CATECHOLAMINE BIOSYNTHESIS PATHWAY POTENTIALLY INVOLVED IN BANANA DEFENSE MECHANISMS TO CROWN ROT DISEASE

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SUMMARY

Variations in Cavendish bananas susceptibility to crown rot disease have been observed (Lassois *et al.*, 2010a), but the molecular mechanisms underlying these quantitative host-pathogen relationships were still unknown. The present study was designed to compare gene expression between bananas (*Musa acuminata*, AAA, 'Grande-Naine') showing a high post-harvest susceptibility (S^+) and bananas showing a low post-harvest susceptibility (S^-) to crown rot disease. This comparison was performed between crowns (S^+ and S^-) collected one hour before standardized artificial inoculations with *Colletotrichum musae*. Fruit susceptibility was evaluated through lesion size on the crown 13 days later. Gene expression comparisons were performed with the cDNA-AFLP technique (Lassois *et al.*, 2009). This revealed that a gene showing a strong homology with a dopamine-β-monooxygenase (DoH) is differently expressed between S^+ and S^- (Lassois *et al.*, 2011). Furthermore, semi-quantitative real-time RT-PCR analyses between S^+ and S^- were applied to confirm the differential expression results for DoH obtained by cDNA-AFLP. Two biological replicates were tested. These semi-quantitative analyses were performed not only on tissues collected one hour before *C. musae* inoculation but also on crown tissues collected 13 days after inoculation. The real-time RT-PCR confirmed that DoH was upregulated in the S^- tissues collected at harvest, just before *C. musae* inoculation. This gene was also highly upregulated in the S^- tissues collected 13 days after crown inoculation. Similar results were obtained for both biological replicates. Our results suggest that catecholamine's could play a role in banana defense mechanisms to crown rot disease.

Key words: Banana, *Colletotrichum musae*, crown rot, cDNA-AFLP, catecholamine, plant defence response.

INTRODUCTION

Crown rot disease affects export bananas in all producing countries and is considered as one of the main post-harvest diseases of bananas (for a review see Lassois *et al.*, 2010b). Losses of up to 86% have been reported for non-chemically-treated bananas from the Philippines (Alvindia *et al.*, 2000). The symptoms appear on the crown, i.e. the tissue joining the fruit pedicels with each other. Although field infections cannot be excluded, infection occurs mainly during harvest and the trimming of clusters from bunches. The disease develops during shipping, ripening, and storage and has a negative impact on the market value of bananas. It results from the development of several relatively nonspecific pathogens, but

many authors agree on the high pathogenicity of *Colletotrichum musae*, which can trigger an infection from a very small inoculum (Finlay and Brown, 1993; Lassois *et al.*, 2008).

Variations, notably geographic and seasonal, have been reported in the development of crown rot disease symptoms (Krauss and Johanson, 2000; Lassois *et al.*, 2008; Lukezic *et al.*, 1967; Shillingford, 1978). These spatiotemporal fluctuations are suggested to reflect variations in the banana fruit quality potential that develops during growth of banana in the field (Lassois *et al.*, 2010b). The quality potential is crucial at each level of the commodity chain and determines the post-harvest onset or absence of crown rot. It depends both on a physiological and a parasitic components, which in turn are influenced by agrotechnical and pedoclimatic factors. The parasitic component reflects the level of crown contamination by the parasitic complex, as well as the pathogenicity of this parasitic complex. The term "physiological component" refers here to the level of fruit susceptibility to crown rot that develops during banana growth. To evaluate the influence of the fruit physiological component in post-harvest development of the disease it is necessary to overcome the influence of the parasitic component. To do this, fruit susceptibility is measured by lesion size after standardized artificial inoculations (de Lapeyre de Bellaire *et al.*, 2008). Although pedo-climatic conditions and agro-technical factors have been found to influence the development of this post-harvest disease (Krauss and Johanson, 2000; Lukezic *et al.*, 1967), few studies have linked such fluctuations to the field susceptibility (Lassois *et al.*, 2008). Yet some pre-harvest factors, such as the source-sink ratio at flowering and the hand position in the bunch, have been shown to affect the post-harvest development of the disease by influencing banana susceptibility to crown rot (Lassois *et al.*, 2010a). The genetic and molecular mechanisms underlying these quantitative host-pathogen relationships have not been identified. Knowledge thereof is crucial to an integrated understanding of complex plant-pathogen interactions, and may contribute to developing new strategies for crown rot control. Such strategies based on plant defence mechanisms may also offer the promise of sustainable agricultural production and improvement of environmental and human health.

