CECÍLIA CHAGAS DE FREITAS

MOLECULAR, BIOCHEMICAL AND MICROSCOPIC ASPECTS OF THE CASSAVA -*Xanthomonas anoxopodis* pv. *manihotis* INTERACTION

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2014

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
F866m
2014

Freitas, Cecília Chagas de, 1987-
Molecular, biochemical and microscopic aspects of the cassava - *Xanthomonas axonopodis pv. manihotis* interaction / Cecília Chagas de Freitas. – Viçosa, MG, 2014.
x, 48f. : il. (algumas color.) ; 29 cm.

Orientador: Luis Claudio Vieira da Cunha.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Referências bibliográficas: f.40-48.

1. Mandioca - Doenças e pragas. 2. *Xanthomonas axonopodis pv. manihotis*. 3. qRT-PCR. I. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-graduação em Fitopatologia. II. Título.

CDD 22. ed. 633.682

CECÍLIA CHAGAS DE FREITAS

MOLECULAR, BIOCHEMICAL AND MICROSCOPIC ASPECTS OF THE CASSAVA -*Xanthomonas anoxopodis* pv. *manihotis* INTERACTION

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Magister Scientiae*.

APROVADA: 27 de Fevereiro de 2014

Francisco Murilo Zerbini Junior

Renata Sousa Resende

Fabrício de Ávila Rodrigues
(Coorientador)

Tocio Sediyama
(Coorientador)

Luís Cláudio Vieira da Cunha
(Orientador)

DEDICATÓRIA

A Deus,

Aos meus pais, José Libério e Maria da Glória

Ao meu avô, 'Seu Chico'

Às minhas irmãs e sobrinhos

Ao meu grande amor, Wanderson

“Dans les champs de l'observation le hasard ne favorise que les esprits préparés.”
(Louis Pasteur)

AGRADECIMENTOS

À Deus, por minha vida e tudo que sou, sem ele eu nada seria.

Aos meus pais, Maria da Glória e José Libério, pelo amor, paciência e força, por estarem sempre do meu lado me ensinando a ser uma pessoa do bem.

Ao meu namorado Wanderson, pela companhia, amor, paciência e por toda ajuda prestada a mim durante toda condução dos experimentos.

Às minhas irmãs Alessandra, Cynthia, Cássia e aos sobrinhos Carolina, Jéssica, Anna Luyza, Jamily, Geise, Juninho, Nathália e Alice pela presença nos momentos difíceis.

Aos meus familiares e amigos, pela torcida.

À Universidade Federal de Viçosa e ao Departamento de Fitopatologia, pela oportunidade de realização do Mestrado.

Ao professor Luís Cláudio pela orientação, amizade, confiança e pelas palavras de apoio a todo o momento.

Ao professor Fabrício pela co-orientação, amizade, confiança e ajuda sempre que eu precisei.

Ao professor Tocio pela co-orientação e por estar sempre disposto a me ceder material de trabalho.

À Renata, que além de amiga querida, foi peça fundamental nas análises bioquímicas.

Ao professor Murilo Zerbini por gentilmente aceitar participar da banca de defesa.

À minha grande amiga Gisele Domiciano, por ter me ensinado tudo que ela sabia.

Aos funcionários do DFP, principalmente ao Macabeu, Bruno e Patrícia, pela amizade.

Ao CNPq, pela concessão da bolsa de estudo.

À EMBRAPA e Saulo, pela concessão dos isolados e materiais de plantio.

À FAPEMIG, pelos recursos do projeto (PROCESSO Nº: CAG - APQ-01907-12).

Ao pessoal do Laboratório de Bacteriologia, principalmente ao Lucas e a Lilian por toda ajuda durante a execução do meu trabalho.

Ao Rafael, Brás e Fernanda, pela disponibilidade e ajuda no RT-qPCR.

Ao Dr. Camilo Lopez pela disponibilidade em me enviar as sequencias dos primers usados no RT-qPCR.

Aos Laboratórios do professor Fabrício, professora Claudine, professor Murilo Zerbini, professor Eduardo Mizubuti, professor Cláudio Mafra, professor Luciano Fietto, pelo suporte e colaboração de todos.

Ao Núcleo de Microscopia e Microanálise da UFV, principalmente a Karla, por toda ajuda e paciência na utilização do microscópio confocal.

A todos que de alguma maneira me ajudaram Jaime, Aline, Frankylin, Murilo, Glória, André, Lucas, Fábio, Silvia, Adriana, Leonardo, Lívio, Isaias e foram responsáveis por este trabalho acontecer.

OBRIGADA A TODOS!!!!

BIOGRAFIA

Cecília Chagas de Freitas, filha de José Libério de Freitas e Maria da Glória das Chagas Freitas, nasceu em 16 de fevereiro de 1987, em Belo Horizonte, Minas Gerais, Brasil.

Em março de 2007 iniciou o curso de Agronomia na Universidade Federal de Viçosa (UFV), Minas Gerais, graduando-se em janeiro de 2012.

Durante a graduação, foi bolsista no Laboratório da Interação Planta Patógeno/ Departamento de Fitopatologia sob a orientação do Prof. Fabrício Ávila Rodrigues.

Em março de 2012, iniciou o Curso de Mestrado em Fitopatologia pela UFV, sob a orientação do Prof. Luís Cláudio Vieira da Cunha.

