrosis surface (INS), variation values (min – max) of INS, grade, number of hands (NH), number of leaves (NL) and black leaf streak disease (BLSD) severity measured during one year in two lowland plantations (Dia-dia and Koumba) and one highland plantation (Ekona). P is the probability.

Location	INS (mm^2)	Grade (mm)	NH	NL	BLSD severity (%)
Dia-dia (80 m–27 °C)	$385.8 \pm 91.1^{a}(311-489)$	32.7 ± 2.0^{x}	8.3 ± 1.7^{y}	8.2 ± 1.8^{x}	33.0 ± 18.2^{y}
Koumba (80 m–26 °C)	$366.4 \pm 94.0^{a}(287-495)$	32.5 ± 2.5^{x}	7.8 ± 0.7^{x}	8.3 ± 1.7^{x}	38.4 ± 18.0^{x}
Ekona (500 m–22 °C)	$313.4 \pm 73.1^{b}(275-368)$	37.4 ± 2.6^{y}	8.2 ± 1.5^{xy}	9.6 ± 2.1^{y}	27.6 ± 17.7^{z}
P	< 0.001	< 0.001	0.006	< 0.001	< 0.001

Note: INS, grade, NH, NL and BLSD severity means was calculated for 12 harvest dates. For each harvest date, 15 bunches were harvested in each location, and three clusters per bunch were inoculated for INS assessment. Data in parentheses are the minimal and the maximal average values of INS observed in the different locations at the different dates. The letters a, b for INS and the letters x, y, z for grade, NH, NL and BLSD severity represent groups of statistically different fruits defined by the Tukey test (5%).

(high-altitude plantation) had the lowest BLSDS and INS values, and the highest values of fruit grade and number of leaves as compared with bananas harvested in Dia-dia and Koumba (low-altitude plantations).

Discussion

One of the objectives of this study was to propose methods of improvements on the methodology for a more accurate assessment of banana susceptibility to crown rot by artificial inoculations. The results show that the standardization of PIEC has increased the reliability of necrotic surface assessments. Increase in necrosis under a continuous temperature of 13 °C followed a typical sigmoid curve. During the first phase of this sigmoid (0 to 7 days), the necrotic extension was discovered to be too slow to assess susceptibility variations. In the last phase (after 15 days) the extension of the crown rot was too high and the crown was almost completely rotten. It was considered that the 13 days lapse of time (end of linear progression) could permit a reliable appreciation of necrotic surface and of fruit susceptibility.

It was hypothesized that the banana cultivation area could influence fruit susceptibility to crown rot. Our data confirmed this hypothesis and provided evidence for wide variations (average INS monthly values ranged from 279 mm^2 to 495 mm^2 according to the location or the period) in the susceptibility of bananas to crown rot as previously seen in fruit susceptibility to wound anthracnose (Chillet *et al*., 2000). Bananas grown in both low-altitude plantations (Dia-dia and Koumba) were more susceptible to crown rot than those grown in the high altitude plantation (Ekona). Similarly, bananas grown in highland plantations in Guadeloupe were found to be less susceptible to wound anthracnose (Chillet *et al.,* 2006, 2007). Because of low temperatures, fruits grown in high altitudes take more time to reach 900 dd than fruits grown in low altitudes. During this long growth period, they accumulate larger amounts of assimilates that could participate in the reduction of the fruit susceptibility level. Low temperatures favour assimilate availability, and affects growth (plant size) more than carbon assimilation (Pollack, 1990; Equiza *et al*., 1997). Such effects of low temperatures on host resistance have also been reported for other pathosystems such as *Bipolaris sorokiniana*/barley, *Puccinia poae-nemoralis*/bluegrass and *Erysiphe necator*/*Vitis vinifera* through the possible triggering of biochemical pathways associated with disease resistance (Moyer *et al*., 2010). As for banana wound anthracnose (Chillet *et al*., 2007), it was hypothesized that in such low-temperature conditions, assimilates are produced in excess as indicated by the bigger grade observed in Ekona at harvest. It has been shown, in the case of leaf rust resistance in wheat, that the setting up of the defence mechanism (phenylpropanoid pathway) represents an important energetic demand, and that several metabolic pathways that contribute to increased carbon flux through the tricarboxylic cycle are triggered concomitantly to the genes involved in phenylpropanoid metabolism (Bolton *et al*., 2008). It can then be hypothesized that the excess of assimilates contributes to this important energetic demand for secondary metabolic pathways, promoting the development of defence mechanisms.

In addition, it is interesting to note that banana plants at Ekona also had the highest number of leaves, leading to a higher source-sink (So-Si) ratio which makes the fruit less susceptible to crown rot disease as compared with fruits from plantations in Dia-dia and Koumba. These observations are in accordance with previous studies which have shown that an increased So-Si ratio enabled a significant reduction of fruit susceptibility to crown rot disease (Lassois *et al*., 2010*a*).

Bananas grown in Ekona had a lower BLSD severity. This might equally account for the higher number of leaves. BLSD is a foliar disease with direct effects on yield and all indirect effects on fruit quality (Jones, 2000). It has been shown that BLSD and Sigatoka Disease (another

related *Mycosphaerella* foliar disease of bananas) strongly reduce the pre-climacteric life (called the greenlife) of bananas harvested at a constant physiological age (Abadie *et al*., 2008; Chillet *et al*., 2009), whereas other abiotic stresses do not have any influence on the greenlife of bananas harvested at this same physiological age (Jullien *et al*., 2008). Because BLSD has a strong effect on greenlife, a potential effect on the susceptibility of fruit to crown rot could also be hypothesized and should be further investigated.

Fruit susceptibility to crown rot may fluctuate in the same cultivation area. Even if seasonal effect was not significant, some variations have been found in the low-altitude plantations where fruit susceptibility was particularly higher for some harvest dates within the rainy season. It has been shown that the incidence of post-harvest diseases follows a seasonal variation, disease incidence being generally higher in the rainy season (Chillet & de Lapeyre de Bellaire, 1996; Krauss & Johanson, 2000). Our results suggest, for low-altitude locations, that fruit susceptibility to crown rot might be higher for certain short periods during the rainy season. This could partially explain the higher disease incidence during this period. Nevertheless, such disease incidence variations could also be explained by higher fruit contamination by the crown rot pathogens during this period (de Lapeyre de Bellaire *et al*., 2000). During the rainy season, an increase of the microbial load in the washing tanks and the airborne spore load was noticed, especially for some species of the pathogenic complex (unpublished data).

In conclusion, we have shown some potential influence of the cultivation area on the susceptibility of bananas to the crown rot disease as well as some variations within the same cultivation area. These findings show that the implementation of alternative control methods in the framework of an integrated management strategy (Lassois *et al*., 2010*b*) should perform better in highlands and particularly during the drier months of the year than in the rainy season. Our results also suggest a possible influence of black leaf streak disease that should be further studied.

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II. Impact of *Mycosphaerella* leaf spot diseases on the banana susceptibility to post-harvest diseases

Besides the influence of abiotic pre-harvest factors on the banana fruit susceptibility to crown rot in field conditions, biotic pre-harvest factors should be taken into consideration. Indeed, banana tree is permanently under the high pressure of *Mycosphaerella* leaf spot disease. These MLSD influence the fruit's physiology and a connection has been made between the severity of these diseases and the greenlife (GL) of bananas fruit. Moreover, it was highlighted, in the study of the spatio-temporal variation in Cameroon, that BLSD can potentially influence the banana's susceptibility to crown rot. A study must be carried out to confirm this potential effect. In the same vein, no information was available on the SD effect on the development of post-harvest diseases. Since the effects of biotic pre-harvested factors on banana's susceptibility to post-harvest diseases have not been properly studied, a complementary study on the influence of *Mycosphaerella* leaf spot diseases on banana susceptibility to two post-harvest diseases (crown rot and anthracnose) in Cameroon for BLSD and in Guadeloupe for SD was made.

The results obtained were submitted for publication to *Fruits*.

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Abstract

Introduction. The susceptibility of banana fruit to crown rot and to anthracnose, the two main post-harvest diseases, is influenced by many pre-harvest abiotic factors. *Mycosphaerella* leaf spot diseases (MLSD) of bananas are biotic pre-harvest factors caused by *Mycosphaerella fijiensis* responsible for Black Leaf Streak Disease (BLSD) and *M. musicola*, which causes Sigatoka Disease (SD). These foliar diseases may also have an influence on fruit physiology. The aim of this study was to determine the influence of these biotic pre-harvest factors on the banana fruit's susceptibility to crown rot and to anthracnose.

Materials and methods. A disease severity gradient was established in two experimental fields (Cameroon for BLSD and Guadeloupe for SD) where, at flowering stage, six different levels of MLSD severity where selected. Fruit susceptibility was determined through necrotic surface assessments after artificial inoculation by *Colletotrichum musae* on the 3rd hand of harvested bunches.

Results and discussion. BLSD significantly influenced banana's sensitivity to crown rot ($P<0.001$) but only had a slight effect on the development of anthracnose ($P=0.041$). SD had no effect ($P>0.05$) on banana's susceptibility to both post-harvest diseases.

These results were discussed with emphasis on the influence of variations in the source-sink ratio on fruit physiology. The influence of BLSD on crown rot disease suggests the need to take into account the management of these foliar diseases for an alternative control method of post-harvest diseases through integrated pest management programs.

Musa spp. / black leaf streak disease (BLSD) / Sigatoka disease (SD) / anthracnose / crown rot / fruit susceptibility

Résumé

Impact des maladies foliaires dues aux *Mycosphaerella* spp. sur la sensibilité des bananes aux maladies post-récolte.

Introduction. La sensibilité des bananes aux maladies post-récolte (pourriture de couronnes et anthracnose), est influencée par plusieurs facteurs abiotiques pré-récolte. Les maladies foliaires de bananes sont des facteurs biotiques pré-récolte causé par *Mycosphaerella fijiensis* pour la maladie des raies noires (MRN) et *M. musicola*, pour la maladie de Sigatoka (MS). Ces maladies foliaires pourraient avoir une influence sur la physiologie du fruit. L'objectif de cette étude était de déterminer l'influence de ces facteurs biotiques pré-récolte sur la sensibilité du fruit à la pourriture de couronnes et à l'anthracnose.

Matériel et méthodes. Un gradient de sévérité de la maladie a été établi dans deux parcelles expérimentales (Cameroun pour la MRN et Guadeloupe pour MS). À la floraison, six niveaux différent de sévérité des ces maladies foliaires ont été selectionés. La sensibilité du fruit a été déterminée en évaluant la surface de couronnes nécrosée après inoculation artificielle des fruits de la 3ème main du régime par *Colletotrichum musae*.

Résultats et discussion. La MRN influence significativement la sensibilité des bananes à la pourriture de couronnes ($P<0.001$), mais a seulement un léger effet sur le développement de l'anthracnose ($P=0.041$). La MS n'a aucun effet ($P>0.05$) sur la sensibilité des bananes à ces deux maladies post-récolte.

Ces résultats ont été discutés avec un accent particulier sur l'influence des variations du ratio source-puits sur la physiologie du fruit. L'influence de la MRN sur la pourriture des couronnes suggère la nécessité de la prise en compte de la gestion des maladies foliaires dans les méthodes alternatives de contrôle des maladies post-récolte au travers des programmes de gestion intégrés.

Musa spp. / maladie des raies noires (MRN) / maladie de Sigatoka (MS) / anthracnose / pourriture de couronnes / sensibilité du fruit

1. Introduction

Mycosphaerella leaf spot diseases (MLSD) are foliar diseases of bananas caused by the ascomycetous fungi *Mycosphaerella fijiensis* and *M. musicola,* respectively for the Black Leaf Streak Disease (BLSD) and Sigatoka Disease (SD). Severe infections of these foliar diseases can lead to a substantial reduction of the leaf area, but also have an indirect effect on the quality of the fruit, especially because bananas harvested from heavily infected plants cannot be exported because of early ripening [1]. On the another hand, *M. fijiensis* is an invasive and a more virulent pathogen as compared to *M. musicola*. *M. musicola* was first prevalent in all banana-growing countries and has been progressively replaced by *M. fijiensis* after it was introduced [2].

Since the beginning of the seventies, two important continental pandemies have been observed throughout the tropics: in Africa and in the Americas [2]. BLSD is still extending and affects most major production areas of banana and plantains around the world. In the Caribbean, *M. fijiensis* was first detected in 1990 in Cuba and has spread recently to Martinica in 2010 and in Guadeloupe and Dominica in 2012 [3]. Beyond the direct impact of BLSD on bananas and plantains production and its economic impact, (yield losses, strong increase of production costs), BLSD has a potential indirect incidence on post-harvest quality of bananas. This could lead to negative impacts on the export banana industry, but also on local trade of plantains that are tolerant to *M. musicola* (except under certain conditions) but susceptible to *M. fijiensis*.

One of the main characteristics of the quality of bananas is the development of post-harvest diseases, especially anthracnose and crown rot that particularly affect the quality of exported bananas. *Colletotrichum musae,* a banana specific pathogen, is responsible for anthracnose lesions [4]. Fruits are mainly contaminated in the field, after flowering, by conidia produced on the floral parts and on senescent leaves [5-6]. Conidia germinate rapidly and form melanized appressoria that remain quiescent until fruit approaches maturity. From there, they form penetration hyphae, which colonize the underlying tissues leading to anthracnose lesions, especially in the case of quiescent anthracnose [7]. If the bananas are bruised, a rot can develop when the fruit is still green, and this later leads into larger lesions [4], technically called wound anthracnose. The etiology of crown rot is more complex since a

broad unspecific and opportunistic fungal complex is involved in this disease. Within this complex, *Colletotrichum musae* is the most pathogenic species [8-10]. Rotting occurs later during shipping, storage, ripening and commercial sale [11].

It has been demonstrated that pre-harvest factors have a strong influence on the incidence and severity of these post-harvest diseases. These pre-harvest factors contribute to determine the fruit quality potential, which depends on agronomic practices and on soil-climate factors [12-13]. Particularly it has been shown that several abiotic factors influence one component of the fruit quality potential as the fruit susceptibility to post harvest diseases. Effectively the fruit susceptibility to wound anthracnose is affected by the production area and the physiological age of fruit (14-15); and the fruit susceptibility to crown rot disease is affected by the production area, the physiological age of fruit and source-sink ratio (16-18).

Until now, the potential impact of biotic factors on the susceptibility of bananas to post-harvest diseases has received limited attention. However, a recent study carried out in Cameroon has highlighted a potential effect of Black Leaf Streak Disease (BLSD) on banana susceptibility to crown rot [18]. Moreover, a relationship was shown between the severity of MLSD and the greenlife (GL) of bananas harvested at a constant physiological age [19, 3]. These results clearly showed that MLSD have a direct effect on the physiology of bananas although the mechanisms involved therein remain unknown.

Then, the main objective of this study is to understand better the potential direct impact of MLSD on fruit quality and particularly to evaluate how these diseases might influence the susceptibility of bananas to anthracnose and crown rot. This study was conducted in 2008 in two different agro-ecological locations (Cameroon and Guadeloupe) where each pathogen (BLSD and SD respectively) was exclusively present.

2. Materials and methods

2.1. Plant material

Banana fruits (*Musa acuminata* [AAA group, *Cav*endish subgroup] cv Grande Naine) were harvested from plants grown in commercial banana farms. The banana plants were selected at the flowering stage (fingers in horizontal position) and were covered with a plastic sleeve. Bunches were tied with a colored belt for them to be recognized at harvest (for each week of selection, a different color was chosen). The third hand of the bunches was harvested and used for analysis.

2.2. Prediction of harvest time

In order to forecast the harvest dates, temperatures were recorded on the different plantations. An electronic probe (Tinytag Plus, Gemini Data Loggers) with regular statements (every 15 minutes) was installed in the experimental plots and an average daily temperature was calculated from all daily data as described by Ganry and Chillet [20]. The bunches were harvested at a constant physiological age [21]; i.e. when the sum of the mean daily temperature accumulated by the fruit at the 14° C threshold, between flowering and harvest, reached 900 degree day (dd).