Plants are continuously exposed to attacks by microbial pathogens. Plant resistance to disease is mediated by both preformed defences and inducible defence responses. Microbial attack generally leads to significant transcriptional reprogramming in the host, and plants respond to it by activation of multiple inducible defence responses. One way to understand the phenomena and reactions involved in variation of banana responses to crown rot is to identify genes involved in these processes via the study of their expression. The cDNA-AFLP technique (Bachem, 1996) has been used to compare genome-wide expression patterns and to identify differentially expressed genes potentially related to plant defence. That technique does not require prior sequence information and allows identification of genes without a priori. It can thus be used for any biological system, especially when genomic resources are lacking. In addition, the mechanisms responsible for compatibility interactions and for the quantitative variation of symptoms after infection remain poorly understood, and it is likely that some of the factors involved remain to be discovered.

The present study was designed to compare gene expression in banana crowns (Musa AAA, Cavendish, Grande Nain), from plants of the same cultivar but showing different susceptibility to crown rot due to *C. musae,* in order to identify genes whose influences banana susceptibility.

MATERIALS AND METHODS

Sampling

Plant material

Banana fruits (Musa sp [AAA group, Cavendish subgroup] cv Grand Nain) were harvested from plants grown on the Dia-Dia commercial plantation (PHP) in Njombé, Cameroon. Bunches were harvested at a constant physiological age (Jullien *et al.*, 2008), i.e. when the mean daily temperature sum accumulated by the fruit at the 14°C threshold between flowering and harvest reached 900 dd. As bananas from plants with 12 leaves and 2 hands are less susceptible to crown rot than bananas from plants with 12 leaves and 8 hands (Lassois *et al.*, 2010a), two plants with each configuration were used as source material (providing biological replicates for real-time RT-PCR, see below). The two 12-leaf/2-hand plants were indicated like "S^- plant 1" and "S^- plant 2" (S^- for low susceptibility), and the two 12-leaf/8-hand plants were called "S^+ plant 1" and "2" (S^+ for high susceptibility). Only the second hand of each bunch was collected and each one was divided into 4 clusters of 4 fingers without defects. Crown tissues were collected at two different times: at harvest, 1 hour before inoculation of C. musae (1 hbi - 1 cluster per group), and 13 days post-inoculation (13 dpi - the other three clusters in each group), after ripening (when symptoms appeared). The three crown samples collected 13 dpi were used for susceptibility testing (section 1.2), which confirmed the expected S^+ or S^- status of each source. Sample collected were immediately frozen in liquid nitrogen, freeze-dried at -80°C for 24 h (Telstar, Cryodos, Barcelona, Spain), and stored at room temperature before RNA extraction (Lassois *et al.*, 2009) and molecular analysis.

Evaluation of susceptibility to crown rot

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

The second hand trimmed from the bunch was divided into four similar clusters. Susceptibility of the second-hand bananas to crown rot was evaluated in three of these clusters. Smoothly and similarly cut crowns were obtained with a sharp knife, leaving as much crown tissue as possible. The latex from the crown tissue was eliminated with absorbent paper and the crowns were surface-sterilized by immersion in 50% ethanol. Fifty microliters of *C. musae* conidial suspension containing 10^4 conidia/ml was applied to the centre of the freshly exposed crown tissue and covered with a small paper filter. Two hours after application of the conidial suspension, the clusters were packed in punched polyfilms normally used in the industry, placed in commercial boxes, and stored at 13°C for 10 days to simulate shipment. Artificial ripening was then initiated by dipping the bananas for 5 seconds in an ethrel solution (480 g/l), after which the clusters remained at 20°C for another 3 days before crown rot assessment. The internal progression of the rot was determined by cutting the cluster crown longitudinally in two and measuring the surface of rot spread into the crown, from the original inoculation point. This "internal necrotic surface" (INS), calculated by assuming a rectan-

gular shape, was expressed in mm^2 (Figure 1). Its average value was taken as a measure of second-hand fruit susceptibility to crown rot.