SUMÁRIO

RESUMO

FREITAS, Cecília Chagas de, M.Sc., Universidade Federal de Viçosa, Fevereiro de 2014. **Aspectos moleculares, bioquímicos e microscópicos da interação mandioca - *Xanthomonas anoxopodis* pv. *manihotis***. Orientador: Luís Cláudio Vieira da Cunha. Coorientadores: Fabrício de Ávila Rodrigues e Tocio Sediyama

A mandioca (*Manihot esculenta*) é cultivada essencialmente nas regiões tropicais e subtropicais e é a quarta cultura alimentar mais importante do mundo. A bacteriose da mandioca, causada pela bactéria *Xanthomonas axonopodis* pv. *manihotis* é umas das mais importantes doenças da mandioca, podendo causar perdas de até 100% na produção. Entretanto, informações sobre mecanismos bioquímicos e moleculares envolvendo a interação *Xam*-mandioca, bem como a translocação vascular no interior dos vasos da planta ainda é limitado. Com o objetivo de entender os mecanismos envolvidos nas respostas de defesa da planta contra *Xam*, cultivares de mandioca, Cacau e Fécula Branca, suscetível e resistente, respectivamente foram usados nas análises das enzimas de defesa, peroxidase (POX), polifenoloxidase (PPO), catalase (CAT), β,1-3-glucanase (GLU), quitinase (CHI) e fenilalanina anônioliase (PAL), bem como na acumulação de peróxido de hidrogênio (H_2O_2) e aldeído malônico (MDA). Usando o RT-qPCR, o perfil da expressão dos genes relacionados com defesa, peroxidase (POX72), catalase (CAT2), β,1-3- glucanase (GLU), qhitinase (CHI), proteína relacionada a patogênese 5 osmotina (PR-5), etileno ERF2 (ET) e proteína CC-NBS-LRR (CC) foram estudados. Adicionalmente, a translocação vascular de *Xam* foi analisada por microscopia confocal, usando um isolado marcado com GFP. A incidência da bacteriose e a população de *Xam* foram significativamente menores no cultivar Fécula Branca quando comparado com Cacau. As atividades da POX, PPO e GLU foram detectadas 24 horas após a inoculação (hai) em Fécula Branca. Em contraste, a atividade em Cacau foi detectada apenas ás 72 hai. Adicionalmente, as atividades da PAL, CHI e CAT foram maiores em Fécula Branca. A concentração de MDA não mostrou nenhuma correlação com o progresso da doença e a concentração de H_2O_2 apresentou um pico ás 120 hai, exatamente no decréscimo do crescimento bacteriano. Baseado no RT-qPCR as expressões de POX, GLU e PR-5 foram detectadas mais cedo no cultivar resistente (24 hai), em contraste com o susceptível (72 hai para POX e GLU e 120 hai para PR-5). As expressões de ET, CAT e CHI foram maiores em Fécula Branca e a expressão de CC não apresentou nenhuma resposta nos tempos de coleta. A translocação de *Xam*-GFP pode ser detectada aos 6 cm para baixo e para cima nos caules de mandioca. Em conjunto, estes resultados mostram que uma resposta mais eficiente de defesa da mandioca baseada na atividade e expressão de proteínas relacionadas com defesa e genes respectivamente, limitou o crescimento bacteriano e contribuíram para resistência da mandioca à bacteriose.

ABSTRACT

FREITAS, Cecília Chagas de, M.Sc., Universidade Federal de Viçosa, February, 2014.**Molecular, biochemical and microscopic aspects of the cassava -*Xanthomonas anoxopodis* pv. *manihotis* interaction.** Advisor: Luís Cláudio Vieira da Cunha. Co-Advisors: Fabrício de Ávila Rodrigues and Tocio Sediyama

Cassava (*Manihot esculenta*), cultivated essentially in tropical and subtropical regions, is the fourth most important food crop in the world.. Cassava bacterial blight (CBB) caused *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) is one of the most important cassava diseases, causing yield losses that can reach 100%. However, information about the molecular and biochemical mechanisms involved in *Xam*-cassava interaction as well as its vascular translocation inside cassava vessels is still limited. To get insight into the mechanisms involved in the defense response of the susceptible and resistant cassava cultivars, Cacau and Fécula Branca, respectively, against *Xam,* were analyzed the activities of the defense-related proteins peroxidase (POX), polyphenol oxidase (PPO), catalase (CAT), β,1-3- glucanase (GLU), chitinase (CHI), and phenylalanine ammonialyase (PAL), including the accumulation of hydrogen peroxide (H_2O_2), and malondialdehyde (MDA). Using RT-qPCR, the expression profile of the defense-related genes peroxidase (POX 72), catalase (CAT2), β,1-3- glucanase (GLU), chitinase (CHI), pathogenesis related protein 5 osmotin (PR-5), ethylene ERF2 (ET) and CC-NBS-LRR like protein (CC) was studied. In addition, the vascular translocation of *Xam* was analyzed by confocal laser scanning microscopy using a GFP-tagged isolate. CCB incidence and *Xam* population was significantly lower on Fécula-branca when compared to Cacau. POX, PPO and GLU activities were detected 24 hours after inoculation (hai) in Fécula-branca. In contrast, activity on Cacau could be detected only about 72 hai. In addition, PAL, CHI, and CAT activity was higher in Fécula-branca. The MDA concentration did not show any correlation with the disease progress and the H_2O_2 concentration presented a peak at 120 hai exactly at the decrease of bacterial growth. Based on RT-qPCR, POX, GLU, and PR-5 expression was detected earlier on the resistant cultivar (24 hai) in contrast to the susceptible one (72 hai for POX and GLU; 120 hai for PR-5). ET, CAT, and CHI expression was higher on Fécula Branca and CC expression did not show any response. *Xam*-GFP vascular translocation could be detected at 6 cm downstream and upstream on cassava stems. Altogether, these results show that a more efficient cassava defense response based on the activity and expression of defense-related proteins and genes, respectively, limited bacterial growth thus contributing to cassava resistance to CBB.