2.3. Experimental design

The experiment was conducted in two commercial banana farms located in two different environments where climatic conditions are favourable for the development of leaf spot diseases:

1. Cameroon: in Njombé (Mbomè), altitude 80 m, average temperature 27° C; mean annual rainfall 3000 mm. Only *M. fijiensis* was present in this plantation.

2. Guadeloupe: in Capesterre-belle eau (Montbelley), altitude 180 m, average temperature 25° C; mean annual rainfall 2800 mm. The experiment was conducted in 2008 and at that time only *M. musicola* was present here.

In each plot, a gradient of leaf spot disease was obtained through a differential chemical control of the foliar diseases, one part of the experimental design being untreated. Moreover, mechanical deleafing of diseased leaves was not done on the plots. Six groups (treatments) of banana plants were selected at different flowering dates (horizontal finger stage) according to their level of BLSD or SD severity:

- A: severity between 0 and 5 %
- B: severity between 6 and 15 %
- C: severity between 16 and 25 %
- D: severity between 26 and 35%
- E: severity between 36 and 45 %
- F: severity > 46 %.

When the gradient of leaf streak disease was obtained in both situations (June 2008), banana trees were selected according to the level of severity to MLSD at flowering stage. In Cameroon, ten banana plants were selected per week for 3 consecutive weeks for each treatment and harvested for 3 consecutive weeks as well after a flowering-to-harvest-period (FHP) of about 77 days. In Guadeloupe, since the banana plot was smaller, such design could not be used and the banana plants were selected for 2 labeling-periods (2 weeks) and banana trees were dispatched for each treatment according to their availability; and they were also harvested for 2 harvest-periods after an average FHP of 82 days (*table I*).

2.4. Assessment of leaf spot diseases

Disease severity was evaluated to characterize the intensity of necrotic surface: disease severity was observed on all selected banana plants from flowering to harvest. The severity index (SI), which is an estimation of the percentage of necrotic leaf area per plant, was then calculated according to the method described by Gauhl et al. [22]. Following this method, the disease severity index of each leaf is scored according the following scale: 0 = no necrotic lesion; 1 = less than 1% necrotic lesions; 2 = 1-5 % necrotic lesions; 3 = 6-15 % necrotic lesions; 4 = 16-33 % necrotic lesions; 5 = 34-50 % necrotic lesions; 6 = more than 50% necrotic lesions. The SI is calculated as SI = (∑scores/6xNTL)x100; where ∑scores is the sum of all infestation indexes of the banana plant and NTL is the number of leaves of the banana plant. The BLSD and the SD severities at flowering and at harvest were respectively named: $BLSDS_F$; $BLSDS_H$; SDS_F and SDS_H.

Number of functional leaves was evaluated to characterize the photosynthetic potential of the banana tree: the number of functional leaves at flowering (NFL_F) and at harvest (NFL_H). The NFL was determined, in our study, with the assumption that functional leaves should have less than 30% of necrotic surface, i.e. an infestation index of the leaf < 5.

Youngest leaf spotted was also assessed. This indicator is commonly used to characterize the efficiency of BLSD control methods [19]: the youngest leaf spotted first at flowering (YLS_F) and later at harvest (YLS_H). The youngest leaf spotted (YLS) was assessed according to the method of Stover and Dickson [23], which involves the monitoring of the youngest leaf (from the top of the plant) bearing at least 10 necrotic lesions.

Fruit diameter (Grade) was evaluated in order to characterize the effect of leaf spot diseases on fruit growth (fruit morphology and fruit filling rate). The diameter (mm) of a fruit was measured in the third hand (median position in internal row) of each bunch harvested.

The percentage of banana tree with at least one ripe fruit at harvest was also measured in both locations for each treatment.

Table I: Number of banana trees selected at flowering (NBTF) and number of banana trees harvested (NBTH) for the different replicates of the experiments conducted in Cameroun for BLSD and in Guadeloupe for SD. Bunches were harvested at a constant physiological age of 900 dd. The duration (days) of the Flowering-to-harvest-period (FHP) is also indicated for each replicate. The NBTH and the number of banana fruit inoculated for analysis are different because of early ripening of fruit before harvest.

		Black Leaf Streak Disease				Sigatoka Disease			
Treatment	Replicate	Date Flowering	NBTF	Harvest date (*FHP*)	NBTH	Date Flowering	NBTF	Harvest date (*FHP*)	NBTH
A	1	11 June2008	10	27 August 2008 (*77 days*)	10	From 11 to 30 June 2008	8	From 1 to 18 September 2008 (*80-82 days*)	7
B	1		10		10		10		8
C	1		10		10		15		13
D	1		10		8		14		9
E	1		10		9		7		7
F	1		7		5				
A	2	19 June 2008	10	3 September 2008 (*76 days*)	10	From 16 September to 3 October 2008	9	From 11 to 23 December 2008 (*81-86 days*)	4
B	2		10		10		10		10
C	2		10		10		13		10
D	2		10		10		12		5
E	2		10		10		9		8
F	2		10		10				
A	3	25 June 2008	10	10 September 2008 (*77 days*)	10				
B	3		10		10				
C	3		10		10				
D	3		10		9				
E	3		10		10				
F	3		10		10				
Total	3		177		171		107		81

2.5. Artificial inoculations for the evaluation of susceptibility to post-harvest diseases

Ripe fruits at harvest were not inoculated. Only green fruit were used for evaluation of susceptibility to both post-harvest diseases starting from the same pathogen (*C. musae*), which can induce anthracnose and crown rot development.

2.5.1. Fungal strains

Two *Colletotrichum musae* strains isolated in Cameroon and in Guadeloupe were used for artificial inoculations. In order to avoid fungal variations, all cultures were initiated 15 days before inoculation, starting from frozen cryotubes. Thereafter, the strain was transplanted in Mathur's medium ($MgSO_4.7H_2O$: 2.5g/l; KH_2PO_4: 2.7g/l; peptone: 1 g/l; yeast extract: 1g/l; saccharine: 10 g/l; agar: 15 g/l) and incubated at 25° C for 10 days. Conidia were recovered with sterile distilled water and later passed through a sterile sieve of 45 µm and suspensions calibrated with a Mallassez cell at a concentration of 10^4 and 10^6 conidia/ml for crown rot and anthracnose inoculations respectively.

2.5.2. Evaluation of susceptibility to crown rot disease

A cluster of four banana fingers was cut at the median of each harvested hand. The cuttings were square, with regular and clear-cut sections in order to obtain similar crowns between the clusters. Once latex ran out, crown tissues were dried up with absorbent paper and sterilized by dipping then in 50° alcohol. Fruits were then laid out at room temperature for 2 hours so as to allow the crowns to dry. A droplet of 50 µl of the *C. musae* suspension (10^4conidia.ml^{-1}) was then deposited at the top of the crown. A sterilized square filter paper of 1 cm^2 was placed on the droplet in order to maintain the inoculum in place. Three hours after inoculation, clusters of the same treatment were packed in perforated plastic bags and in commercial boxes. They were stored under stable conditions at 13° C for 13 days [18]. At the

end of this storage period, an evaluation of the internal progression of the rot within the crown was done. The clusters were divided into two different parts and the transverse cutting of the crown allowed a visualization of spread of the rot on the crown. The "Internal Necrosis Surface" (INS) expressed in mm^2 was calculated by assuming a rectangular shape to the internal necrosis. Its value was taken as a measure of fruit susceptibility to crown rot.

2.5.3. Evaluation of susceptibility to wound anthracnose

A median internal finger without defect was selected from each harvested hand. On each finger, two zones were located using a fine marker. A droplet of 25 µl of the inoculum suspension (10^6 conidia.ml^{-1}) was deposited at the center of these zones. Fruits were laid at room temperature to allow the droplets to completely dry up. Once dried, a sterile humidified cotton swab was deposited on each inoculation point and wrapped with a plastic film. Bananas of the same treatment were packed thereafter in perforated plastic bags placed in commercial boxes. The boxes were stored for 2 days at 25° C to allow the development of appressoria [24]. Then, the inoculated areas were bruised by applying a standardized compression. In Guadeloupe, standardized compressions were carried out using a penetrometer with a rounded probe of 1cm of diameter, which exerted during 4 sec a deformation of 5mm with a speed of 5 $mm.sec^{-1}$. The probe was controlled by an analyzer of texture TA-XT2 coupled with software X-TRAD. In Cameroon, this equipment was not available so wounds were inflicted manually with a rounded probe of 1 cm. Bananas were then packed and stored at 13° C for 10 days in order to simulate industrial export conditions as described by de Lapeyre de Bellaire et al. [24]. At the end of this storage period, fruits were laid at 20° C and the length (L) and width (w) of the necrotic surface were measured at various dates. Evaluations were made 12, 14, 16 and 18 days after harvest (dah) in Cameroon and 20 dah in Guadeloupe. The Necrotic surface (NS, mm^2) was then calculated by the ellipse area formula (Length × width × Π/4), and its value was taken as a measure of fruit susceptibility to wound anthracnose at these different dates.

2.6. Statistical Analysis

For both locations, the effect of the different treatments on leaf spots severity (BLSDS, SDS); on the number of functional leaves (NFL) and on the youngest leaf spotted (YLS) at flowering and at harvest; on the grade of fruit and on the INS and NS were analyzed by analysis of variance (ANOVA). The average values of $BLSDS_F$, $BLSDS_H$, SDS_F SDS_H, NFL_F, NFL_H, YLS_F, YLS_H, grade, INS, and NS (at different date of evaluation calculated for the both wounds) were subjected to a fixed two-way ANOVA performed with Minitab software v.15.1, with Treatment and Date as factors. Separations of means were based on Tukey's multiple range tests at a 5% probability level. $BLSDS_F$, $BLSDS_H$, SDS_F SDS_H, NFL_F, NFL_H, YLS_F, YLS_H, grade, INS; NS at different dates of evaluation were correlated via linear regression using Pearson correlations calculations and significance tests. The analysis was conducted on Minitab software v.15.1.

3. Results

3.1. Pre-harvest effects of leaf spot diseases on the banana plants and fruits

A higher degree of disease severity at flowering as measuring by Severity Index (SI) (treatment F, SI > 45 %) could not be achieved in Guadeloupe for SD (*table I*). During the flowering-to-harvest-period (FHP), different effects of leaf spot diseases were observed on banana plants and on fruit morphology. In both locations, banana trees selected for the various groups (treatments) showed different significant disease levels as shown for the SI, the NFL and the YLS (*tables II*, *III*). The gradient of SI selected at flowering has been maintained at harvest. Nevertheless, at harvest stage, the differences between all the treatments for SI, NFL and YLS were not the same as in the flowering stage (*tables II, III*).

During the FHP, leaf spot diseases led to an important reduction of the leaf area in the different treatments. In all the cases, the SI increased dramatically especially for BLSD in Cameroon (*table II*). The evolution of the leaf spot disease was also confirmed by an important decrease of the position of the YLS and by an important decrease of the NFL (*tables II, III*). As such (especially for BLSD in Cameroon), banana trees selected at flowering with high SI (treatments E and F) showed complete necrosis of leaves leading to the absence of functional leaves on the banana plant at harvest (*table II, figure 1*).

Morphological differences were observed between the fruits of the different groups (treatments) that were harvested at a constant physiological age of 900 dd in Cameroon (*table II, figure 2*). Fruits with lower disease severity at flowering (treatments A, B and C) had bigger grade than those with high disease severity (treatments D, E and F) (*table II*). Morphological differences between fruits of the different groups (*table III*) were not observed in Guadeloupe.

In some cases, bunches harvested on heavily infested banana trees were fully ripe at harvest in both locations (*figure 3, table IV*). Fruits of treatments A and B were not ripe at harvest as compared to fruits of other groups (treatments C, D, E, and F). The highest percentage of ripe fruits was found in treatments D and E. In addition, the percentage of ripe

fruits increased with the level of leaf spot disease at flowering but for the higher disease level of BLSD in Cameroon (treatment F, severity at harvest > 46%). The percentage of *M. fijiensis* in ripe fruits at harvest was less important as compared to *M. musicola* (*table IV*).

3.2. Effects of leaf spot diseases on banana susceptibility to crown rot disease

In Cameroon, BLSD influenced the banana fruit susceptibility to crown rot disease. The INS continuously increased from treatment A to F, and the treatments had a significant effect on fruit susceptibility to crown rot (P<0,001). As such, higher levels of BLSDS corresponded to higher levels of fruits susceptibility to crown rot (INS) disease. Three statistically different groups were distinguished amongst the different treatments (*table II*).

SD in Guadeloupe did not influence (P>0,05) fruit susceptibility to crown rot (*table III*). Because of the high amount of ripe banana fruits at harvest for treatment E (SI between 36 and 45%), this group was not taken into consideration for inoculations assessments.

3.3. Effects of leaf spot diseases on banana susceptibility to wound anthracnose

BLSD in Cameroon influenced banana fruit's susceptibility to wound anthracnose at the earliest dates of observation (12 dah). Among the various groups, only treatment F had a significant effect on NS (P=0,001) on the first date of evaluation (12 dah). Two statistically different groups were distinguished (*table II*). Higher levels of BLSDS (treatment F) corresponded to a higher level of susceptibility to wound anthracnose unlike with other groups (treatments A, B, C, D, E). However, for later dates of evaluation (14 dah, 16 dah and 18 dah), treatment F had more important values of NS but the differences between the groups were not significant (P>0,05) (*table II*).

SD in Guadeloupe did not influence (P>0,05) fruit susceptibility to wound anthracnose (NS, 20 dah) the only date of obsevation (*table III*).

Table II: BLSD severity in % at flowering ($BLSDS_F$) and at harvest ($BLSDS_H$), number of functional leaves at flowering (NFL_F) and at harvest (NFL_H), youngest leaf spotted at flowering (YLS_F) and at harvest (YLS_H), fruit grade (mm), crown rot (INS in mm²), anthracnose (NS in mm²) mean values and standard deviations during the six months assessment in Cameroon. *P* is the probability obtained with ANOVA.

	Banana tree						Banana fruit					
Treatment	$BLSDS_F$	$BLSDS_H$	NFL_F	NFL_H	YLS_F	YLS_H	Grade	INS	NS 12 dah	NS 14 dah	NS 16 dah	NS 18 dah
A	3,1 ± 1,6^{a}	61,8 ± 16,4^{a}	12,6 ± 1,8^{a}	5,2 ± 2,2^{a}	8,2 ± 3,1^{a}	1,8 ± 1,0^{a}	37,5 ± 0,1^{a}	274,8 ± 52,3^{a}	69,1 ± 50,0^{ab}	229,8 ± 125,2^{a}	359,5 ± 176,7^{a}	518,4 ± 230,0^{a}
B	11,5 ± 3,0^{b}	71,2 ± 14,6^{b}	12,8 ± 1,6^{a}	4,4 ± 2,1^{a}	6,5 ± 1,2^{b}	1,2 ± 0,5^{b}	37,6 ± 0,1^{a}	256,9 ± 53,2^{a}	61,6 ± 41,3^{a}	182,6 ± 118,4^{a}	285,3 ± 170,1^{a}	458,1 ± 241,7^{a}
C	21,0 ± 2,7^{c}	89,4 ± 11,8^{c}	13,3 ± 1,6^{a}	1,6 ± 2,0^{b}	5,8 ± 1,2^{b}	1,0 ± 0,0^{b}	36,2 ± 0,2^{a}	303,2 ± 63,3^{ab}	81,9 ± 64,6^{ab}	222,5 ± 127,7^{a}	330,5 ± 163,9^{a}	516,1 ± 209,8^{a}
D	31,6 ± 3,2^{d}	94,4 ± 11,8^{c}	11,6 ± 1,6^{ab}	0,7 ± 1,6^{bc}	5,5 ± 0,8^{b}	1,0 ± 0,0^{b}	35,5 ± 0,2^{ab}	360,4 ± 53,2^{bc}	68,8 ± 59,6^{ab}	245,6 ± 173,4^{a}	370,3 ± 222,9^{a}	578,9 ± 289,9^{a}
E	40,1 ± 3,6^{e}	99,9 ± 0,6^{d}	8,8 ± 3,0^{b}	0,0 ± 0,0^{c}	4,3 ± 1,0^{bc}	1,0 ± 0,0^{b}	34,1 ± 0,2^{b}	349,5 ± 76,5^{b}	78,2 ± 55,1^{ab}	217,6 ± 149,0^{a}	329,2 ± 187,5^{a}	515,6 ± 230,0^{a}
F	52,1 ± 5,6^{f}	100,0 ± 0,0^{d}	7,4 ± 1,3^{b}	0,0 ± 0,0^{c}	3,8 ± 0,6^{c}	1,0 ± 0,0^{b}	34,9 ± 0,2^{ab}	393,7 ± 60,2^{c}	105,6 ± 80,0^{b}	254,6 ± 142,0^{a}	368,5 ± 165,9^{a}	592,0 ± 259,5^{a}
P	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.041*	*0.248*	*0.301*	*0.178*

Means are the result of 10 replicates represented with standard deviation in the table.
For each column, different letters represent statistically different groups defined by the Tukey test (5%).