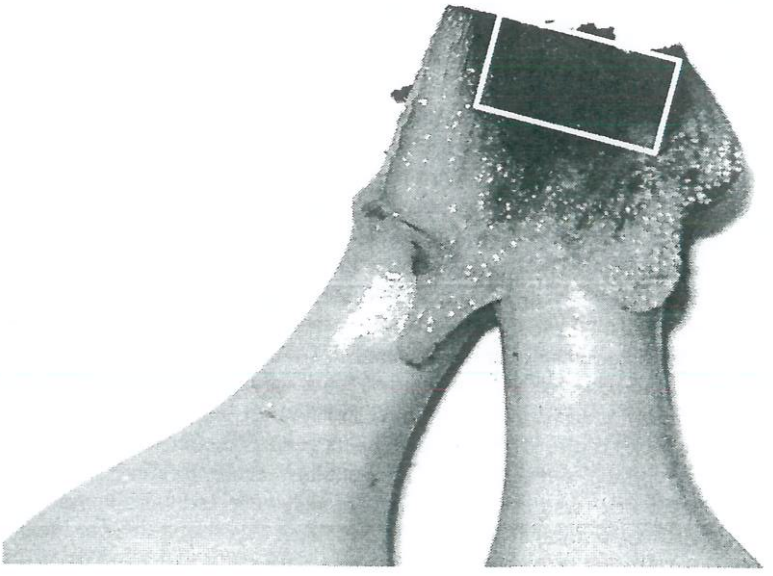

Figure 1. Evaluation of crown susceptibility. The internal necrotic surface (INS) was calculated assuming a rectangular shape and is expressed in mm^2.

Identification of genes differentially expressed according to the crown-rot susceptibility

The RNA isolation and reverse transcription protocols were previously described in Lassois *et al.* (2011). The cDNA pools derived from S^+ plant 1 and S^- plant 1, collected 1 hbi, were subjected to cDNA-AFLP and compared.

cDNA-AFLP

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

Isolation and reamplification of differentially expressed transcription-derived fragments (TDFs)

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

Cloning and sequencing of reamplified differentially expressed TDFs

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

Sequence analysis

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

Transcript derived fragment validation by real-time RT-PCR

The results obtained by cDNA-AFLP were confirmed by real-time RT-PCR with two independent biological replicates. In these confirmation tests, expression-level differences between S^- and S^+ samples were determined for both collection times (1 hbi and 13 dpi). Template-free controls (water instead of cDNA) were included. Three technical replicates were done for each biological replicate. Forward and reverse primers were designed with the online *Primer3* software under default settings and synthesized at Eurogentec (Seraing, Belgium). Real-time RT-PCR was carried out with StepOne+ Real-Time PCR systems (Applied Biosystems, Foster City, CA, USA). Each PCR amplification was performed in 20 µl reaction mixture consisting of 10 µl Maxima Sybr Green qPCR Master Mix 2X (Fermentas), 2 µl each of the forward and reverse primers (10 µM), 1 µl cDNA template (1ng/µl), and 5µl PCR-grade wa-

ter. The cycling conditions were: pre-incubation for 10 min at 95°C, followed by 40 cycles, each consisting of 30 s denaturing at 95°C, 40 s annealing at 52°C, and 45 s elongation at 72°C, the last cycle ending with a final 10-min extension at 72°C. Melting curve analysis (60 to 95°C) was performed to validate the gene specificity of the primers. Differences in expression were calculated according to the "Delta-delta method" (Pfaffl, 2001), elongation factor 1-alpha (Ef1a) being used as an internal control for normalization. RT-PCR analyses and subsequent calculations were performed with the StepOne™ software (Applied Biosystems, Foster City, CA, USA).