1. INTRODUCTION

Cassava (*Manihot esculenta*) is a woody shrub of the Malphigiales order, within the Euphorbiecea family. It is the only one among the 98 known species within the Euphorbiaceae family, grown for feeding purposes. Some studies have shown that cassava is native from vegetation associated with rivers, in the Amazon rainforest and Cerrado transition, near Peru and Brazil (Cardoso *et al.*, 2006) . The latest agricultural and archaeological data indicate the Amazon region as the probable cradle of cassava, while alternative versions consider its emergence in Peru (Andes) or even in Africa. Native from tropics, cassava is favorably cultivated in tropical and subtropical regions, with ideal temperature ranging from 24 to 25°C.

Currently, cassava is the fourth most important food crop in the world mainly in the tropical region. Cassava root and its sub products are a source of food for more than 800 million people around the world (Fao, 2012). Highly adapted to nutrient poor soils, it is also considered a good source of calories feeding and energy production. The cassava root is used as feedstock for many industrial products, such as: starch, flour, chips and pellets to animal feeding and culinary use. The cassava starch an industrial product extracted from its roots represents around 30% of the Brazilian market. In the world, it is the second most important source of feedstock for starch production, after corn (Vilpoux, 2006).

The five principal producers in order of importance are: Nigeria, Indonesia, Brazil, Thailand and Congo (Fao, 2012). Brazil´s production corresponds to 9% of total produced in the world. Currently the Brazilian production is around 23 million ton of roots, which corresponds 43% of Nigeria’s production. The production is concentrated on the states of Pará, Bahia, Maranhão, Paraná e Ceará (Ibge, 2013). It is estimate that the activities from cassava cultivation and the flour and starch processing generate approximately 1 million direct jobs (Cardoso *et al.*, 2006). The large domestic demand for cassava flour, starch and other derivatives is sufficient to absorb almost all of the national production, and starch derivatives represent an important product for exportation.

Under certain environmental conditions and, depending on the cultivar susceptibility, some diseases can cause significant cassava yield losses and, affect root quality that can compromise its use for industrial processing. Among them, the disease known as cassava bacterial blight (CBB), caused by the Gram negative bacteria *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) is considered one of the most important diseases that affect the crop. *Xam* is a gram-negative bacterium, unpigmented in culture medium with a single polar flagellum. The bacteria are catalase positive, hydrolyse esculin and milk aerobically and are capable of using sodium polipectato. It also asparaginase, deaminase, urease and phenylalanine negative. This pathogen was first detected in Brazil in 1912 and was later reported in other countries in Latin America, Asia and Africa. It is currently distributed in all cassava producing regions of the world. The pathogen was first named as *Bacillus manihotis* (Arthaud - Berthet) Starr. Later it was called *Phytomonas manihotis* (Arthaud – Berthetand Bondar) Viegas, and subsequently included in the genus *Xanthomonas* as *X. manihotis* (Arthaud - Berthet) Starr. In 1974 with the eighth publication of Bergey 's Manual of Determinative Bacteriology , the bacterium was considered a pathovar of *X. campestris*, varying only by the specificity of cassava plants , receiving the name of *X. campestris* pv. *manihotis* (Berthet - Bondar). In 1996 was proposed a division into two species, one part remained *X. campestris* and the other part *X. axonopodis*, changing its name to *X. axonopodis pv . manihotis (Young et al., 1996).*

In the field, the spread of the disease is done mainly by water drops from rain. Contaminated tools also help in the spread of the disease, especially when harvesting is carried out together with the preparation of the material for planting (Lozano, 1986). The pathogen penetrates the host tissue through stomata or wounds, then crosses the spongy parenchyma and reaches the plant vascular system. The ideal conditions to disease development are high moisture and temperature between 22-26°C, under this conditions the bacterium takes about 12 hours to reach the xylem and 11-13 days to cause symptoms. The disease is most severe during periods when large fluctuations of day-night temperature of 15-30°C occur in the rainy season (Lozano and Sequeira, 1974).

The disease normally starts with inoculums from contaminated stem cuttings and can lead to both systemic and foliar symptoms. The systemic infection is more severe and the disease intensity is related with the plant genetic resistance and plant age as well as the environmental conditions and the bacterial virulence/ aggressiveness. When the bacteria reach the vascular system, it can cause vascular obstruction, either by accumulation of bacterial cells or formation of callose by the plant and tissue discoloration. It is possible to observe the presence of canker in the stem, in the petioles and, in the veins on the leaf, through which intense bacterial exudation can occur (Miura and Monteiro, 1997). Subsequently, symptoms evolve to total or partial wilting, leaf drop and sometimes plant death. In the aerial part of the plant, symptoms initially appear as soaked and necrotic angular lesions. Later these lesions develop and become irregular; leaves covering large tracts, acquiring light brown to brown, with blue coloration in the abaxial side (Miura and Monteiro, 1997).

The occurrence of this disease has increased substantially over the years in various countries in Latin America and Africa (Verdier *et al.*, 2004). In Brazil this disease has caused significant losses in the field, mainly in the Southern regions states of Mato Grosso and Paraná, Distrito Federal and Northeast. CBB can be managed through the application of cultural practices to reduce the spread and survival of the pathogen, use of healthy materials and the use of resistant cultivars. The most efficient control strategy is the use of resistant cultivars (Fukuda, 2006). The difference between resistant and susceptible cultivars is expressed as a variation in the pathogen's colonization rate. For this reason, the sources of resistance known until this moment are usually considered quantitative. Resistance based on hypersensitivity response has not been discovered yet. Yield losses in the order of 100% and 5-7% have been reported in susceptible and resistant cultivars, respectively (Lozano, 1986). Therefore, for the sustainability of the production system, a detailed knowledge of the response of different cultivars to *Xam* is a key point to the development of more effective control strategies.