Table III: SD severity in % at flowering (SDS_F) and at harvest (SDS_H), number of functional leaves at flowering (NFL_F) and at harvest (NFL_H), youngest leaf spotted at flowering (YLS_F) and at harvest (YLS_H), and anthracnose (NS in mm²) mean values and standard deviations during the assessment period in Guadeloupe. *P* is the probability obtained with ANOVA.

	Banana tree						Banana fruit		
Treatment	SDS_F	SDS_H	NFL_F	NFL_H	YLS_F	YLS_H	Grade	INS	NS 20 dah
A	3,8 ± 1,9^{a}	33,5 ± 18,6^{a}	10,7 ± 1,4^{a}	8,0 ± 2,6^{a}	8,8 ± 2,2^{a}	1,5 ± 0,9^{a}	35,0 ± 2,8^{a}	92,7 ± 25,0^{a}	434,8 ± 221,6^{a}
B	10,2 ± 2,1^{b}	47,1 ± 28,5^{a}	10,7 ± 1,9^{a}	5,7 ± 3,3^{a}	7,1 ± 1,6^{b}	1,1 ± 0,3^{ab}	35,3 ± 1,1^{a}	63,5 ± 34,1^{a}	295,2 ± 173,1^{a}
C	20,0 ± 3,2^{c}	73,1 ± 20,1^{b}	10,3 ± 2,4^{a}	3,3 ± 3,1^{b}	5,1 ± 1,3^{c}	1,2 ± 0,4^{ab}	34,9 ± 2,1^{a}	81,0 ± 35,4^{a}	441,4 ± 273,1^{a}
D	30,3 ± 2,3^{d}	94,3 ± 7,2^{c}	9,4 ± 1,3^{ab}	0,2 ± 0,6^{c}	4,7 ± 0,8^{c}	1,0 ± 0,0^{b}	34,8 ± 2,0^{a}	100,8 ± 46,2^{a}	429,0 ± 207,7^{a}
E	39,5 ± 5,9^{e}	96,3 ± 9.9^{c}	8,5 ± 1.6^{b}	0,3 ± 1.1^{c}	4,1 ± 0,6^{c}	1,0 ± 0,0^{b}	33,6 ± 1,5^{a}	-	-
P	*0,000*	*0,000*	*0,001*	*0,000*	*0,000*	*0,032*	*0,575*	*0,133*	*0,139*

Means are the result of 10 replicates represented with standard deviation in the table.
The letters a, b, c, d, e, f represents groups of statistically different fruits defined by the Tukey test (5%).

Figure 1: Effects of BLSD on the foliage of banana plants lightly infested (a. treatment A, SI at flowering < 5%) and banana plants severely infested (b. treatment F, SI at flowering > 46%).

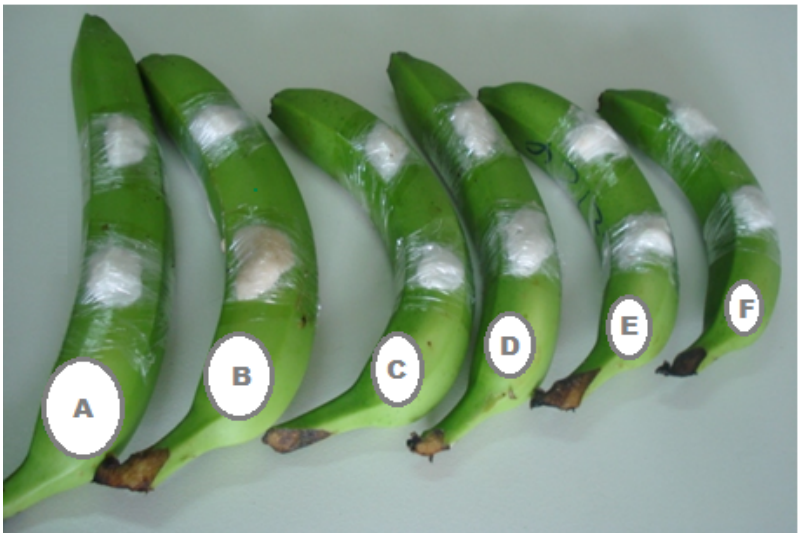

Figure 2: Effects of different levels of BLSD severity (treatment A-F) on the morphology of bananas harvested at the same physiological age (900dd). White parts on banana fruit represent a sterile humidified cotton swab deposited on each inoculation point (for anthracnose) and wrapped with a plastic film.

Figure 3: Effects of BLSD on early ripening of fruit before harvest: in case of light infection fruits are not ripe at harvest (a. treatment A, SI at flowering < 5%); in other cases with severe infection, fruits are ripe at harvest stage (b. treatment E, SI at flowering > 46%).

Table IV: Percentage of ripe fruits at harvest (%) in Cameroun (Black Leaf Streak Disease) and Guadeloupe (Sigatoka Disease).

Treatment	Cameroon	Guadeloupe
A	0	0
B	0	0
C	12	23
D	15	38
E	29	71
F	5	-

3.4. Relationships between leaf spot diseases and banana susceptibility to post-harvest diseases

For SD and particularly for BLSD, disease parameters were well correlated to one another and better correlations were encountered for disease parameters observed at the same stage, i.e. at flowering or at harvest (*tables V, VI*). The NFL and the YLS were inversely proportional to disease severity (*tables V, VI*). In addition, an important correlation (P<0,001) was found between Leaf Spot Disease Severity (SDS, BLSDS) at flowering and at harvest (*tables V, VI*). A significant negative correlation (P<0,001) was found between the grade of fruits and the $BLSDS_F$ and $BLSDS_H$ (*table V*) but such relation was not found for SD (*table VI*).

$BLSDS_F$ and $BLSDS_H$ were therefore positively correlated with the development of crown rot and of anthracnose on banana fruits. The relationship between INS and BLSDS (at flowering and at harvest) was significant (P<0.001) and linear (*table V*). Thus, high levels of $BLSDS_F$ and $BLSDS_H$ were correlated with high levels of INS. NS was slightly correlated (P<0.001) to high levels of BLSDS. However, this effect is true only for the date of evaluation corresponding to 12 dah. No correlation (P>0.05) was found between the BLSDS and NS 14 dah, 16 dah and 18 dah (*table V*).

SDS_F and SDS_H were not correlated (P>0.05) with crown rot and wound anthracnose (NS) 20 dah (*table VI*).

Table V: Analysis of correlation between BLSD severity and some characteristics of banana tree (number of functional leaves, youngest leaf spotted) and fruit (fruit grade, crown rot (INS) and anthracnose (NS) 12, 14, 16, 18 dah) at flowering and harvest.

		BLSDS at flowering		BLSDS at harvest	
Source	d.f.	*r*-value	*P*-value	*r*-value	*P*-value
BLSDS at harvest	169	0,74	<0,001	-	-
NFL at flowering	169	-0,67	<0,001	-0,50	<0,001
NFL at harvest	169	-0,73	<0,001	-0,94	<0,001
YLS at flowering	169	-0,64	<0,001	-0,53	<0,001
YLS at harvest	169	-0,40	<0,001	-0,54	<0,001
Fruit grade at harvest	169	-0,54	<0,001	-0,44	<0,001
Crown rot (INS)	169	0,58	<0,001	0,49	<0,001
Anthracnose (NS 12 dah)	169	0,22	0,006	0,18	0,029
Anthracnose (NS 14 dah)	169	0,09	0,258	0,17	0,026
Anthracnose (NS 16 dah)	169	0,06	0,446	0,17	0,027
Anthracnose (NS 18 dah)	169	0,13	0,088	0,21	0,006

d.f. is the degree of freedom, *r* is the Pearson correlation value and *P* its probability.

Table VI: Analysis of correlation between SD severity and some characteristics of banana tree (number of functional leaves, youngest leaf spotted) and fruit (fruit grade, crown rot (INS) and anthracnose (NS) 20 dah) at flowering and harvest.

		SDS at flowering		SDS at harvest	
Source	d.f.	*r*-value	*P*-value	*r*-value	*P*-value
SDS at harvest	79	0,75	<0,001	-	-
NFL at flowering	79	-0,44	<0,001	-0,47	<0,001
NFL at harvest	79	-0,72	<0,001	-0,90	<0,001
YLS at flowering	79	-0,70	<0,001	-0,62	<0,001
YLS at harvest	79	-0,26	0,019	-0,37	0,001
Fruit grade at harvest	79	-0,23	0,048	-0,16	0,160
Crown rot (INS)	64	0,16	0,264	0,03	0,814
Anthracnose (NS 20 dah)	64	0,15	0,244	0,17	0,175

d.f. is the degree of freedom, *r* is the Pearson correlation value and *P* its probability.

4. Discussion

The experimental designs put in place in Cameroon for BLSD and in Guadeloupe for SD generated disease gradients at flowering which were almost maintained until harvest, although the structure of these gradients were different at harvest stage. The severity levels reached at harvest for treatments A and B (disease severity < 15% at flowering) were higher for BLSD in Cameroon than for SD in Guadeloupe, because of differences in their kinetics.

Leaf Spot Diseases had the expected impacts on the banana trees through an important reduction of the leaf surface. An important reduction of the number of functional leaves at harvest was observed for highly infected plants, especially for treatments D, E and F (severity at flowering > 26%) that had less than one functional leaf at harvest. This foliar reduction has been widely reported in previous studies [25, 2, 19]. As expected, BLSD's impact on leaf surface was more important than SD's impact. Indeed, *M. fijiensis* is a more aggressive pathogen than *M. musicola* [26, 27].

Since the reduction in the photosynthetic area generates stresses during fruit filling, an impact on fruit morphology was also expected [28, 11]. Then, morphological differences in the diameter of fruit's were observed as a result of heavy BLSD infestation between fruits of the different treatments harvested at a same physiological age. However, heavy SD infestation did not lead to a reduction in the diameter of fruit harvested at a constant physiological age as previously reported [20]. This was probably because the level of stress induced by SD on fruit filling was lower than for BLSD, which induced faster defoliations.

MLSD's effects have been previously reported to lead to a reduction in yield, as well as in premature ripening of fruits in the field [29, 25, 2, 19]. As expected, in both locations, bananas harvested from severely infected plants (treatments C, D, E and F) were at times already ripe at harvest.

Our results confirmed leaf streak disease's direct effects on fruit physiology [19]. If BLSD's effect on fruit ripening has been widely documented, this is the first report of BLSD's effect on banana susceptibility to crown rot disease. This result confirms previous observations suggesting that some biotic factors had a potential effect on banana's susceptibility to crown rot [18].

The presence of biotic stresses during fruit filling induced a reduction in sources (NFL) of the banana tree but not in sinks (fruits), leading to a reduction of the source-sink (So-Si) ratio. We can then hypothesize that such a decrease could account for a reduced susceptibility to crown rot as previously shown [11]. Since the reduction of the source was more important for BLSD than for SD, this could account for the lack of increase in fruit's susceptibility to crown rot which was observed with increasing SD severity. Also noteworthy is the fact that the values of Internal Necrotic Surfaces measured in Guadeloupe were very low as compared to earlier studies [7], which could also explain the absence of SD's effect on the susceptibility of the banana to crown rot.

In the case of banana anthracnose, there was a slight effect of BLSD on bananas harvested from heavily infested plants (P=0.041) just for the first date of evaluation (12 dah). This effect was not found in the following dates of evaluation (14 dah, 16 dah, 18 dah). So, we can hypothesize that SD had no effect on fruit susceptibility to wound anthracnose, probably because the only evaluation date (20 dah) was rather too late. The lesser effect of BLSD on wound anthracnose could be explained by the fact that the modification of So-Si ratio had less effect on fruit's susceptibility to banana anthracnose as compared to crown rot. Effectively, in previous studies, the changes in So-Si ratio were found to influence the banana fruit susceptibility to crown rot [11], but not to wound anthracnose [13].

Stresses generated by the effects of BLSD seem to trigger physiological changes on the banana fruit resulting from a disequilibrium between the availability of assimilates during the fruit growth. This disequilibrium could lead to a reduction of the formation of secondary metabolites like phenolic compounds. Phenolic compounds have effectively been shown to be involved in the defense mechanisms of different banana tree tissues [30, 31, 32, 33, 34]. Moreover, products arising from the decomposition of banana dopamine seem to have a fungitoxic activity against *C. musae* [35, 36]. Since several phenolics are involved in the banana tree's defense mechanisms, they might also be involved in fluctuations observed in banana's susceptibility to the crown rot disease.

In summary, the aim of this study was to evaluate the MLSD influence on the susceptibility of bananas to post-harvest diseases. Our results have highlighted a new effect of the BLSD on the bananas fruit's quality. However, the physiological mechanism involved in the relationship between BLSD severity and post-harvest diseases are still unknown. The

management of BLSD should also be considered in the elaboration of alternative control methods of post-harvest diseases mainly through an integrated strategy.

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Chapter IV. Analysis of phenolics role in susceptibility variation mechanisms

I. Severe modifications in source-sink ratio influence the susceptibility of bananas to crown rot and its phenolic content

The influence of abiotic and biotic pre-harvest factors on banana's susceptibility to post-harvest diseases, mainly crown rot, has been shown. However, the biochemical mechanism underlying the fruit susceptibility variations is not yet understood. Since the accumulation of phenolics in banana tissues have previously been correlated to banana's resistance to biotic stress, it was necessary to study the influence of the crown phenolic content on fruit's susceptibility to crown rot. Prior to the characterization of the phenolic content of banana crown in relation to the susceptibility variation, it was important to have samples with different levels of susceptibility. A previous study had shown that source-sink ratio modification at flowering induced high difference in the level of fruit susceptibility. Thus, a three weeks study was set up in the field where a severe modification in source-sink ratio at flowering was observed. This was done in order to obtain banana crowns of differential susceptibility to crown rot and to have sufficient amount for biochemical analysis. GC-MS method was applied on samples collected during the 3th week for catecholamines analysis. The amount of banana crown powder for this harvest week was however not enough for all analyses, especially for the 13dpi. That was in fact the key problem during this sampling, since we were collecting only the healthy parts of the crown (parts without necrosis). Therefore, HPLC and LC-MS was applied on samples collected during the 2th week for the analysis of polyphenols.

The results obtained are in a manuscript and will soon be submitted for publication under *Plant Pathology*.

Ewané C.A., Nott K., Ngoh Newilah G., Paquot M., Wathelet J.P., Lepoivre P., de Lapeyre de Bellaire L. *The severe source-sink ratio change affects the susceptibility of banana to crown rot and its phenolics content*.