RESULTS

Banana samples

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

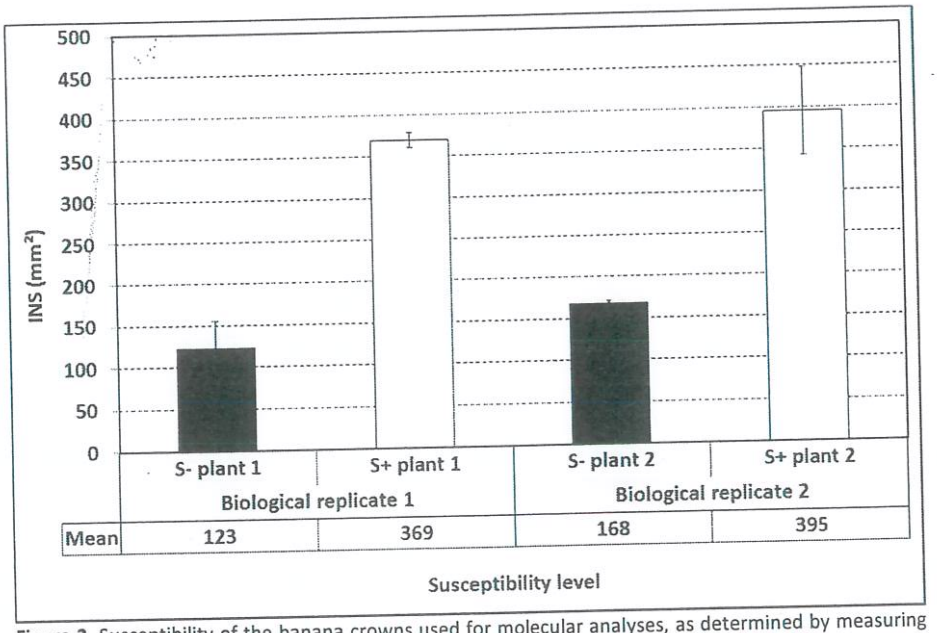

Figure 2. Susceptibility of the banana crowns used for molecular analyses, as determined by measuring the internal necrotic surface (INS, expressed in mm^2). The INS average and standard deviation of the 3 clusters of the same second hand are given for the 2 independent biological replicates and for the 2 different susceptibility level banana plants. 12-leaf/2-hand plants were indicated like "S^- plant 1" and "S^- plant 2" (S^- for low susceptibility), and the two 12-leaf/8-hand plants were called "S^+ plant 1" and "2" (S^+ for high susceptibility)

Identification of the potentially involvement of the cathecholamine biosynthesis pathway in banana defence mechanisms

The cDNA pools from S^- plant 1 and S^+ plant 1 collected 1hbi were subjected to cDNA-AFLP screening. A total of 8 combinations of EcoRI+2/MseI+2 primers were used and more than 1500 bands were amplified from the cDNA pools. The cDNA-AFLP technique revealed a total of 157 amplicons differentially expressed between the high- and low-susceptibility samples collected 1 hbi (i.e. about 10% of the total amplicons on the corresponding profile). Only bands showing a clear intensity difference between S^+ and S^- upon visual inspection, without other bands nearby and with a size superior to 100 bp, were excised from the gels and sequenced. A total of 16 bands were successfully recovered from the gels, reamplified, cloned and sequenced. The nucleotide sequences were then subjected to a BlastX homology search. This revealed that a gene showing a strong homology with a dopamine-β-monooxygenase (DoH) combined with a cytochrome B561 (CB) is differently expressed between S^+ and S^- (Table 1). This gene expression was confirmed by real-time RT-PCR from the same cDNA-AFLP samples but also from another couple of S^+ and S^- banana plants (S^+ plant 2 and S^- plant 2), providing two independent biological replicates (Figure 2). Furthermore, real time RT-PCR also showed that this gene was differently expressed on crown collected 13 days post-inoculation (Table 1). The dopamine-β-monooxygenase appeared upregulated in the S^- tissue collected 1 hbi, becoming highly upregulated in the S^- tissue collected 13 dpi.

Table 1. Characteristics of the differently expressed transcript derived fragments (TDF) identified by comparing the cDNA-AFLP profiles of highly susceptible and less susceptible banana crown tissues collected 1 hour before inoculation. The table also shows the number of cases in which differential expression was confirmed by real-time RT-PCR (two independent biological replicates). BR1: biological replicate 1; BR2: biological replicate 2. The rightmost column provides, on a semi-quantitative scale, an estimate of the extent of differential transcription of the selected genes, as measured in confirmatory real-time RT-PCR assays performed on material collected at both times. A "+" sign means upregulation in S^- as compared to S^+ crown tissue. The "regulation levels" defined for S^- tissue is the mean of the two biological replicates and corresponding: +1: less than 2-fold upregulation; +10: more than 10-fold upregulation.

Genbank accession numbers	Size (bp)	Homology	Annotation	Organism origin	E-value (%similarity)	Real Time RT-PCR confirmation		Regulation level	
						BR1	BR2	1hbi	13dpi
HO058947.1 (Lassois *et al.*, 2011)	348	EEF29330	dopamine beta-monooxygenase	*Ricinus communis*	5e-34 (74%)	yes	yes	+1	+10

DISCUSSION

In the present study, the cDNA-AFLP technique has provided useful information allowing a step towards understanding the mechanisms involved in the physiological component of the fruit quality potential, which determines the banana response to crown rot disease and susceptibility variations. Because of the compatibility of the interaction, we expected significant transcriptional changes to occur among genes associated with cellular changes involved in general defence responses. cDNA-AFLP analysis has enabled us to identify a gene showing homology with a dopamine-β-monooxygenase (DoH) combined with a cytochrome B561 (CB). DoH-CB proteins form a recently identified group of proteins, likely to play a key role in