Defense responses in plants

During evolution plants have evolved numerous defense responses mechanisms, specific against various forms of biotic and abiotic stress. This made possible the existence of features that allow the survival of the species in nature. In this sense, the plants can change their developmental process plan and circumvent unfavorable situations, such as the attack of pests or pathogens (Pinto *et al.*, 2010). The survival of plants, therefore, depends on their ability to adapt these to adverse conditions, which acts as a selective pressure. The adaptation and resistance are reflected by changes in the metabolism of the plant cell, including the synthesis of defense related proteins. These proteins have multiple roles in the resistance and survival of the plant, either acting directly, fighting the pathogen, or indirectly by altering the structure of the cell and cellular functions (Mysore and Ryu, 2004; Jones and Dangl, 2006). Plants subjected to biotic or abiotic stresses, undergo changes in their pattern of protein expression, either inhibiting or inducing the synthesis of certain protein constituents (Ryan, 2000).Among the defense proteins induced in plants, the polyphenol oxidases (PPO) (Constabel and Ryan, 1998) peroxidases (POX), phenylalanine ammonia-lyase (PAL), and pathogenesis-related (PR) proteins (Linthorst and Van Loon, 1991; De Wit, 2007), are best described for their roles in plant defense (Chen, 2008).

Changes in the synthesis of pathogenesis related proteins (PRs) resulting from the interaction between plant and pathogen, are one of the most noticeable and well studied (Linthorst and Van Loon, 1991; Kang *et al.*, 2005; Soosaar *et al.*, 2005). First identified in tobacco leaves, during a hypersensitive response to tobacco mosaic virus (TMV) infection, these proteins have subsequently been described in other plants in response to various pathogens (Joosten *et al.*, 1990; Linthorst and Van Loon, 1991).

The enzyme phenylalanine ammonialyase (PAL) has the amino acid phenylalanine as a substrate. This is a key enzyme for all routes of synthesis of phenolic compounds, which are involved in resistance to pests and pathogens. This enzyme is responsible for the first of a series of metabolic reactions, which generates numerous natural phenylpropanos based products, including lignin, some pigments and protectors against UV light. The production of this enzyme is regulated during plant growth, but it is also induced by various environmental stimuli such as infection, injury, heavy metal contamination, light, and hormones (Rahman and Punja, 2005).

A number of PPO and POX accumulate in infected tissues thus playing an important role in the defense mechanisms of plants against pathogens (Pinto *et al.*, 2010). These enzymes promote the oxidative degradation of phenolic compounds near the site of infection, resulting in the appearance of dark coloration as a result of from the oxidative polymerization of quinines (Macheix *et al.*, 1986; Bolwell *et al.*, 2002) Different reports have shown a positive correlation between the POX and PPO activities and host resistance against pathogens.

β, 1-3 glucanases are enzymes that hydrolyze glycosidic type β - 1, 3 present in β - D - glucans, releasing glucose as the main product. The glucans are produced by fungi and bacteria, which participate in the formation of the cell wall, the main structural feature of β - glucans is to assist in maintaining the rigidity and integrity of the cell wall. The β -glucans can be degraded by β - glucanases, which are multifunctional enzymes that hydrolyze polysaccharides such as cellulose and other β -glucans (Pitson *et al.*, 1993; Kumar and Deobagkar, 1996). These hydrolases participate directly in the control of disease process because hydrolyze β -1, 3 - 1, 6- glucans cell wall constituents of some pathogens (Fleuri and Sato, 2008; Iorio *et al.*, 2008).

Chitinases are enzymes produced by a wide variety of plants as a defense mechanism against insects, fungi and bacteria attacks. This enzyme uses chitin as a substrate producing biologically active oligosaccharides of different sizes. Evidence indicates that chitinases have a direct role in protection by directly attacking the polymers of chitin, which is the major cell wall component of most fungi (Collinge *et al.*, 1993).

Catalase is an enzyme responsible for the breakdown of H_2O_2 into water and molecular oxygen. Hydrogen peroxide also has an important role in defense mechanisms, because in addition to its direct antimicrobial effect, it can stimulate (via redox signaling) other defense mechanisms such as the production of lignin and phytoalexins and hypersensitivie response, leading to cell death, thus preventing the growth and development of the pathogen.

The hormone molecule ethylene (ET) plays a critical role in the activation of plant defenses against different biotic stresses through its participation in a complex signaling network that involves jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA). Pathogen attack, wounding, and herbivores trigger asymmetric activation of this defense signaling network, thereby affecting the final balance of interactions between its components and establishing a targeted response to the initial threat. Ethylene's contribution to the modulation of this defense network relies on the complexity of multigene families involved in ET biosynthesis, signal transduction, and crosstalk and enabling the plant to fine-tune its response (Adie *et al.*, 2007).

Microscopic aspects

Development of systemic infections caused by gram-negative vascular plant-pathogenic bacteria has been previously investigated (Ramey *et al.*, 2004; Danhorn and Fuqua, 2007). These studies were most thoroughly performed with *Pantoea stewartii* subsp. *stewartii*, causing wilt and leaf blight in sweet corn (Koutsoudis *et al.*, 2006; Herrera *et al.*, 2008), and *Xylella fastidiosa*, a host-specific pathogen of grape that causes leaf scorching and berry desiccation (Newman *et al.*, 2003; Baccari and Lindow, 2011). Other vascular bacteria which have been studied include *Erwinia amylovora*, the causal agent of fire blight in apple and pear (Koczan *et al.*, 2009); *Xanthomonas campestris* pv. *campestris*; the causal agent of black rot on crucifer plants (Dow *et al.*, 2003); and *Ralstonia solanacearum*, which causes lethal wilt on many plants (Kang *et al.*, 2002).