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Abstract

Banana susceptibility to crown rot is influenced by many biotic and abiotic pre-harvest factors which includes the source-sink (So-Si) ratio. The banana plant's resistance to biotic stress has been previously correlated to its phenolic content. It is hypothesized that there is a potential influence of the crown's phenolic content on fruit's susceptibility. The aim of this work was to investigate on the influence of severe So-Si ratio modifications, via the removal of leaves and hands, and the involvement of phenolics in the fruit's susceptibility to crown rot. Fruit susceptibility was evaluated 13 days post-inoculation (13dpi) with *Colletotrichum musae*. Banana crowns obtained on the day of harvest before inoculation (dhbi) and the 13dpi stages were analyzed for changes in phenolic compounds using GC-MS, HPLC and LC-MS devices. The severe So-Si ratio modifications had a significant effect ($P< 0,001$) on fruit's susceptibility, fruits of low So-Si ratio being most susceptible. The severe So-Si ratio modifications also significantly influenced ($P< 0,001$) some tree and some fruit characteristics. The less susceptible (S^-) crowns had higher amount of phenolics compared to the more susceptible (S^+) ones. Dopamine was identified as the major phenolic in banana crown. Norepinephrine and normetanephrine levels were higher in crowns the dhbi, especially the S^+ ones. Hydroxycinnamic acids, notably ferulic acid and its derivatives were accumulated at significant level ($P< 0,001$) in the S- crowns as compared to the S^+ ones the dhbi, but decreased in 13dpi. Phenolics have a possible role in the biochemical defense of the banana crown and could be used by the producers as chemical criterion for estimation of the level of bananas susceptibility to crown rot.

Keywords: *Musa* spp., source-sink ratio modifications, crown rot, fruit susceptibility, phenolics, chromatographic methods (GC-MS, HPLC, LC-MS).

1. Introduction

Crown rot is a disease of the tissue uniting the peduncles. Caused by a broad, unspecific parasitic complex, it is the most important post-harvest disease affecting the quality of exported bananas in Cameroon. Within this complex, *Colletotrichum musae* (Berk. & Curt.) Arx is the most commonly described pathogenic species (Finlay & Brown, 1993; Krauss & Johanson, 2000; Lassois *et al*., 2010a). The disease develops during shipping, ripening, commercialization, storage and has a negative impact on the market value of the bananas (Slabaugh & Grove, 1982). Crown rot control relies mainly on the application of post-harvest fungicides (de Lapeyre de Bellaire & Nolin, 1994). Nevertheless, seasonal and spatial variations in the performance of these practices have been observed. It has been suggested that spatiotemporal fluctuations may represent variations in the quality potential of the fruit which is influenced by pre-harvest factors such as agronomic practices and agro-ecological conditions (Chillet & de Lapeyre de Bellaire, 1996; Lassois *et al*, 2010a).

The fruit's susceptibility to the crown rot disease is an important component in determining its quality potential (Lassois *et al.*, 2010a). The So-Si ratio modifications were found to significantly influence (P= 0,004) the banana fruit's susceptibility to crown rot (Lassois *et al*. 2010b). Moreover, the effects on the metabolic pathway potentially involved in So-Si ratio modifications on fruit susceptibility have been reported (Lassois *et al.*, 2011). This study suggested phenolics, notably dopamine, could be involved in the quantitative response of bananas to crown rot.

Dopamine and products which derive from its oxidation are the main phenols in bananas and these phenols have a fungitoxic activity against *Colletotrichum musae* (Muirhead *et al.*, 1984), and antioxidant properties (Kanazawa & Sakakibara, 2000; Someya *et al*., 2002). Besides the high concentration of phenols, phenylalanine ammonia-lyase, oxidative enzymes like peroxidase, polyphenol oxidase (PPO), were reported in many resistant banana tissues such as in the interaction between *Musa* spp. and *Fusarium oxysporum* F sp. Cubense (Panama disease) (Kavino *et al.*, 2009). Amongst the many diversities of secondary metabolites involved in the banana's resistance to different pathogens, preformed and induced phenolics have been widely reported in the literature as potential participants in the banana tree defense mechanisms (Ewané *et al.*, 2012).

Phenolics were shown to be involved in preformed defense mechanism of the banana tree against Black Leaf Streak Disease through certain cells of the mesophyll specialized in their important storage in partially resistant cultivar tissues as compared to the susceptible ones (Beveraggi, 1995; El Hadrami, 1997). Phenolics were also involved in induced defense mechanisms; for instance, after a wound or an inoculation by *C. musae*, several phytoalexins (phenylphenalenones, irenolone and emenolone types) were induced in the tissues of the green bananas (Luis *et al.*, 1993; Kamo *et al.*, 1998; Kamo *et al.*, 2001). Higher contents of several phenolics have been observed in the root's tissues of the banana cultivars resistant to phytophagous nematodes and the role of these phenolics in defense mechanisms has been reported (Valette *et al*., 1998; Collingborn *et al.*, 2000; de Ascensao & Dubery, 2003; Wuyts *et al.*, 2007). However, the mechanisms involved in the fruit's susceptibility to crown rot remain unexplained and such variations have not yet been linked to the phenolic content of the tissues of the banana crown.

The objective of this work was to characterize the effect of the severe modifications of the So-Si ratio and the subsequent role of phenolics on fruit's susceptibility variation to crown rot disease. In order to obtain banana crowns with differential levels of susceptibility to crown rot, the effect of the severe modifications of So-Si ratio on some banana trees and fruits characteristics at harvest, and on fruit's susceptibility to crown rot 13 days post-inoculation (13dpi) were investigated. In previous studies, the modifications of the So-Si ratio (moderate modifications) was an important pre-harvest factor which influenced the morphology of the banana tree and fruit (Jullien *et al.*, 2001; Chillet *et al.*, 2006); the rate of fruit filling (Jullien *et al.*, 2001), and also the fruit's susceptibility to crown rot i.e. the fruit's quality potential (Lassois *et al.*, 2010b). To evaluate the involvement of phenolics in variations of banana susceptibility to crown rot disease, the phenolic content of both the susceptible (S^+) and the less susceptible (S^-) banana crowns at two stages (dhbi and 13dpi) were characterized.

2. Materials and methods

2.1. Plant material

Banana fruits (*Musa acuminata* [AAA group, Cavendish subgroup] cv Grande Naine) were harvested from banana trees which had undergone severe So-Si ratio modifications at the flowering stage (fingers on horizontal position) by removing sources (leaves, L), and sinks (hands of the bunch, H) according to the following modifications :

- 12L/1H: 12 leaves and 1 hand remaining, source-sink ratio= 8;
- 12L/2H: 12 leaves and 2 hands remaining, source-sink ratio= 4;
- 12L/8H: 12 leaves and 8 hands remaining, source-sink ratio= 1 (reference);
- 2L/8H: 2 leaves and 8 hands remaining, source-sink ratio= 0.17;
- 1L/8H: 1 leaf and 8 hands remaining, source-sink ratio= 0.08.

The values of So-Si ratio were calculated as described by Lassois *et al.*, (2010b). The last leaves to have emerged on the banana tree and the first, second and eighth hands to have appeared on the bunch were those remaining at the flowering stage.

Each bunch selected at flowering was covered with a plastic sleeve and tied with a colored belt in order to be recognized at harvest. Bunches were harvested at a constant physiological age (Jullien *et al*., 2008), i.e. when the sum of the daily mean temperature accumulated by the fruit at the 14°C threshold, between flowering and harvest, reached 900 degrees day (dd). In order to predict the time of harvest, temperatures were recorded on the experimental plot via an electronic probe (Tinytag Plus, Gemini Data Loggers) with regular data capture (every 15 minutes) and the calculation of an average daily temperature from all the daily data.

2.2. Experimental design

This experiment was conducted within five months in a commercial banana farm belonging to the PHP (Plantations du Haut Penja) banana company, specifically on a first cycle plot located at Bouba, about 100 m altitude beside Njombé in the Littoral Region of Cameroon. The average temperature of the locality is 27°C and the mean annual rainfall is 2500 mm.

Between June and July 2009, five banana trees were selected, per week, for three successive weeks for each modality. For each harvested banana tree, two clusters consisting of four banana fruits were cut off from the banana hand and used at two different stages. The first cluster was used as control on the day of harvest before inoculation (dhbi): the banana crown was immediately frozen in liquid nitrogen and freeze-dried after removal of the green parts for further biochemical analyses. The second cluster was used for *C. musae* inoculation and the evaluation of the fruit's susceptibility to crown rot was carried out 13 days after inoculation (13dpi). Crowns were also frozen in liquid nitrogen, and freeze-dried after removal of the green and necrosed parts (Fig. 1).

In addition to assessing fruit's susceptibility, at harvest (900 dd) some fruit and tree characteristics were measured. The grade and length of the same median fruit harvested on the second hand as well as the number of leaves at harvest (NLH) for each tree

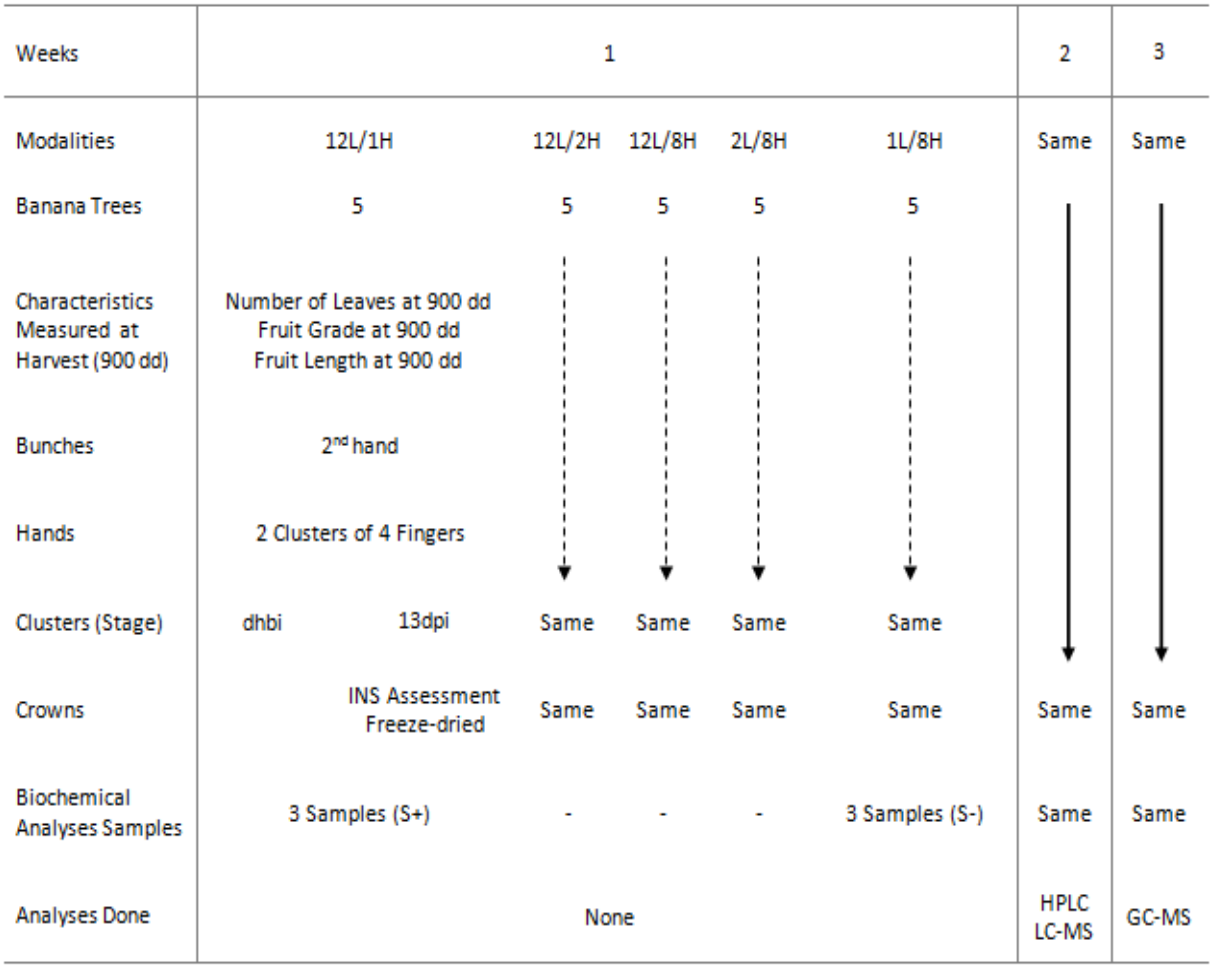


Figure 1: Experimental design of the So-Si ratio effect on the susceptibility of the banana crown.

2.3. Evaluation of susceptibility to crown rot disease

The susceptibility of bananas to crown rot was evaluated through the artificial inoculation of banana clusters with *Colletotrichum musae* isolate Co-CMR-65. Bunches were harvested the day of experimentation and their second hands were transported to the laboratory. The median part of the hand was cut into two clusters of four banana fingers. The cuttings were squared, with regular and clear-cut sections in order to obtain similar crowns between the clusters. Once latex ran out, crown tissues were dried with absorbent paper and sterilized by dipping them into 50° alcohol. Fruits were then laid out at room temperature for 2 hrs so as to allow the crowns to dry. A droplet of 50 µl of the *C. musae* suspension (10^4 conidia.ml^{-1}) was then deposited at the top of the crown. A sterilized square filter paper of 1

cm^2 was placed on the droplet in order to maintain the inoculum in place. Three hours after inoculation, the clusters were packed in perforated plastic bags in commercial boxes and stored under stable conditions at 13°C for 13 days (Ewané *et al*., 2012b). At the end of this storage period, an evaluation of the internal progression of the rot within the crown was carried out. The clusters were divided into two parts and the transverse cutting of the crown allowed a visualization of the surface of rot spread into the crown. The "Internal Necrosis Surface" (INS) expressed in mm² was calculated by assuming a rectangular shape to the internal necrosis (Ewané *et al*., 2012b). Its value was taken as a measure of fruit's susceptibility to crown rot.

2.4. Phenolic constituents of banana crown

2.4.1. Sample preparation

Two extreme crowns treatments: S^+ (1L/8H) and S^- (12L/1H) were selected as samples for the analyses of the phenolic constituents: the reason being their difference at the levels of susceptibility to crown rot. Phenols were analysed by gas chromatography coupled with mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) and liquid chromatography coupled with mass spectrometry (LC-MS). The crown treatments were collected at two stages: the day of harvest before inoculation (S^+/dhbi and S^-/dhbi) and 13 days post-inoculation of the fruit (S^+/13dpi and S^-/13dpi). To minimize the influence of environmental variation on the results of the analyses, the crowns studied were collected the same harvest week. However, for these analyses, the limiting factor was the amount of plant material available especially for infected crowns (13dpi). Indeed, the removal of green and necrosed parts of the crown was carried out before freezing the crowns in liquid nitrogen and lyophilization. Therefore, conditions of extraction of phenolics were optimized for small samples of freeze-dried crown powder of about 100 mg. For GC-MS analysis, three S^+ and three S^- banana crowns harvested on week N° 3 were selected for both stages (dhbi/13dpi). For HPLC and LC-MS analyses, three S^+ and three S^- crowns harvested on week N° 2 were selected for the two stages (dhbi/13dpi).

Prior to these analyses, the freeze-dried crowns were milled in a coffee grinder. Preliminary tests enabled the set-up of the extraction (ultrasound, solvent) and chromatographic separation (choice of column, solvents, injection volume) conditions. Ultrasound caused no evaporation and did not degrade the phenolic standards.

2.4.2. Methanolic extraction of phenolics

2.4.2.1. Extraction for GC-MS analyses

Extraction samples were carried out according to the method reported by Szopa *et al*. (2001) except that L-ascorbic acid (1.7 %, w/v) was used as an antioxidant and was added to the extraction solvent. This method allows the extraction of the free soluble phenolic compounds and of those bound by glycosylation or esterification to the cell wall. 4 mL of methanol per g FW was added to the milled tissue. After 15 min of heating at 70°C, the extract was centrifuged (5 min, 12.000 *g*). The internal standard, ribitol (30 µg/g FW), was directly added to the homogenate sample. The supernatant was diluted with one volume of water and extracted with two volumes of $CHCl_3$. An aliquot of water phase was freeze-dried. The dried extract was dissolved in 100 µL methoxyamine hydrochloride (20 mg/mL), and incubated at 37°C for 90 min. The samples were then derivatized with 100 µL of N-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at 37°C for 90 min.