catecholamine action in plants (Tsubaki *et al.*, 2005; Verelst and Asard, 2004). Catecholamine's are biogenic amines possessing a 3,4-dihydroxy-substituted phenyl ring. Among them are phenyl ethylamine, tyramine, dopamine, norepinephrine, and epinephrine (Kuklin and Conger, 1995). Dopamine hydroxylation by dopamine-β-monooxygenase leads to synthesis of norepinephrine, which is subsequently methylated to epinephrine. In banana fruits, dopamine is formed only through hydroxylation of tyramine (Smith, 1980). From a broad range of plants, bananas, and particularly Cavendish bananas, have the richest content in catecholamine's and particularly in dopamine: 100 µg/g FW in Cavendish banana peel as compared with 7 µg/g FW for potato, the second richest plant of this list (Kulma and Szopa, 2007). This observation suggests that catecholamine's might play an important role in banana physiology.

Catecholamine biosynthesis and the catecholamine content depend on various factors: plant development stage (Swiedrych *et al.*, 2004b; Szopa *et al.*, 2001), growth conditions (Swiedrych *et al.*, 2004b), and environmental signals (Kulma and Szopa, 2007) such as the pH of the environment (Homeyer and Roberts, 1984), brightness (Protacio *et al.*, 1992), temperature (Swiedrych *et al.*, 2004), and stress conditions including pathogen attack (Facchini *et al.*, 1996), drought, ABA treatment, UV light, and wounding (Swiedrych *et al.*, 2004b; Swiedrych *et al.*, 2004). Wound tissue formation is accompanied by a rise in the dopamine concentration, and wounded tissues appear to stimulate dopamine production in the surrounding healthy tissue (Steelink *et al.*, 1967). The dopamine content of banana is also determined by the extent of the contribution of the "acuminate" genome to the genotype (Griffiths, 1961).

Various functions, including plant pathogen resistance, have been proposed for catecholamine's (Swiedrych *et al.*, 2004; reviewed in Kulma and Szopa, 2007). Effects are probably due to the antioxidant properties of catecholamine's themselves and also to cell-wall deposition and to the antioxidant properties of derivatives (Kulma and Szopa, 2007). Whether catecholamine's can stimulate some defence responses more directly remains to be seen (Kulma and Szopa, 2007).

Catecholamine's are metabolized via at least three pathways, involving methylation, conjugation with other phenolic compounds or oxidation (Kulma and Szopa, 2007). This can lead to formation of derivatives active in plant defence, such as alkaloids and phenolic compounds (Kulma and Szopa, 2007).

It is well known that dopamine and tyramine (Yang *et al.*, 2004) are major substrates of polyphenol oxidase in banana fruit. The enzyme and its substrates are believed to be located, normally, in separate cell compartments, but they can come in contact with each other after membranes have been disrupted (Promyou *et al.*, 2008; Vaughn and Duke, 1984; Wuyts *et al.*, 2007). The intermediate quinines, formed by the action of polyphenol oxidase on dopamine, are more reactive and possibly toxic (Appel, 1993). Subsequently there occurs polymerization to polyphenols responsible for the darkening of tissue during lesion formation and believed to seal off the tissue to limit secondary infection or the further spread of pathogens. Enzymes produced by pathogens are inactivated by quinines and plant proteins become unavailable for nutrition.

Catecholamine, dopamine, or oxidation products thereof are suggested to be involved in banana resistance mechanisms (Mace, 1963; Muirhead and Deverall 1984, Valette *et al.*, 1998; Wuyts *et al.*, 2006; Wuyts *et al.*, 2007). Studies have also linked resistance to *C. musae* to a brown necrotic reaction within the peel (Brown and Swinburne, 1981), suggestive of an involvement of dopamine oxidation. It is noteworthy that the peripheral necrosis observed here in the more tolerant banana crowns showed a specific red-brown colour.

It would be interesting to learn more about the role of dopamine-β-monooxygenase and other catecholamine's in the response of bananas to crown rot. We have shown that the dopamine-β-monooxygenase gene is overexpressed in bananas less susceptible to the disease; however, we do not yet have information on this enzyme and its substrates in the crowns. By correlating the levels of various catecholamine's' in crowns with different levels of susceptibility it might be possible to gain insight about their respective roles. Furthermore, quantification of the potential antifungal activities of catecholamine's could indicate whether they are active by themselves in defense mechanisms or whether they are precursors of other active compounds.

Finally, it is necessary to evaluate the expression levels in other situations of susceptibility variation. To be sure that they are really involved in quantitative regulation of banana defence responses and not in other pathways that might be triggered by a change in the source-sink ratio quite independently of any plant defence response.

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