The introduction of green fluorescence protein (GFP) as a marker of gene expression (Prasher *et al.*, 1992; Chalfie *et al.*, 1994) has facilitated research in localization and identification of the GFP tagged bacteria in the infected cells and plant tissues. The use of GFP has become a powerful and valuable tool for addressing most of the biological issues in plant –microbe interactions (Chalfie *et al.*, 1994). The enormous advantage of using GFP over conventional microscopic probes is that it allows the observations of the protein in living cells. Bacterial cells tagged with GFP can be enumerated *in situ* and samples do not need to be fixed, hybridized or stained (Tombolini *et al.*, 1997).

In spite of the importance of cassava for nourishment and yield losses caused by CBB, little is known about the interaction between *Xam* and its host plants, especially the mechanisms controlling the defense response of cassava cultivars. We hypothesize that different cassava cultivars have distinct defense responses against *Xam* infection.

Thus, this study aimed at characterizing the biochemical and molecular responses of cassava cultivars during its interaction with *Xanthomonas axonopodis* pv. *Manihotis*. First, we sought to understand the defense responses of susceptible (Cacau) and resistant (Fécula Branca) cassava cultivars, in relation to infection by this pathogen, through analysis of biochemical activity of defense related protein as well as the expression profile of defense related marker genes by Reverse Transcriptase real time PCR (RT-qPCR). Second, we aimed at investigating how it is the bacterial behavior inside the cassava tissues by confocal laser scanning microscopy (CLSM).

2. MATERIAL AND METHODS

2.1. Cassava cultivars and bacterial isolates

The cassava cultivars Fécula Branca (resistant to CBB) and Cacau (susceptible to CBB) were selected for this study. The bacterial isolates were kindly provided by Dr. Saulo Oliveira, from EMBRAPA Mandioca e Fruticultura (CNPMF). Isolates were cultivated in 523 (Kado and Heskett, 1970) solid medium and incubated at 28°C during 16-24 hours. Cassava plants were obtained by planting cassava buds on commercial substrate (ProPlant). After 15 days the seedlings were transplanted to 1 kg pots. Fourty-five days old plants were inoculated on stems using the toothpick method and on leaves by spraying a suspension of 10^7 CFU/ml (OD600=0,1). Plants were incubated in a mist chamber for a period of 24 hours before and after inoculation. After removal plants were kept at 28°C and disease incidence was evaluated up to 12 days after inoculation. The experiments were conducted on Bacteriology Laboratory at UFV (Viçosa, Minas Gerais, Brazil).

2.2. Bacterial transformation, plasmid stability and quantification

Bacterial quantification on tissues was carried out by serial dilution plating the bacteria in 523 medium containing the desirable antibiotic. Two isolates of *Xam* were transformed with the four selected plasmids (Table 1). Plasmid miniprep (Maniatis et al., 1982) preceded the transformation to obtain a higher concentration of the plasmid. For this, the cells were growth in 4 ml of liquid Luria-Bertani medium (LB), containing the respective antibiotics for each plasmid. The cells were shaken for 24 hours. A centrifugation was made for 3 minutes at 14500 *g* and the supernatant was discarded. The pellet was washed with a Lysis Solution I. A new centrifugation was taken and the supernatant was discarded. Followed again by adding Lysis Solution I, in addition to the Lysis Solution II and Lysis solution III. Performed by centrifugation for 5 minutes at 14500 *g*, 500 µl of the supernatant transferred to a new Eppendorf tube. The plasmid was decanted by centrifugation adding 500 µl of ice-cold isopropanol (4°C) in the supernatant. After 3 successive washes of the pellet with 70% ethanol, this was air dried for 15 min, ressuspended in TE and subsequently treated with RNase.

Table 1. List of plasmids used for transformation

Plasmids	Marker Genes	Insertion	References
pVSP61	Kanamycin resistance	Replicative	(Loper & Lindow, 1994)
pProbe-AT-GFP	Ampicillin resistance and GFP expression	Replicative	(Miller et al., 2000)
pRZT3-GFP	Tetracycline resistance and GFP expression	Replicative	(Bloemberg et al., 2000)
pAG-408 (miniTn5-GFP)	Gentamicin resistance and GFP expression	Integrative	(Suarez et al., 1997)

For preparation of competent cells, isolates were grown in 50 ml LB medium until OD_{600}= 0.6 was reached. Then, bacterial cells were cooled on ice for 15-30 minutes and centrifuged at 4°C for 10 minutes at 1950 *g*. The supernatant was discarded and the bacterial pellet washed twice with 30 ml of Milli-Q ice water (4°C), dissolved gently and pelleted again using the same conditions. A new centrifugation was performed and then the pellet was resuspended in 550 µl of 10% glycerol. Aliquots were flash frozen and immediately stored at -80°C. Electrocompetent cells of each isolate were transformed by electroporation, using Bio-Rad apparatus, 2.5 kV, 25 mF and 200V in 0.2 cm cuvettes. The transformants were incubated in liquid LB medium, without antibiotic, for four hours, under constant shaking and then plated on solid LB medium containing the required antibiotics of each plasmid and incubated at 28°C. Individual colonies of each isolate was selected and stored at -80°C in 30% glycerol. Growth curve assays comparing the wild type and transformed isolates were carried out incubating the isolates in liquid 523 medium under agitation and measuring the OD_{600} periodically up to stabilization of growth. The wild and transformed isolates were inoculated on stems of susceptible cassava plants and disease progress was evaluated. Plasmid stability tests were conducted by inoculating stems of the susceptible cultivar Cacau with transformed isolates. Seven days after inoculation stems were collected, homogenized in 10mM $MgCl_2$ and plated in 523 solid medium with and without antibiotic. Fourty-eight hours after incubation at 28°C, the colonies were counted on both plates. An aliquot of homogenized tissue was taken and reinoculated in healthy cassava plants. This procedure was conducted for a period of 15 days. This experiment was performed using a completely randomized design with 5 replications and all variables were analyzed by the analysis of variance (ANOVA), and the means from treatments for each isolate were compared by the *t* test ($P < 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC).