2.4.2.2. Extraction for HPLC and LC-MS analyses

Samples for HPLC and LC-MS analysis were extracted following two methods: one for some phenolics in a free soluble form, the second for others bound to the cell walls. Standards were prepared under the same conditions as crown samples to monitor degradation and avoid underestimation (Wuyts *et al*., 2007). For the soluble phenolics, 100 mg of dried and powdered crown tissues were placed in a pyrex tube with a Teflon-coated cover and extracted

with 3 mL of aqueous methanol 80% (v/v) containing 1.7% L-ascorbic acid (w/v). The mixture was ultrasonicated for 2 h at 40°C and centrifuged at 16 000 *g* for 10 min. The supernatant was passed through 0·45 μm PTE filters into HPLC-vials.

Bound phenolics were extracted through acid hydrolysis following the method used by Wuyts *et al.* (2007). 100 mg of dried and milled crown tissue and 2 mL of a solvent containing 62·5 % aqueous methanol and 6N HCl (80:20, v/v) were suspended in a pyrex tube with a Teflon-coated cover, placed under nitrogen gas flow to prevent oxidation and incubated 30 min at 90°C with regular shaking. Samples were then cooled on ice and 1 mL of methanol was added. The tubes were vortexed and the extract passed through 0·45 μm PTE filters into HPLC-vials.

2.4.3. Chromatographic analysis of banana crown methanolic extracts

2.4.3.1. GC-MS analyses

GC-MS analyses were performed on a HP-6890 GC system coupled with a 5973N mass detector and equipped with a HP-5MS fused silica capillary column (30 m x 0.25 mm, 0.25 μm film thickness). Injection temperature was 230° C, the interface set to 250° C and the ion source adjusted to 180° C. The temperature program started at 70° C for 5 min, followed by a 5° C/min temperature ramp up to 300°C and 310°C was maintained for 1 min. The system was then equilibrated at 70°C for 6 min before the next injection. The flow rate of helium, the carrier gas, was 1 mL/min. Mass spectra were recorded at 2 scan/s with an *m/z* 50–600 scanning range. 2 μL of sample was used for analysis. The chromatograms and mass spectra were evaluated using the MSD ChemStation program (Hewlett Packard). Dopamine, epinephrine, norepinephrine and normetanephrine (Sigma) were used as standards. The extracts were spiked with dopamine, epinephrine, norepinephrine and normetanephrine and estimated recoveries were 78, 65, 83 and 80 % respectively. Each catecholamine was quantified by internal standardization with ribitol and their respective calibration curve, using the following ions: ribitol (m/z 307; 319), dopamine (m/z 174; 338; 426), epinephrine (m/z 174; 294; 355) norepinephrine (m/z 174; 355; 514), normetanephrine (m/z 174; 297; 456).

2.4.3.2. HPLC analyses

HPLC analyses were performed on an Agilent HP 1100 system using a C18 Inertsil ODS2 (250 x 2.1 mm, 5 µm) reverse phase analytical column, with a diode array detector. Phenolics were monitored at five different wavelengths: 254, 280, 330, 366 and 450 nm and the peak spectra were recorded. Fluorescence detection was also carried out with an excitation wavelength of 305 nm and emission at 410 nm. Methanolic extracts were separated with a mobile phase consisting of acetonitrile and MilliQ water acidified at pH 3·0 with phosphoric acid and a flow rate of 0·3 mL/min at 35°C. A gradient of acetonitrile was programmed (0 % for 5 min, increase to 50% in 70 min, 50 to 100 % in 3 min and 100 % for 7 min). The column was then re-equilibrated with 0% acetonitrile for 10 min. The injection volume of the extracts was of 20 µL. Retention time (R_T) and UV absorption spectra of the peaks of the extracts were compared with the ones obtained with the phenolic standards from Sigma-Aldrich, (ascorbic, gallic, chlorogenic, caffeic, coumaric, ferulic and sinapic acids; catechin, rutin, naringin) and Fluka (quercitrin, hesperidin, ploridzin, quercetin, cinnamic acid, naringenin and kampherol) previously analyzed under the same HPLC conditions, in order to confirm their identification. The identified compounds were quantified by external calibration with the corresponding standard. The unidentified compounds were quantified in dopamine equivalents and the hydroxycinnamic acids in ferulic acid equivalents. For each treatment (S^+ and S^-), three samples were extracted at two stages (dhbi and 13dpi) and the results were statistically analyzed.

2.4.3.3. LC-MS analyses

LC-MS analyses were carried out with an Agilent HP 1100 system coupled to an HCT mass spectrometer (Bruker Daltonics) to corroborate the identifications made by the HPLC. The extracts were diluted with methanol in order to obtain a total concentration of 10 µg/ml. The column and elution gradient used were the same as described above for the HPLC-UV analysis. After the column, the flow was split and 0.05 ml/min were sent to the electrospray source (ESI). Column effluent was monitored in negative and positive ion modes. The ESI conditions were: Pressure N_2 = 35 psi, N_2 flow rate = 8.5 l/min and 365° C. The ionic trap was

set to scan from 150 to 1000 m/z (scan rate of 26 000m/z/s). The trap was emptied either after 200 ms or as soon as a total ionic current of 100 000 was reached. The R_T and mass spectra of the peaks of the extracts were compared with those of standards previously analyzed under the same LC-MS conditions in order to confirm their identification.

2.5. Statistical analysis

The effects of severe So-Si ratio modifications on banana susceptibility to crown rot was analyzed by the subjection of INS values calculated for the three clusters and each fruit grade and length values to a partially nested mixed three-way ANOVA (Minitab software v.15.1). Each tree was taken as an experimental unit and Treatment, Week and Bunch as factors. Multiple comparisons of the different means were performed by applying Tukey test at a 5% probability level. Data of crown phenolics quantified by HPLC analyses were subjected to a three-way ANOVA (Minitab software v.15.1), with Treatment, Stage and Replicate as factors.

3. Results

3.1. Effects of severe so-si ratio modification on susceptibility to crown rot and on some fruit and tree characteristics

The modifications of the So-Si ratio at flowering had a significant effect ($P< 0.001$) on the fruit's susceptibility to crown rot disease (Table 1). The level of the fruit's susceptibility to this disease was consistently higher in 2L/8H and 1L/8H So-Si ratio modifications as compared to the 12L/1H and 12L/2H ones (Fig. 2); with INS average value of 524.0 mm² and 513.5 mm² compared to 246.2 mm² and 278.7 mm² respectively (Table 2). From week to week within a So-Si ratio modifications, significant effects were found ($P= 0.001$). Moreover, within the different So-Si ratio modifications, no bunch effect was found ($P= 0.062$). However, a low but significant interaction was observed ($P= 0.035$) between So-Si ratio modifications and the week (Table 1). There was no susceptibility difference between (i) 12L/1H and 12L/2H So-Si ratio modifications and (ii) 2L/8H and 1L/8H So-Si ratio modifications. Three statistically different groups were distinguished amongst fruits that have undergone So-Si ratio modifications during flowering by the removal of both leaves and hands (Table 2). Samples of extreme treatments (12L/1H and 1L/8H) with differential susceptibilities to crown rot were then selected for chromatographic analyses (Table 3).

The severe So-Si ratio modifications which occurred during flowering had a very significant effect ($P< 0.001$) on fruit's grade and length (Table 1). Fruit grade, fruit length and the number of leaves at harvest (NLH) were different for each So-Si ratio modification. The ranking of these modifications shows that fruits with higher So-Si ratio have a significantly higher grade ($P< 0.001$) and were significantly longer ($P< 0.001$) than those with a low So-Si ratio (Table 2). The NLH was higher in trees with high So-Si ratio as compared to those with low So-Si ratio. At 900 dd, fruit length was higher for high So-Si ratio (8 and 4) as compared to the ones with low So-Si ratio (1, 0.17 and 0.08). There was no difference between the grade and the length of fruits harvested from banana trees with 12L/1H and 12L/2H So-Si ratio modifications, and between the ones with 2L/8H and 1L/8H So-Si ratio modifications. Thus,

three statistically different groups were distinguished between fruit grades and lengths that have undergone severe So-Si ratio modifications (Table 2).

Table 1: Analysis of variance of the effect of modification in So-Si ratio on banana's susceptibility to crown rot (INS), fruit grade and length.

		INS		Grade		Length	
Source	d.f.	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Treatment	4	24,25	<0,001	121,66	<0,001	121,20	<0,001
Week	2	8,88	0, 001	1,99	0,151	1,29	0,288
Treatment *Week	8	2,36	0,035	0,33	0,948	2,38	0,034
Bunch (Treatment)	20	1,77	0,062	1,10	0,383	1,60	0,104

d.f. is the degree of freedom.

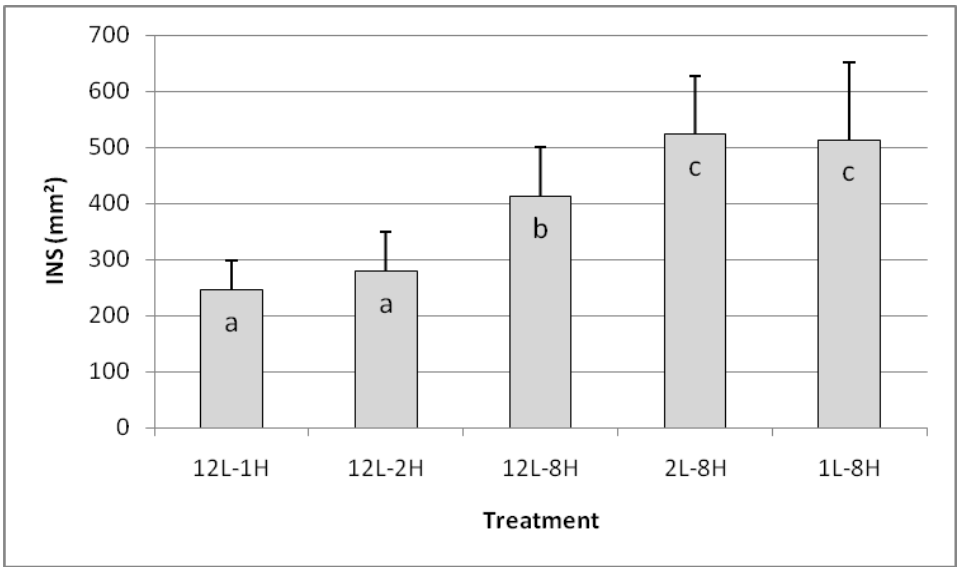


Figure 2: Internal necrosis surface (INS) 13dpi for different treatments. Each point represents the average of fifteen replicates for a three week assessment.

Table 2: Mean values and standard deviations of INS, fruit grade, length and number of leaves at harvest (NLH) after a 3 week assessment.

Treatment	So-Si ratio	INS (mm²)	Grade (mm)	Length (cm)	NLH
12L/1H	8	246,2 ± 51,7[a]	39,0 ± 1,5[a]	33,1 ± 1,4[a]	6,9
12L/2H	4	278,7 ± 71,5[a]	38,3 ± 1,5[a]	32,3 ± 1,4[a]	6,7
12L/8H	1	412,9 ± 87,0[b]	34,8 ± 1,2[b]	29,5 ± 1,1[b]	6,2
2L/8H	0,17	524,0 ± 103,1[c]	30,1 ± 1,3[c]	25,3 ± 0,7[c]	2
1L/8H	0,08	513,5 ± 138,1[c]	29,5 ± 1,8[c]	24,7 ± 1,4[c]	1

Means are the result obtained after 3 weeks here represented with standard deviation in the table. The different letters a, b, c and d represent groups showing statistically significant differences.

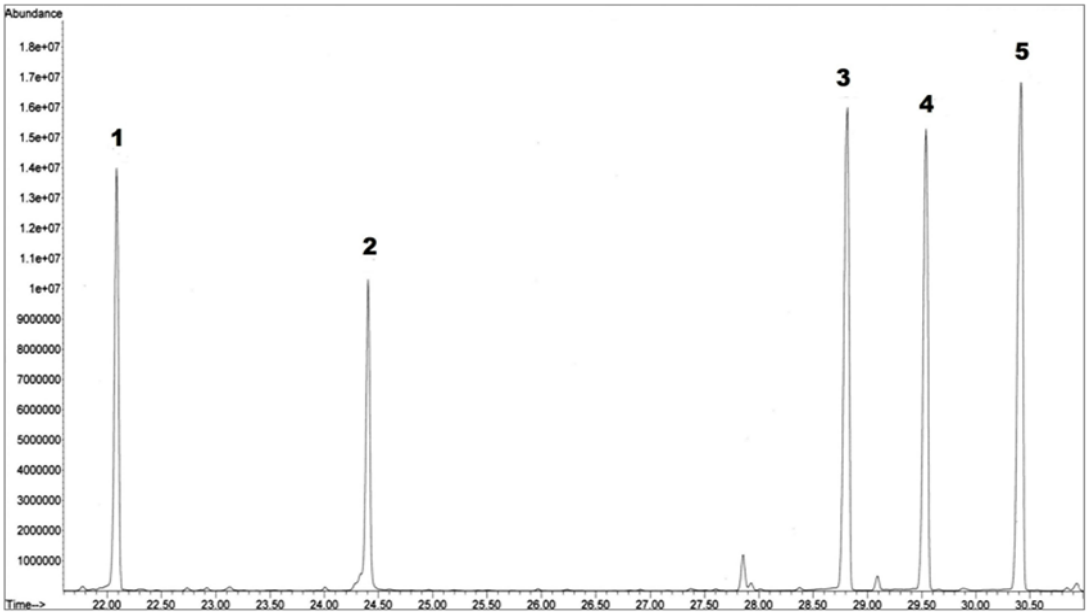
Table 3: Banana crown samples used for chromatographic analysis, harvested in the third successive week for GC-MS analyses and in the second successive week for HPLC and LC-MS analyses.

	INS (mm²) for GC-MS		**INS (mm²) for HPLC & LC-MS**	
Samples	**S^-**	**S^+**	**S^-**	**S^+**
1	294	513	252	646
2	176	494	264	620
3	252	560	231	576

3.2. Phenolic constituents of banana with differential susceptibility to crown rot disease

3.2.1. GC-MS analyses

The GC-MS method developed by Szopa *et al.* (2001) allowed a better separation (Fig. 3), identification and quantification of the four catecholamines under study (dopamine, epinephrine, norepinephrine, normetanephrine). Their presence in the methanolic extracts of crowns was clearly confirmed on the basis of the R_T and mass spectra of their standards (Fig. 3). For both stages studied (dhbi and 13dpi), dopamine content was not significantly ($P > 0.05$) different in the S^- (12L/1H) and S^+ (1L/8H) crown samples. For each treatment, a slight decrease of dopamine content was noticed in the crown samples from the dhbi to the 13dpi (Table 4). Norepinephrine and normetanephrine contents significantly ($P < 0.05$) differed between the S^- and S^+ banana crowns on the dhbi. Their average amount was six and two times higher, respectively, in the S^+ banana crowns as compared to the S^- ones (Table 4). Their concentration decreased in banana crowns 13dpi. They were detected in very low amounts in the crowns (for both treatments) but their concentrations were impossible to quantify. Epinephrine was not detected in banana crowns. Octopamine, another derivative of tyrosine, and other polar metabolites such as phenolic acids, carbohydrates (polysaccharides), free amino acids, fatty acids, phytosterols and derivatives were also identified in the crown extracts (not shown).



Peak N°	1	2	3	4	5
R_T (min)	22.09	24.40	28.78	29.52	30.39
m/z ( )	307; 319	174; 294; 355	174; 338; 426	174; 355; 514	174; 297; 456

Figure 3: GC-MS profile of catecholamines standards (Sigma). 1. Ribitol (Internal standard), 2. Epinephrine, 3. Dopamine, 4. Normetanephrine, 5. Norepinephrine.

Table 4: Catecholamine contents for different treatments of banana crown. (S^-, S^+) and sampling stages (dhbi, 13dpi). GC-MS chromatographic conditions are described in the text.

Catecholamine	Treatment	S^- (mg/g crown FW)	S^+ (mg/g crown FW)
Dopamine	dhbi	21.23 ± 0.68 ax	19.76 ± 0.29 ax
	13dpi	20.46 ± 0.28 ax	17.91 ± 2.00 ax
Norepinephrine	dhbi	1.03 ± 0.53 a	6.34 ± 1.35 b
	13dpi	-	-
Normetanephrine	dhbi	1.73 ± 0.09 a	3.22 ± 0.58 b
	13dpi	-	-

Means are the result of 3 replicates represented with standard deviation in the table. The different letters a and b for treatment (S^+/S^-), x and y for sampling stages (dhbi/13dpi) represent groups showing statistically significant differences.

3.2.2. HPLC and LC-MS analyses

The HPLC method developed enabled a good separation of the 21 phenolic standards (see SC R_T in Table 5 which corresponds to the R_T of the pure compounds). Our LC-MS conditions used allowed the detection and confirm the identification of only 17 of these standards, the m/z of the base peak of the spectra, which correspond to the $[M-H]^-$ ion are presented in table 5 (SC $[M-H]^-$). The R_T of standards identified with HPLC was not different to the one obtained with LC-MS. Under the conditions used for extraction of the bound phenolic compounds, some of the standards gave one chromatographic peak at the same R_T as under the extractions conditions used for the soluble phenolics. It was assumed that the hydrolytic conditions did not affect these molecules (dopamine, norepinephrine, normetanephrine, quercitrin, quercetin, naringenin and kaempferol). They were therefore futher studied nevertheless for gallic acid, catechin, caffeic acid, coumaric acid, ferulic acid, sinapic acid, naringin and cinnamic acid; two peaks were observed for each compound after they were been subjected to the extraction conditions for the bound phenolic compounds. One peak corresponded to the unmodified standard (same R_T) and the other to an acid extraction by-product. For chlorogenic acid, in addition to the unmodified molecule, two supplementary peaks were detected. Under the extractions conditions used for the bound phenolics, two by-products could be detected at 280 nm. Rutin, hesperidin and phloridzin were very much affected by the extraction conditions used for the bound phenolics and the peak of the starting molecule was no more detected and a new peak was observed. The R_T of the unique (the unmodified or single acid extraction by-product) or the multiple peaks (several by-products and eventually the original molecule) of each standard after HPLC are presented in Table 5 (AHC R_T). The base peak m/z observed for the spectra of each peak after LC-MS (in other to confirm their identification) are equally shown in Table 5 (AHC m/z).

Table 5: HPLC retention time (R_T) in minutes and LC-MS spectral information of 21 authentic phenolic standards separated on the Inertsil ODS 2 column under soluble conditions (SolC) and after acid hydrolytic conditions (AHC).

				HPLC analyses		LC-MS analyses	
N°	Standard	Linear formula	Molar mass	SolC R_T	AHC R_T	SolC $[M - H]^-$	AHC $[M - H]^-$
1	**Dopamine**	$C_8H_{11}NO_2$	153.08	2.5	2.5	151.8	151.9
2	**Norepinephrine**	$C_8H_{11}NO_2$	153.08	2.5	2.5	ND	ND
3	**Epinephrine**	$C_9H_{13}NO_3$	183.09	2.5	2.5	ND	ND
4	**Normetanephrine**	$C_9H_{13}NO_3$	183.09	2.5	2.5	ND	ND
5	**Ascorbic acid**	$C_6H_8O_6$	176.03	4.1 - 5.7	ND	174.7	ND
6	**Gallic acid**	$C_7H_6O_5$	170.02	11.5	11.8 - 25.8	168.9	168.9 - 182.7
7	**Catechin**	$C_{15}H_{14}O_6$	290.08	27.0	27.0 - 45.8	288.9	288.9 - 427.0
8	**Chlorogenic acid**	$C_{16}H_{18}O_9$	354.10	28.1	28.6 - 35.7 - 43.6	352.9	ND - 366.9 - ND
9	**Caffeic acid**	$C_9H_8O_4$	180.04	28.5	28.8 - 43.5	178.9	179 - 192.7
10	**Coumaric acid**	$C_9H_8O_3$	164.05	34.2	33.5 - 51.1	162.9	ND - 176.7
11	**Ferulic acid**	$C_{10}H_{10}O_4$	194.06	36.7	36.7 - 52.9	192.9	192.5 - 377.9
12	**Sinapic acid**	$C_{11}H_{12}O_5$	224.07	37.2	37.2 - 52.4	222.8	222.6 - 236.6
13	**Rutin**	$C_{27}H_{30}O_{16}$	610.15	38.7	51.2	609.1	ND
14	**Quercitrin**	$C_{21}H_{20}O_{11}$	448.10	41.7	41.7	447.9	ND
15	**Naringin**	$C_{27}H_{32}O_{14}$	580.18	41.8	42.3 - 55.1	579.2	270.9 – 433.0
16	**Hesperidin**	$C_{28}H_{34}O_{15}$	610.19	42.8	57.8	609.1	ND
17	**Phloridzin**	$C_{21}H_{24}O_{10}$	436.14	44.5	58.5	435.0	ND
18	**Quercetin**	$C_{15}H_{10}O_7$	302.04	50.7	50.7	300.9	ND
19	**Cinnamic acid**	$C_9H_8O_2$	148.05	51.2	51.4 – 69.1	ND	ND - ND
20	**Naringenin**	$C_{15}H_{12}O_5$	272.07	55.3	55.3	271.0	ND
21	**Kaempferol**	$C_{15}H_{10}O_6$	286.05	57.5	57.3	284.9	285.0

ND=Not detected

For the extracts of the soluble phenolics (ESP), chromatographic profiles were very similar for both treatments (S^+ and S^-) at both stages (dhbi and 13dpi). Only a major compound was eluted after 2.5 min (peak 1). It was identified as dopamine by comparison to its R_T, its UV spectrum and its major m/z (152) observed in ESI-MS (negative mode) with those obtained from the pure dopamine standard. Dopamine was also detected through the GC-MS method used in this study (see above). This cathecolamine was equally the major soluble compound detected in the methanolic extracts of banana roots (Wuyts *et al.* 2007). In the chromatographic profile of the ESP, dopamine peak co-eluted with ascorbic acid that was added as a protective into the solvent as previously reported (Wuyts *et al*. 2007). Due to poor resolution, dopamine was quantified by external calibration based on the fluorescence peak height. The results obtained are presented in Table 6. The same method of quantification was applied for dopamine in the extracts of the bound phenolics (EBP).

Chromatograms obtained from the EBP of the S^- and S^+ treatments at both stages (dhbi and 13dpi) are shown in Figure 5. The differences observed for the EBP are much more pronounced than for the ESP. However, the chromatographic profile of banana crown EBP differs quantitatively, but not qualitatively depending on the level of susceptibility to crown rot (S^+ and S^-) and the stage (dhbi and 13dpi). Attention was focused on ten major peaks that are common to the EBP at both stages and for both treatment (peak 1 to 10 in Figure 5). Peak 1 was identified as dopamine just like in the ESP (R_T, UV, ESI-MS). It was the major compound detected in the EBP. Dopamine concentration in the crown was higher in ESP as compared to the EBP (Table 6). For the ESP, there was no significant ($P > 0.05$) difference in dopamine level between both treatments (S^- and S^+) at each stage (dhbi and 13dpi). However, this level was significantly different ($P= 0.002$) from one sample to another in the ESP compare to the EBP (Table 6). For the EBP, there was no significant ($P > 0.05$) difference in dopamine level the dhbi between both treatments (S^- and S^+). However, from dhbi to 13 dpi, a significant ($P> 0.05$) decrease was observed in the S^+ crown while this amount remained unchanged in the S^- crown.

The UV spectra of the compounds of peaks 2 to 10 are shown in Figure 4. Compounds 2 to 4 had a maxima in their spectrum at 280 nm and compounds 5 to 10 close to 310-330 nm. Peaks 2 to 4 were quantified in dopamine equivalents (λ_{max} 280 nm) while peaks 5 to 10 in ferulic acid equivalents (λ_{max} 330 nm). Thus, calibration curves of pure dopamine and ferulic

acid were as such determined. In the ESP and EBP of the crowns, vanilline and rutin were detected in traces as well as traces of many other phenolics and derivatives (not shown).

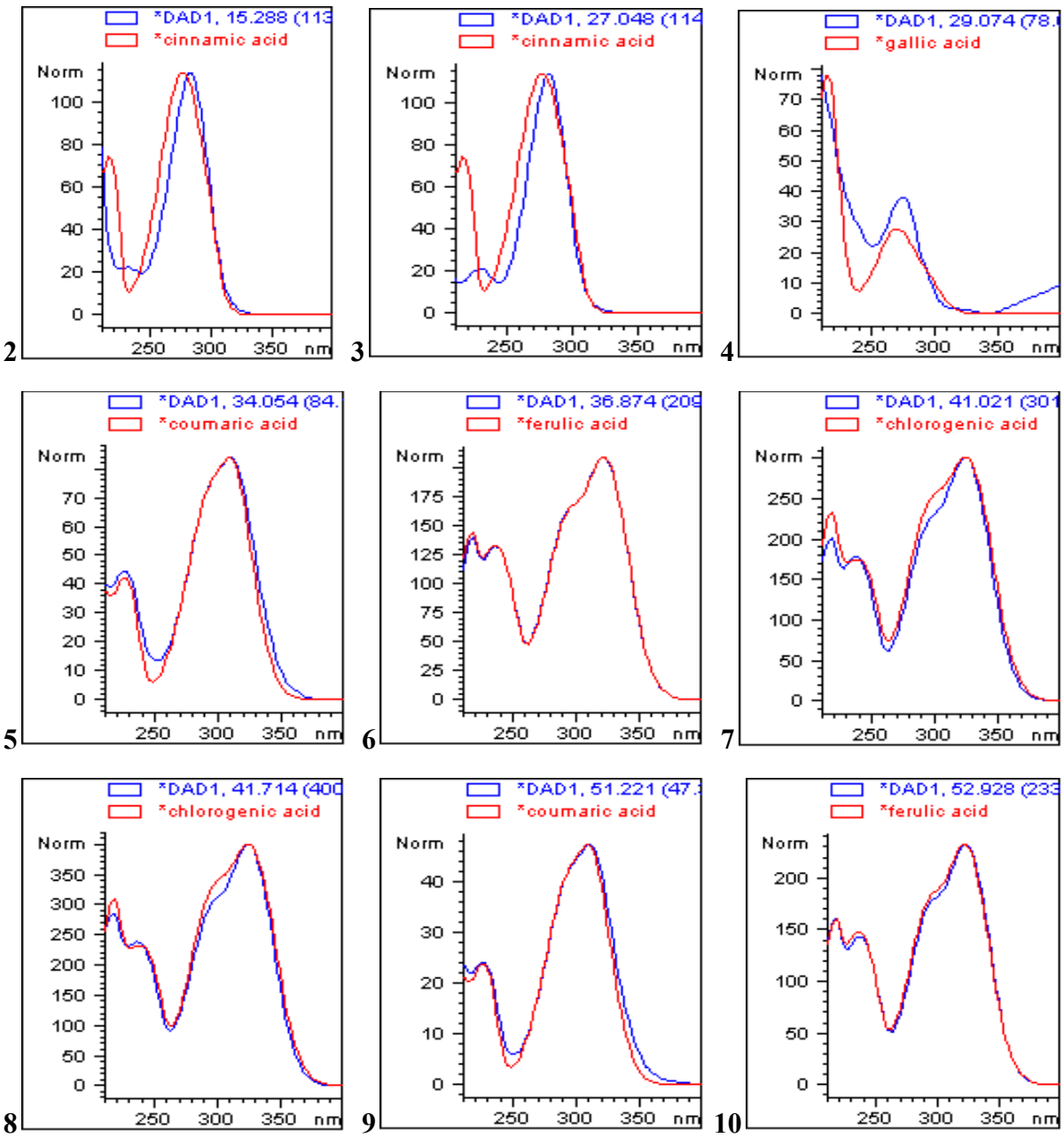


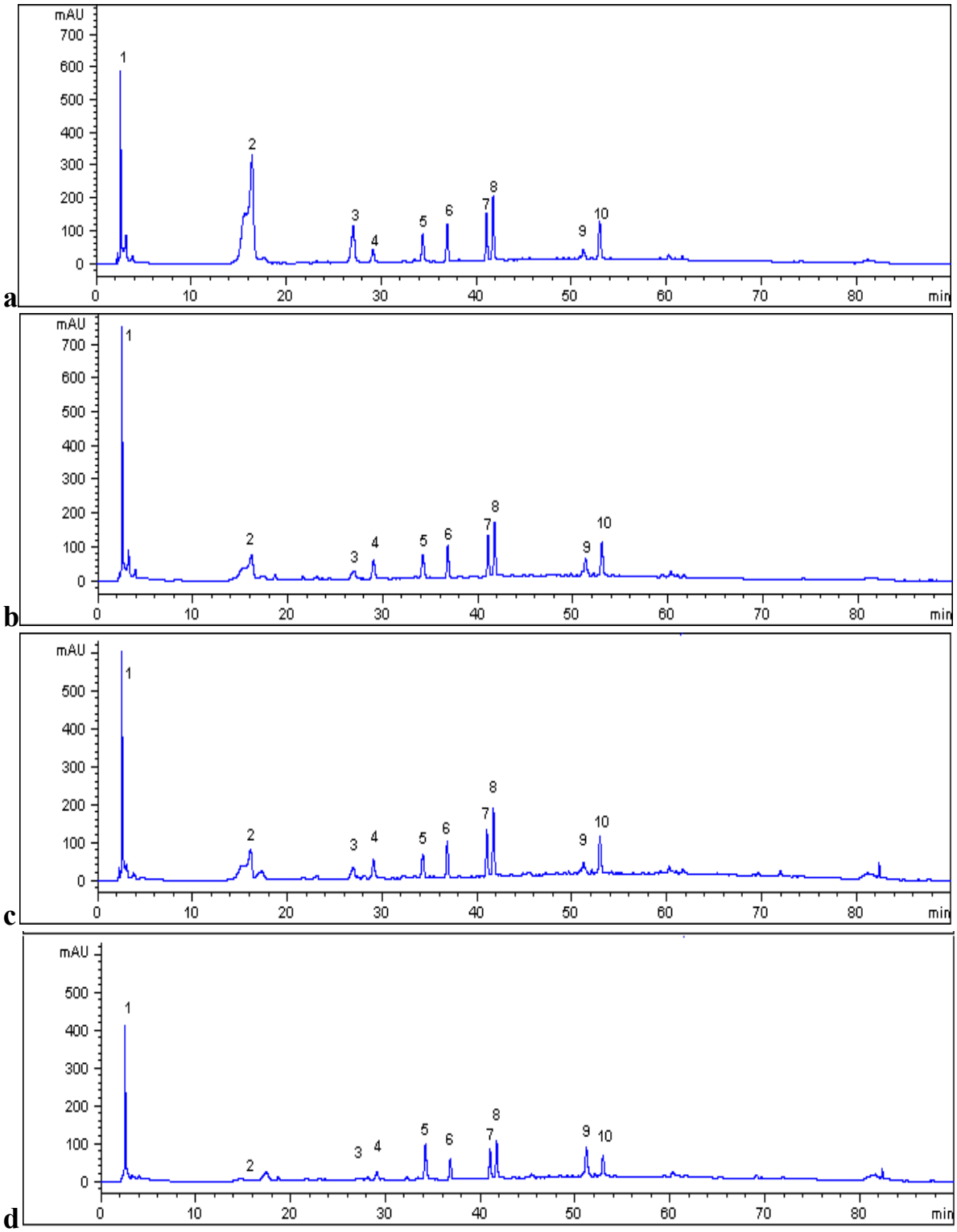
Figure 4: HPLC peaks (2-10) UV spectrum: A=f (λ) obtained during identification of peaks, superposition of phenolic compounds present in the banana crown sample (Blue) with the ones of the corresponding phenolic standard (Red) whose layout was carried out under the same experimental conditions.