2.3. Resistance evaluation

A total of fifteen plants of each cultivar were inoculated with the virulent bacterial isolate. Tewnty-four hours before and after the inoculation plants were incubated in a mist chamber and then transferred to a growth chamber at 28°C (UR= 90%, photoperiod of 12 hours with light and 12h without light). Cassava stems were inoculated using the toothpick method and disease incidence and average wilt time were evaluated during 10 days. This experiment was performed using a completely randomized design and all variables were analyzed by the analysis of variance (ANOVA), and the means from treatments for each isolate were compared by the *t* test ($P < 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC).

2.4. Biochemical analysis

2.4.1. Tissue collection and samples maceration

Stem tissue was collected 0, 24, 72, 120 and 168 hours after inoculation (hai). A total of 0.3 g of stem tissue from cultivar Cacau and Fécula Branca, inoculated or noninoculated with *Xam* (only toothpick wounded; free of bacteria) was weighed and immediately frozen in liquid nitrogen and macerated in a mortar to obtain a fine powder. The powder was homogenized in 2 ml of 100mM potassium phosphate buffer (pH=6.8) containing 1mM of phenylmethylsulfonyl fluoride (PMSF) (Sigma), 0.1mM of Ethylenediamine tetraacetic acid (ETDA) (Sigma) and polyvinylpyrrolidone (PVP) 1% (w/vol) (Sigma). After maceration the samples were centrifuged at 15000 *g* for 25 minutes at 4°C. The supernatant, containing the total protein extract was divided into aliquots and stored at -80°C.

2.4.2. Peroxidase (POX) activity

The POX activity was determinate according Kar and Mishra (1976) by a colorimetric assay using pyrogallol as substrate and hydrogen peroxide. A mixture of 300μL of distilled water, 190μL of 100mM potassium phosphate buffer (pH 6.8), 250μL of 100 mM pyrogallol and 250μL 100 mM hydrogen peroxide was added to 10μL of the extract. The absorbance was measured in spectrophotometer (Evolution 60, Thermo Scientific, Waltham, MA, USA) at 420nm every 10 s for 1 min after addition of the extract to the mixture in a total of five readings. The molar extinction coefficient of 2.47mM^{-1} cm^{-1} was used to calculate POX activities (Chance & Maehly, 1955), which were expressed in mmol purpurogallin produced min^{-1} mg^{-1} of protein.

2.4.3. *β*- 1, 3-glucanase (GLU) activity

The GLU activity was determined as described by Lever (1972). The reaction was initiated by the addition of 20μL aliquots of the supernatant to a mixture of 230μL of buffer 100 mM sodium acetate (pH 5.0) and 250μL of the substrate laminarin (Sigma-Aldrich, Sao Paulo, Brazil) in a concentration of 4 mg mL^{-1}. The reaction mixture was incubated in a water bath for 30 min at 45◦C. After the incubation period, the amount of reducing sugars was determined by adding 250μL of dinitrosalicylic acid (DNS) to the mixture and then incubating the resulting mixture in a water bath for 15 min at 100◦C. The reaction was interrupted by cooling the samples in an ice bath to 30◦C. In the control samples, the reaction mixture was the same, except that the DNS was added before heating the mixture at 45°C. The absorbance of the product released by GLU was measured at 540 nm and the activity of GLU was expressed in absorbance units min^{-1} mg^{-1} of protein

2.4.4. . Polyphenoloxidase (PPO) activity

The PPO activity was determinate according to methodology written by Kar and Mishra (1976), using as substrate the Pyrogallol. The mixture was composed of 450μL of distilled water, 250 μL of 100mM potassium phosphate buffer (pH 6.8) and 200 μL of 100mM pyrogallol, which was added to 50 μL of the extract. The absorbance was measured in spectrophotometer (Evolution 60, Thermo Scientific, Waltham, MA, USA) at 420nm every 10 s for 1 min after addition of the extract to the mixture in a total of five readings. The molar extinction coefficient of 2.47mM^{-1} cm^{-1} was used to calculate POX activities (Chance and Maehley, 1955), which were expressed in mmol purpurogallin produced min^{-1} mg^{-1} of protein.

2.4.5. Chitinase (CHI) activity

The CHI activity was determined by the method of Harman et al. (1993). The reaction was initiated by the addition of 20μL aliquots of the supernatant to a mixture of 470μL of buffer 50 mM sodium acetate (pH 5.0) and 10μL of the substrate p-nitrophenyl-β-DN-N-diacetilquitobiose (Sigma-Aldrich, Sao Paulo, Brazil) at a concentration of 2 mg mL^{-1}. The reaction mixture was incubated in a water bath at 37◦C for 2 h. The reaction was interrupted by adding 500µL of 0.2 M sodium carbonate. In the control samples, only the sodium carbonate was used after adding the extract to the reaction mixture and the samples were incubated in a water bath at 37◦C for 2 h. The absorbance of the final product released by the chitinase was determined at 410 nm. The molar extinction coefficient of 7×104mM cm^{-1} was used to calculate CHI activity, which was expressed in µmol of p-nitrophenyl produced by min^{-1} mg^{-1} of protein

2.4.6. Phenylalanine ammonialyase (PAL) activity

The reaction was started after addition of 100 µl of crude enzyme extract to a mixture containing 350 µl of 24 mM Tris-HCl buffer (pH8.8) and 550µl of 100 mM L-phenylalanine substrate solution. The reaction mixture was incubated in a water bath at 40°C for 3 h. In the control samples was constituted by 100 µl of crude extract and 900 µl of Tris-HCl buffer. The reaction was terminated by adding 200 µl of 6N HCl. Absorbance of the transcinnamic acid derivatives was recorded at 290 nm after adding the crude enzyme extract to the substrate mixture. An extinction coefficient of 10^4 mM^{-1} cm^{-1} (Zucker, 1965) was used to calculate PAL activity, which was expressed as µmol min^{-1} mg^{-1} protein.

2.4.7. Catalase (CAT) activity

Catalase activity as determinate according to methodology written by Havir e Mchale (1987) using H_2O_2 as a substrate. The reaction was constituted by 500 µl of potassium phosphate pH 6.8, 100 µl of H_2O_2 12.5 mM, 350 µl of MilliQ water and 20 µl of vegetal extract. The absorbance readings were performed at 240 nm in spectrophotometer (Evolution 60, Thermo Scientific, Waltham, MA, USA) every 10 seconds for two minutes. A unit of CAT is defined as the amount of enzyme required to decompose 1 µmol of H_2O_2 min-1.

For each enzyme, six separate aliquots were performed using samples from each treatment. Soluble protein concentration was measured using the Bradford method (Bradford, 1976).

2.4.8. Determination of the concentration of malonic aldehyde (MDA)

The oxidative damage of lipids was estimated as the content of the total 2-thiobarbituric acid (TBA)-reactive substances and expressed as equivalents of malondialdehyde (MDA). Pieces of stems around the inoculated area of plants from cultivars Fécula Branca and Cacau were collected at 0, 24, 72, 120 and 168 hours after inoculation. The quantification of MDA was made according to methodology written by

Cakmak e Horst (1991). In total, 200 mg of leaf tissue was ground into a fine powder using a mortar and pestle with liquid nitrogen. The fine powder was homogenized in 2 ml of 0.1% (wt vol–1) trichloracetic acid (TCA) solution in an ice bath. The homogenate was centrifuged at 10000 *g* for 15 min at 4°C. After centrifugation,
0.333 ml of the supernatant was reacted with 1 ml of TBA solution (0.5% in 20% TCA) for 20 min in a boiling water bath at 90°C. After this period, the reaction was stopped in an ice bath. The samples were centrifuged at 13000 *g* for 4 min, and the specific absorbance was determined at 532 nm. The nonspecific absorbance was estimated at 600 nm and subtracted from the specific absorbance value. An extinction coefficient of 155 mM^{-1} cm^{-1} (Lima et al., 2002) was used to calculate the MDA concentration, which was expressed as micromoles per kilogram of fresh weight.

2.4.9. Hydrogen peroxide (H_20_2) determination

Pieces of stems of cultivars Fécula Branca e Cacau were collected at 0, 24, 72, 120 and 168 hours after inoculation. The samples were placed in liquid nitrogen and were stored at -80°C until the analysis. In total, 200 mg of leaf tissue was ground into a fine powder using a mortar and pestle with liquid nitrogen. The fine powder was homogenized in an ice bath in a volume of 2 ml of a mixture containing 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine. The homogenate was centrifuged at 10000 *g* for 15 min at 4°C (Kuo & Kao, 2003). In total, 100 µl of the supernatant was added to a reaction mixture containing 100 µM ferric ammonium sulfate ($FeNH_4[SO_4]$), 25 mM sulfuric acid, 250 µM xylenol orange, and 100 mM sorbitol in a volume of 2 ml (Gay & Gebicki, 2000). The samples were kept in the dark for 30 min and then the absorbance was determined at 560 nm. The controls for the reagents and crude extracts were prepared under the same conditions and subtracted from the sample. The H_2O_2 concentration, estimated based on a standard curve of H_2O_2, was expressed as micromoles per kilogram of fresh weight.

2.4.10. Experimental design and data analysis

A 2x2 factorial design, consisting of two cassava cultivars (Cacau and Fécula Branca) with or without bacterial inoculation, was arranged in a completely randomized design with four replications. Each experimental unit consisted of one pot with one plant. The data was analyzed by the analysis of variance (ANOVA), and the compared by the *t* test ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). For the enzymes activities assays as well as H_2O_2, and MDA concentrations, a 2x2x5 factorial design was considered for the ANOVA, consisting of two cassava cultivars, with or without inoculation and five sampling times (0, 24, 72, 120, and 168 hai).

2.5. Gene expression profiling

2.5.1. RNA extraction and cDNA synthesis

Total RNA was extract from cassava stems 24, 72 and 120 hours after inoculation. RNA extraction was performed using the TRizol® Plus RNA Purification Kit (Invitrogen) according to manufacturer's instruction. RNA concentration was estimated using NanoDrop 2000c (Thermo Scientific) and its quality assayed by agarose gel electrophoresis. First strand cDNA synthesis from total RNA was carried out using the SuperScript® III First-Strand Synthesis Kit (Invitrogen), according manufacturer's instruction.