Table 6: Dopamine (Peak 1) contents of ESP and EBP expressed in mg/g crown FW for different treatments of banana crown (S^-/S^+) and sampling stages (dhbi/13dpi). HPLC chromatographic conditions are described in the text. *P* is the probability obtained with ANOVA: *P* value 1 for Treatment, *P* value 2 for Stage and *P* value 3 for Replicate.

	Stage	S^- crown	S^+ crown	*P* value 1	*P* value 2	*P* value 3
ESP	dhbi	11.86 ± 7.84 ax	11.90 ± 3.80 ax	*0.925*	*0.848*	*0.002*
	13dpi	11.77 ± 4.69 ax	11.47 ± 4.19 ax			
EBP	dhbi	7.89 ± 1.05 ax	7.20 ± 0.10 ax	*0.051*	*0.219*	*0.652*
	13dpi	8.06 ± 2.21 ax	4.72 ± 0.25 by			

Means are the result of 3 replicates represented with standard deviation in the table. The different letters a and b for treatment (S^+/S^-), x and y for sampling stages (dhbi/13dpi) represent groups showing statistically significant differences.

Peaks **2**, **3** and **4** (Fig. 5) did not correspond to any of the 21 phenolic standards selected in this study. Their UV/visible spectrum showed λ_{max} at 280 nm (Fig. 4). The UV/visible spectrum of peaks **2** (R_T 16 min) and **3** (R_T 27 min) were closest to the ones of cinnamic acid amongst the 21 phenolic standards. The spectrum of peak **4** was closest to that of gallic acid. The base peak obtained for their ESI-MS spectra (negative mode) were respectively ions of m/z 531, 545, and 143 (Table 7). Peak **2** (MW = 532amu) is not a distinct peak but a large one, which seems to be constituted by more than one product. Peak **2** was the second most important compound in the EBP after dopamine. It amount was significantly different (*P*= 0.014) in the S^- crowns as compared to the S^+ ones. Concerning the dhbi, its average amount was about 10 times more important in the S^- crowns than in the S^+ ones (153.8 and 15.6 µg *dopamine equivalents* per g FW respectively). This level significantly decreased (*P*= 0.044) at 13dpi to 36.4 and 4.3 µg *dopamine equivalents* per g FW respectively (Table 8). The amount of peak **3** was significant different (*P*= 0.032) in the S^- and the S^+ crown samples. This amount decrease significantly (*P*< 0.05) 13dpi in the S^- crown samples. For peak **4**, no significant difference (*P*= 0,144) was noticed in the S^- and the S^+ crown samples at both stages.



Peak N°	1	2	3	4	5	6	7	8	9	10
R_T (min)	2.5	16.0	27.0	29.1	34.3	36.9	41.0	41.7	51.2	52.9

Figure 5: HPLC chromatogram at 280 nm of bound methanolic fraction of banana crown for the different treatments (S^+/S^-) and sampling stages (dhbi/13dpi). a) S^-/dhbi, b) S^+/dhbi, c) S^-/13dpi and d) S^+/13dpi. Chromatographic conditions are described in the text. Numbers over the peak indicate: 1-Dopamine; 5-Coumaric acid; 6-Ferulic acid; 9-Coumaric acid methyl ester; 10-Ferulic acid HCl extraction by-product; numbers 2, 3, 4, 7 et 8 indicate unknown phenolics.

Peaks **5** and **9** respectively had, the same R_T, UV spectrum and the base peak in ESI-MS (*m/z* 163 and m/z 177, attributed to $[M-H]^-$) than the coumaric acid standard and its acid extraction by-product, (coumaric acid methyl ester) resulting probably from the methylation of the acid function by the methanol used for the extraction in the presence of HCl. Based on this hypothesis, peaks **5** and **9** were identified as the coumaric acid and its methyl-ester respectively. Similarly, peaks **6** (R_T 36.9 min) and **10** (R_T 52.9 min) were identified by HPLC-UV and LC-MS as a ferulic acid and its acid extraction by-product respectively. Ferulic acid showed the $[M-H]^-$ deprotonated ion (*m/z* 193) as previously reported in banana roots (Wuyts *et al.*, 2007) while the ion at *m/z* 378 corresponded to ferulic acid extraction by-product (Fig. 4 & Table 7). Peaks **7** (R_T 41.0 min) and **8** (R_T 41.7 min) were both identified by HPLC as chlorogenic acid derivatives based on the comparison of their UV absorption spectra with the one of chlorogenic acid standard. However, LC-MS analysis did not corroborate the nature of these molecules (Fig. 4 & Table 7). A significant difference (P= 0.008) was found between average contents of coumaric acid (peak **5**) in the S^- and the S^+ crown samples, while there was no significant difference (P> 0.05) between both stages (dhbi and 13dpi). For its methyl ester (peak **9**), no significant difference (P= 0,385) was noticed in the S^- and the S^+ crown samples at both stages. The average content of ferulic acid (peak **6**), its acid extraction by-product (peak **10**), peak **7** and **8** were significantly (P< 0.001) important in the S^- crown samples as compared to the S^+ ones. This level was significantly reduced 13dpi as compared to the dhbi stage more especially in the S^+ crown samples (Table 8).

Table 7: HPLC/LC-MS analysis data of phenolic compounds in banana crown extract, according to their retention time, UV absorption spectra and their negative ionization mass spectra.

Peak (λ_{max})	R_T (min)	HPLC Compound	$[M - H]^-$	LC-MS Compound
1 (280 nm)	2.5	Dopamine	151.6	Dopamine $[M-H]^-$
2 (280 nm)	16.0	Unknown	530.7	Unknown $[M-H]^-$
3 (280 nm)	27.0	Unknown	544.8	Unknown $[M-H]^-$
4 (280 nm)	29.1	Unknown	142.5	Unknown $[M-H]^-$
5 (330nm)	34.3	Coumaric acid	162.6	Coumaric acid $[M-H]^-$
6 (330nm)	36.9	Ferulic acid	192.6	Ferulic acid $[M-H]^-$
7 (330nm)	41.0	Chlorogenic acid	416.7	Unknown $[M-H+64]^-$
8 (330nm)	41.7	Chlorogenic acid	338.7	Unknown $[M-H-14]^-$
9 (330nm)	51.2	Coumaric acid ME	176.6	Coumaric acid by product
10 (330nm)	52.9	Ferulic acid Ebp	377.6	Ferulic acid Ebp

ME = Methyl Ester

Ebp = Extraction by-product

Table 8: Phenolic contents of EBP for different treatments of banana crown (S^-/S^+) and sampling stages (dhbi/13dpi). HPLC chromatographic conditions are described in the text. Peak 6 is ferulate and expressed in µg/g crown FW. Peaks 2, 3 and 4 are expressed in µg of dopamine equivalents/g FW while peaks 5, 7, 8, 9 and 10 are expressed in µg of ferulate equivalents/g FW. *P* is the probability obtained with ANOVA: *P* value 1 for Treatment, *P* value 2 for Stage and *P* value 3 for Replicate.

Peak N°	Stage	Less susceptible crown (S^-)	Susceptible crown (S^+)	*P* value 1	*P* value 2	*P* value 3
2 (unknown)	dhbi	152.80 ± 0.66 ax	15.64 ± 0.03 bx	*0.014*	*0.044*	*0.502*
	13dpi	36.42 ± 0.15 ay	4.30 ± 0.01 by			
3 (unknown)	dhbi	86.00 ± 0.99 ax	3.63 ± 0.00 bx	*0.032*	*0.051*	*0.412*
	13dpi	13.11 ± 0.11 ay	0.79 ± 0.00 bx			
4 (unknown)	dhbi	5.60 ± 0.03 ax	5.89 ± 0.01 ax	*0.144*	*0.092*	*0.056*
	13dpi	5.56 ± 0.03 ax	1.81 ± 0.01 ax			
5 (Coumaric acid)	dhbi	11.86 ± 0.05 ax	7.26 ± 0.02 bx	*0.008*	*0.057*	*0.005*
	13dpi	8.73 ± 0.05 ax	5.56 ± 0.02 bx			
6 (Ferulic acid)	dhbi	23.15 ± 0.02 ax	12.23 ± 0.02 bx	*<0.001*	*<0.001*	*0.153*
	13dpi	16.51 ± 0.02 ay	5.46 ± 0.01 by			
7 (unknown)	dhbi	27.82 ± 0.08 ax	15.85 ± 0.01 bx	*<0.001*	*0.008*	*0.077*
	13dpi	20.92 ± 0.05 ay	7.42 ± 0.02 by			
8 (unknown)	dhbi	35.89 ± 0.11 ax	20.17 ± 0.02 bx	*<0.001*	*0.008*	*0.049*
	13dpi	26.87 ± 0.07 ay	9.28 ± 0.02 by			
9 (Coumaric acid ME)	dhbi	7.43 ± 0.01 ax	7.31 ± 0.01 ax	*0.843*	*0.358*	*0.381*
	13dpi	8.09 ± 0.01 ax	7.94 ± 0.02 ax			
10 (Ferulic acid Ebp)	dhbi	38.32 ± 0.10 ax	20.15 ± 0.07 bx	*<0.001*	*0.001*	*0.002*
	13dpi	28.74 ± 0.09 ay	9.38 ± 0.03 by			

Means are the result of 3 replicates represented with standard deviation in the table. The different letters a and b for treatment (S^+/S^-), x and y for sampling stages (dhbi/13dpi) represent groups showing statistically significant differences.

ME = Methyl Ester
Ebp = Extraction by-product

4. Discussion

The severe modification in So-Si ratio was found to influence the susceptibility of fruits to crown rot disease. Banana's susceptibility to crown rot increased when So-Si ratio severely decreased and vice-versa. Therefore, bananas with high So-Si ratio were less susceptible to crown rot than those with low So-Si ratio. Our results were in accordance with Lassois *et al*. (2010b) that revealed an important effect of So-Si ratio modifications on the fruit quality potential. The severe sink reduction (88% and 75%) and the severe source reduction (92% and 83%) lead to a lesser susceptibility and a higher susceptibility, respectively, of banana fruits to the development crown rot. As expected, the removal of some hands at flowering induced less competition and increased availability of assimilates for the hands remaining on the bunch (Lassois *et al*., 2010b), and vice-versa for the severe defoliation. The severe defoliation appeared to influence the processes determining fruit susceptibility to crown rot development. However, it was not the case for moderate mechanical defoliation that also reduced the photosynthetic activity of banana tree (Lassois *et al*., 2010b). This therefore suggests a concept of doorstep level above which the mechanical defoliation has little or no effect.

The So-Si ratio modification during the growth of the bunch significantly influences the banana tree and fruit characteristics at harvest. The results of this study provided evidence of wide variation in banana fruit's morphology which was similar to previous reports (Jullien *et al.*, 2001; Mouen Bedimo *et al.*, 2003; Chillet *et al.*, 2006; Lassois *et al*., 2010b). From flowering to harvest, the number of leaves on banana tree decreases. These morphological differences could be explained by differential development associated with cell division and fruit filling characteristics. As previously stated, an empirical increase in So-Si ratio resulted in an increased in fruit filling rate (Jullien *et al.*, 2001). Therefore, when So-Si ratio increased, fruit's grade and length also increased and vice-versa.

Catecholamines were found in S^- and S^+ banana crowns. GC-MS, HPLC and LC-MS enabled them to be identified as dopamine and its derivatives (norepinephrine and normetanephrine). This study is the first to report catecholamine content in banana crowns. The dhbi, the higher amount of norepinephrine and normetanephrine found in S^+ crowns as compared to the S^- ones, can indicate a potential stress in S^+ crown tissues; while in S^- one, the release of such stress compound can be regulated by their phenolic content. Our results are

opposed to a previous study, where the surexpression of dopamine-β-monooxygenase gene was highlighted in bananas less susceptible (S^-) to crown rot (Lassois *et al.*, 2011). This revealed the degree of complexity of dopamine regulation mechanisms. However, it has been shown that in response to a mechanical wound, a light increase in catecholamine level was observed in the potato leaves; but the highest rise was observed in the case of normetanephrine (Szopa *et al*., 2001). Catecholamines were suggested to be new biochemical markers of biotic and abiotic stress in plants (Szopa *et al*., 2001; Świedrych *et al*., 2004; Kulma & Szopa, 2007), and they can play a major role in alternative control methods.

Dopamine level (GC-MS and ESP-HPLC conditions) was almost the same between the susceptible and the less susceptible crown regardless of the stage (dhbi and 13dpi). These results corroborate with a previous study, which showed that the dopamine level did not differ between healthy and infected banana roots of resistant and susceptible cultivars (Wuyts *et al.*, 2007). The amount of dopamine in our banana tissues did not correlate with the level of susceptibility of our samples. It calls into question the hypothesis of dopamine potential implication in defense response. However, the formation of banana cluster, which is a process that goes through mechanical wound, can be perceived by the crown as a stress factor that triggers an increase in the accumulation of dopamine and derivatives in the tissues regardless of the level of susceptibility. In the same way, catecholamines are required in very small quantities and they are readily modified (methylated) during course of action (Kulma & Szopa, 2007). Dopamine could as such get involved in the banana crown's response to pathogen attack as a mediator of plant defense. It was indeed found to be a very good substrate for PPO isolated from banana root and thus, constitutes a potential chemical barrier to nematode infection (Wuyts *et al*., 2007). Since dopamine it's a catecholamine, it could get involved in nitrogen detoxification (Kulma & Szopa, 2007).

In addition, dopamine amount (EBP-HPLC conditions) remains unchanged in the S^- crown samples 13dpi while this amount decreases significantly in the S^+ crown. This supports our hypothesis of dopamine been involved in defense response of bananas to crown rot. Recently, the surexpression of dopamine-β-monooxygenase gene was highlighted in bananas less susceptible (S^-) to crown rot in comparison to the susceptible (S^+) ones; the implication of dopamine in the quantitative response of bananas to crown rot has been suggested (Lassois *et al.*, 2011). However, physiological mechanism involves in dopamine accumulation and probably transformation has not yet been researched.

Dopamine was the major phenolic present in banana crown with between 17.9 - 21.2 mg per g crown FW (GC-MS analyses) and 4.7 - 11.8 mg per g crown FW (HPLC analyses) regardless of the level of susceptibility (S^- and S^+) and the stage (dhbi and 13dpi). These different amounts could be inherent in the extraction conditions, the analysis methods, and/or on the environmental conditions from flowering to harvest (bunches marked and labeled in weeks 2 and 3). Dopamine has also been reported as the major component of banana roots with 2.8 - 8.4 mg per g FW (Wuyts *et al*., 2007), of banana peel with 3.8 - 13 mg per g FW and of banana pulp with fewer milligrams (Kanazawa & Sakakibara, 2000).

Among the 10 compounds identified in the EBP of crown tissues, only compounds **1**, **5**, **6**, **9** and **10** have been previously identified in banana tissues and have been related to defense mechanisms (Valette *et al*., 1998; Kanazawa & Sakakibara, 2000; de Ascenceo & Duberry, 2003; Wuyts *et al*., 2007). Dopamine and anthocyanidin related products could form physical barriers to nematode infection through PPO action in decompartimentalized cells (Wuyts *et al*., 2006a); while ferulic acid and its esters can be toxic for root nematodes (Wuyts *et al*., 2006b) or bound to cell walls for their protection against enzymatic attacks (Wuyts *et al*., 2007). Since hydrosoluble tannins contain gallic acid and its ester, compound **4** could be a compound hydrolyzed from tannins and might be related to mechanisms of defence. Gallic acid tannins were identified (Mendoza *et al*., 1992; del Mar Verde Mendez *et al*., 2003) in banana tissues roots and related to defense responses (Valette *et al*., 1998, Collingborn *et al*., 2000). Compounds **2**, **3**, **4**, **7** and **8** remain unknown. However, they remain interesting for their potential role in banana crown defense and should be further extracted, purified and completely characterized by MS and NMR techniques.

The dhbi, almost all the phenolics were found in higher amounts in the S^- crowns as compared to the S^+ ones. This can be explained by the fact that modification in So-Si ratio leads to a change in partitioning of assimilates between various sinks which influenced the formation of secondary metabolites involved in plant-pathogen interaction (Lassois *et al*.; 2010b). Our results support a previous report detailing an increased amount of phenylpropanoids in resistant cultivars of banana roots than in the susceptible ones (Wuyts *et al*., 2007). However, the amount of phenolics was more important in the banana crown samples than in the roots samples.

Moreover, it should be emphasized that for almost all the phenolics, a significant decrease in the level was noted from the dhbi to the 13dpi stage especially in the S^+ crown.

Possible antifungal effects and/or maturation effects can explain this decrease but cannot be confirmed at this stage. As afore-mentioned in the literature, the amount of phenolics decreases during the stage of the fruit's maturity (ripening) (Kanazawa & Sakakibara, 2000). Therefore, there is a need to access the maturation effect on the phenolic composition of banana crown by analyzing the non-infected banana crown tissues 13 days after harvest.

Given that some phenolics were present in uninfected banana crowns (dhbi) as well as in the infected ones (13dpi), they were considered constitutive compounds in contrast to *de novo* produced ones. A lack in this study lies in the fact that the 13dpi seem to be almost too late for the assessment of the biochemical events occurring in banana crown tissues in the first hours and days after inoculation and the establishment of infections; and it cannot be determined if there was fluctuations in the quantity of phenolics between the dhbi and 13dpi and/or the synthesis of *de novo* compounds. Putting to question, the origin of phenolics in crown tissues (preexistent or induced by mechanical wound and/or infection).

There are good reasons to speculate that all phenolics in banana crowns may create a toxic environment for fungi ingress and for the multiplication, notably in the S^- crown samples. Phenolics identified in banana roots (dopamine, ferulic acid …) have been considered as chemical or physical barriers and they play key roles in the defense against nematode infection (Valette *et al*., 1998; Collingborn *et al.*, 2000; de Ascensao & Dubery, 2003; Wuyts *et al.*, 2007). The phenolic content of banana crown could thus be part of the biochemical response of the mechanism involved in banana susceptibility variation. In the present study, in our LC-MS conditions, it was impossible to identify all the phenolics involved in banana susceptibility to crown rot. Indeed, to confirm identification, pure reference molecule for each detected peak should have been available. In addition, they were not biochemically tested for their fungicidal activity *in vitro*, consequently their particular antifungal properties cannot be speculated.

Finally, the influence of severe So-Si ratio modifications on the banana susceptibility to crown rot and on some fruit and banana tree characteristics, as well as a differential accumulation of phenolics in crown tissues has been demonstrated. The results suggest an efficient effect of So-Si ratio modifications on the banana fruit quality potential in terms of phenolic accumulation and present, a valuable implication of phenolics in the biochemical basis of banana susceptibility variation. Hence, this study should be the starting point to understanding the function(s) of these intriguing molecules in the banana crown's defence.

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Chapter V. Conclusions and Perspectives

The understanding of the physiological component involved in crown rot development of bananas could be essential and crucial to build-up alternative control methods in the framework of an integrated control strategy. A formal study on the physiological component of banana fruit was focused on the influence of some environmental pre-harvest factors and the genetic determinant implied in the variation of susceptibility to crown rot. This study has shown the influence of temporal effect, source-sink (So-Si) ratio modification at flowering and hand position on the bunch on crown rot development. Moreover, the study on So-Si ratio influence suggested that phenolics, notably dopamine, could be involved in the quantitative response of bananas to crown rot. However, the influence of some other biotic and abiotic pre-harvest factors on crown rot development as well as on the involvement of the phenolics pool in the mechanism of susceptibility variations could not be assessed. This study was thus focused on two aspects of the fruit physiology under Cameroonian agro-ecological conditions.

In the first part, we highlighted **(I)** the influence **(A)** of geographical and seasonal variations and **(B)** of *Mycosphaerella* leaf spot diseases on the fruit physiological component at harvest vis-à-vis crown rot disease development of bananas. The second part of this research **(II)** was the characterization of the phenolic content of banana crowns of different level of susceptibility to crown rot which have undergone severe So-Si ratio change.

(I)

(A) In part one, the first step in the research was the improvement on the standardization of post-inoculation environmental conditions. The methodology for assessing banana susceptibility to crown rot by artificial inoculations has been enhanced so that little variations in banana fruit's susceptibility to crown rot could be determined and therefore, the reliability of necrotic surface assessments increased. Secondly, an evaluation of the influence of some abiotic pre-harvest factors: production area (low and high-altitude plantations) and seasonal variations (dry and rainy season) on the susceptibility of bananas to crown rot in Cameroon was made. Fruit susceptibility was thus, found to be significantly influenced by the production area. Bananas grown in low-altitude plantations (Dia-Dia and Koumba) were more susceptible to crown rot than those grown in the high altitude plantation (Ekona). Seasonal effect was not significant but some variations, due to a temporal effect, were found in high-altitude production areas where lower disease incidence was found in certain short periods during the dry season. It was therefore suggested that a potential positive effect of highlands

and particularly during the dry months of the year could have an effect on the lesser development of crown rot disease. It appears that the amounts of assimilates accumulated is essential for the regulation of fruit susceptibility. In plantations with high altitude, because of lower temperatures, the speed of fruit filling is slower and it takes more time to reach 900 dd than in lower altitudes. During the long period of growth, fruits accumulate larger amounts of assimilates which, in excess, could contribute to the important energetic demand for secondary metabolic pathways, essential for the formation of defense mechanisms. Furthermore, the higher number of sources (high number of leaves) as well as the low black leaf streak disease (BLSD) severity of banana trees in high-altitude plantation (Ekona) as compared to both low-altitude ones contribute in the reduction of the level of fruit susceptibility. Since BLSD have a strong effect on greenlife, a potential effect of BLSD on banana susceptibility to crown rot is now suggested. However, the physiological mechanisms that could explain the variations of fruit's susceptibility highlighted here are under study.

(B) Due to the potential effect of BLSD on banana's susceptibility to crown rot, it was necessary to assess the relationship between the presence of biotic pre-harvest factors (*Mycosphaerella* leaf spot diseases) on banana trees in Cameroon for BLSD and in Guadeloupe for Sigatoka disease (SD) and the crown rot disease development. The results confirmed MLSD direct effect on the fruit's physiology and highlighted a new effect of BLSD on fruit quality. BLSD was found to significantly influence banana's susceptibility to crown rot and a positive linear correlation was found between the two diseases. Banana fruit grown on plants with light BLSD pressure were less susceptible to crown rot than the ones grown under heavy pressure. On the other hand, it was realized that SD has no effect on banana's susceptibility to crown rot. These observations suggested that BLSD's impact on fruit's susceptibility was more important than SD's impact. Heavy BLSD infection induces differences in leaf surface (source reduction) as well as morphological differences in diameter for fruits under different treatments harvested at the same physiological age. Leaf spot diseases reduce source-sink (So-Si) ratio and induce high competition between banana fruits and the poor availability of assimilates during growth, leading to different level of susceptibility to crown rot. However, heavy SD pressure did not lead to the same effect on fruit's physiology vis-à-vis crown rot development. Physiological mechanisms involved in

host/pathogen interactions such as pathogenic fungus invasion and fruit susceptibility to crown rot disease could be different for BLSD and SD.

PROSPECTS

Some abiotic and biotic pre-harvest factors are known to influence the development of crown rot disease through fluctuations that could be linked either to fruit susceptibility or to fruit contamination. Abiotic factors often weaken plants, making them more susceptible to biotic factors (fungi, nematodes, bacteria and viruses). These pre-harvest factors are keys in the post-harvest development of crown rot disease and its control. Since the quality potential of the fruit depends on pre-harvest factors that influence the level of fruit's susceptibility at harvest, a better understanding of these pre-harvest factors could limit the risk of development of crown rot disease. However, not very much is known about other pre-harvest factors that can influence the level of fruit contamination such as nutrients deficiencies (N, P, K …), amounts of moisture available, mechanical and chemical injury, and light intensity for photosynthesis. Since the So-Si ratio was highlighted along this study as influencing the fruit susceptibility variation, it would be particularly important to assess the light intensity effect.

The altitude influence must be confirmed by setting up experiments in different plantations of variant altitudes especially high-altitude. The presence of BLSD on banana tree could be a limiting factor for the physiological component of the fruits quality. Therefore, it would be significant to define threshold, levels of the BLSD severity without any effect on the bananas susceptibility to post-harvest diseases. These results also question the mechanism involved in banana crown rot susceptibility variations. This question has been studied in second part (**II**).

On the other hand, it is essential to assess one another important part of the fruit quality potential : the parasitic component. Right now, we have no idea on the type of interactions (synergic and/or antagonist) occurring within the broad unspecific and opportunistic parasitic complex responsible of the crown rot disease. Moreover, fungal endophytes have been reported to protect plants (tomato, barley, banana …) against pathogens and pests. The banana

crown interaction with some endophytic microorganisms like the non-pathogenic *Fusarium oxysporium* is not yet study and could be useful in this research topic. It would be thus, important to study the different interactions within the parasitic complex in other to understand the level of fluctuation of banana susceptibility to crown rot and select some potential successful biocontrol agents.

PRACTICAL APPLICATIONS

The two major results (of this part) imply that the application of the fungicides on the banana fruits in order to reduce post-harvest diseases incidence should be directed to the production zones, the seasons of the year and the MLSD pressure.

Based on our results, a reliable methodology for the evaluation of fruit's susceptibility to crown rot is the storage of banana fruits at 13° C for 13dpi (days post-inoculation by *Colletotrichum musae* without artificially ripening with ethylene). Furthermore, the choice of high-altitude production zone and dry season can be more adopted in the context of an integrated strategy reducing or avoiding post-harvest chemical treatment. However, these alternative methods for the control of the development of crown rot disease should also consider the management of BLSD.

(II)

The second part of this work was focused on the phenolic content variations of banana crown which may be linked to fruit's susceptibility variation that have undergone severe modification in So-Si ratio. The influence of severe modification in So-Si ratio was highly significant on fruit's susceptibility to crown rot, fruits with low So-Si ratio being more susceptible than the ones with high So-Si ratio. The severe modification in So-Si ratio was also found to significantly influence some banana trees and fruit characteristics leading to wide variation in banana fruit's morphology. It was suggested that the cutting off of some hands at flowering induces less competition and good availability of assimilates during the growth of the hand left on the bunch. On the other hand, the severe mechanical defoliation at

flowering reduces the photosynthetic activity of the banana tree and also appears to influence the processes determining fruit's susceptibility to the development of crown rot disease.

Samples of extreme treatments with differential susceptibility to crown rot, i.e. high So-Si ratio (12L/1H and S^-) and low So-Si ratio (1L/8H and S^+) were selected for GC-MS, HPLC and LC-MS analyses. Chromatographic analyses were used to characterize the phenolic content of banana crowns and to evaluate their involvement in variations of banana susceptibility in both susceptible (S^+) and less susceptible (S^-) banana crowns at two stages (dhbi and 13dpi). The first focus was on catecholamines characterization by GC-MS, followed by phenolics characterization by HPLC and LC-MS. So far, there is no report in the literature on the phenolic content of banana crowns (S^- and S^+) at two stages (dhbi and 13dpi). GC-MS method allowed a good separation, identification and quantification of dopamine, norepinephrine and normetanephrine (catecholamines). HPLC method enabled a good separation of phenolic standards while LC-MS conditions used allowed the ESI-MS detection of most these standards.

The amount of phenolics accumulated by banana crowns less susceptible (S^-) to crown rot was higher as compared to the susceptible (S^+) ones. Dopamine was identified as the major compound in banana crown. In GC-MS and ESP-HPLC conditions, no difference was found in dopamine content between the healthy and infected crown the dhbi and 13dpi. But in EBP-HPLC conditions, this amount was significantly decreased in the S^+ (1L/8H) crown samples 13dpi, while it remain unchanged in the S^- (12L/1H) at both stages. Norepinephrine and normetanephrine were found in high quantity the dhbi in banana crown, especially in the S^+ crowns. The number of phenolics is more important in the EBP than in the ESP. However, the concentration of dopamine in the crown was significantly higher in ESP as compared to the EBP. The chromatographic profile of banana crown EBP differs quantitatively, but not qualitatively, with regard to the level of susceptibility to crown rot (S^+ and S^-) and the stage (dhbi and 13dpi). In the EBP for both treatment and at both stages, ten major peaks were detected and identified for some, while others remain unidentified. Accumulation in a highly significant level of hydroxycinnamic acids (ferulic acid, coumaric acid and their derivatives) and other unidentified compounds the dhbi, were associated with less susceptibility (S^-) of banana crown samples to crown rot as compared to the susceptible (S^+) ones. However, the level of these phenolics decreased significantly at the 13dpi stage.

Fluctuations in dopamine level between the S^- and the S^+ crown samples, and the dhbi and 13dpi stage can be explained by the potential involvement of dopamine in the banana crown defense response to pathogens attack as a mediator of plant defense. Moreover, the higher amount of norepinephrine and normetanephrine in S^+ crowns can indicate a potential stress in crown tissues. However, physiological mechanisms involve in dopamine accumulation are not yet understood. Dopamine, coumaric acid and its methyl ester, ferulic acid and its acid extraction by-product have been previously identified in banana tissues and they were related to defense mechanisms. Compounds **2**, **3**, **4**, **7** and **8** remain unknown and like the identified compound, the dhbi, they were found in higher amounts in the S^- crowns as compared to the S^+ ones and this level significantly decreased at 13dpi especially in the S^+ crown, except for compound **4** and coumaric acid methyl ester. Maybe all these phenolics have a potential role in banana crown defense. Possible antifungal effects and/or maturation effects can explain the decrease observed at 13dpi stage but this cannot be confirmed with certainty at this stage. The phenolic content of banana crown could thus be part of the biochemical response of the mechanism involved in banana susceptibility variation.

PROSPECTS

Some phenolics are known to be involved in banana tissues response to pathogenic attack. The present results give fundamental information on the implication of phenolics in the biochemical basis of banana susceptibility variation to crown rot disease. However, with the current state of knowledge, this information cannot be useful to direct the crop management. Hence, this study should be the starting point to understand the function of phenolics in banana crown defense. The unknown but interesting compounds obtained during this study having a potential implication in the banana crown defense will be extracted, purified and completely characterized. The fungicidal activities of all these phenolics will then be biochemically tested *in vitro*.

The mechanisms involved in banana crown tissues susceptibility fluctuation are not yet understood. We need to continue the study of the crown defense mechanism in order to understand the biochemical events that occur in banana crown tissues in the first hours and

days after pathogen penetration, before the infection establishment, and till 13dpi stage. It is also important to understand the mechanism of the biosynthesis of phenolics in banana crown in other to have an idea on the fluctuations in the quantity of these phenolics between the dhbi and 13dpi. This will be helpful to determine if these compounds were preexisting and/or *de novo* synthesized (induced by mechanical wound and/or infection, notably wounding during the formation of banana clusters in the packing station). Moreover, it will be important to assess maturation effect (13 day after harvest) on phenolics composition of healthy banana crown.

PRACTICAL APPLICATIONS

Phenolics could be used as chemical criterion for estimation of the level of bananas susceptibility to crown rot. These indicators could be used later in a model of integration capable of providing a decision-making aid to producers and various actors of the banana industry**.** This can lead to the reduction of the pesticides used in banana culture, and more particularly in the elimination of post-harvest fungicidal treatments.