2.5.2. Real time PCR (RT-qPCR)

Gene expression analyses was carried out for seven defense-related genes (Table 2), by RT-qPCR (Reverse Transcriptase quantitative real Time PCR), using a gene specific primers. A temporal analysis of the relative gene expression profile from inoculated stems of both cultivars was compared to the non inoculated control at 24, 72, 120 hours after inoculation in a StepOne Real Time PCR System (Applied Biosystems) using the Fast SYBR ® Green PCR Master Mix (Applied Biosystems). All reactions were analyzed in triplicates with 3 repetitions, and the average values used for quantification. The relative expression level of the targeted genes was normalized to constitutive endogenous gene Elongation Factor 1A (EF1 A) (Li et al., 2010), based on the Ct comparative method (ΔΔCt) (Schmittgen & Livak, 2008). Following RT-qPCR analyses, the dissociation curve was generated to ensure quality and specificity of amplification. The complete reaction was composed of 5 µl of Sybr Green Mix, 0.6 µl each primer, 1 µl of cDNA diluted 5 times and 2.8 µl of MilliQ water with the following cycling program: 95 °C for 10 min followed by 40 cycles of 3 s at 95 °C, 30 s at 60 °C, and 15 s at 95°C.

Table 2. Gene specific primers used for RT-qPCR analyses

Primers	Sequences	Tm °C	Length (bases)	References
EF1 A F	5’-CATGAGGCTCTCCAGGAAGC-3’	57.7	20	(Li et al., 2010)
EF 1 A R	5’-TGCCACGAATCCTCTCTTGAG-3’	56.7	21	(Li et al., 2010)
POX 72 F	5’-GGACATATTGGCTTCAAAGGG-3’	53.8	21	(An et al., 2012)
POX 72 R	5’-CCTAGGACCTGTCAATGGAG-3’	54.3	20	(An et al., 2012)
CAT2 F	5’-GAGCGTGTTGTCCATGCTAG-3’	56.0	20	(An et al., 2012)
CAT 2 R	5’-CATGAATAACAGTGGAGAAACGGAC-3’	55.9	25	(An et al., 2012)
PR-5 F	5’-TAAGCATCTGGGCACCT-3’	53.4	17	(Lopez et al., 2005)
PR-5 R	5’-AGTATTATCCAACAGTGTCCT-3’	50.6	21	(Lopez et al., 2005)
CHI F	5’-AAGTCAACACATCGGC-3’	49.6	16	(Lopez et al., 2005))
CHI R	5’-AGGGAGGTTCAGACCAA-3’	52.5	17	(Lopez et al., 2005)
CC-NBS-LRR F	5’-AAGAGGTGAAATCACTACCA-3’	50.7	20	(Lopez et al., 2005)
CC-NBS-LRR R	5’-AATTCGTCAGCATGTCC-3’	49.7	17	(Lopez et al., 2005)
ET F	5’-CCAAGCTCTACAGAGGAGTC-3’	54.5	20	(An et al., 2012)
ET R	5’-CTCAGCTGTGTCGAAAGTGC-3’	56.4	20	(An et al., 2012))
GLU F	5’-CTCAATTCTGCTGGTCTTGGAG-3’	55.6	22	(Owiti et al., 2011)
GLU R	5’-CCAGCAATGGAGATTGATTGTTC-3’	54.5	23	(Owiti et al., 2011)

2.5.3. Experimental design and data analysis

A 2x2 factorial experiment, consisting of two cassava cultivars (Cacau and Fécula Branca) with or without inoculation, was arranged in a completely randomized design with three replications. Each experimental unit consisted of one pot (1kg) with one plant. The data from all variables were analyzed by the analysis of variance (ANOVA), and the means from the none inoculated and inoculated plant treatments for each cultivar were compared by the *t* test ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). For the gene expression profiling, 2x2x3 factorial design was considered for the ANOVA, consisting of two cassava cultivars, with or without inoculation, and three sampling times (24, 72 and 120 hai).

2.6. Bacterial growth curve assays

Bacterial growth was measured in all samples used for biochemical and gene expression analyses. For bacterial quantification stem tissue was weighted and ground in 200 µl of 10mM $MgCl_2$. After gridding, the suspension was centrifuged at 1000 *g* for 2 minutes at room temperature. The supernatant was used to make a 10-fold serial dilution of up to 10^{-8} (20 µl of the anterior dilution on 180 µl of $MgCl_2$). Then, the dilutions were plated on solid 523 culture medium amended with 5µl/ml tetracycline and 10µl/ml cycloheximide and incubated at 28°C for 48 hours for colony counting.

2.7. Plant Inoculation, Tissue Collection and microscopic observation

Plants of cultivar Cacau were inoculated on the stems with the toothpick method with the bacterial isolate CMF 53 transformed with the plasmid pRZT3-GFP. A single hole was done in the middle of the stem to follow the bacteria upward and downward. Cassava plants were putted on the mist chamber during 24 hours before and after the inoculation and were kept at 28°C chamber until the last collect. The collects were done 0, 5, 8 and 10 days after inoculation (dai). The tissue collection was made in transverse cuts on the stem, at the inoculation point at 0 dai, at 2, 4, 6, 8 cm from de inoculation point downward and upward at 5, 8 and 10 dai and fresh visualized in confocal laser scanning microscopy (Zeiss LSM 510 Meta). Plants inoculated with the bacterial wild isolate and none inoculated plants were used as a control.

3. RESULTS

3.1. Bacteria isolate

To perform all experiments, we selected the virulent *Xam* isolate CMF53. This isolate was selected based on pathogenicity tests as being the most virulent/aggressive to cassava plants, among 20 tested ones. Plants inoculated with this isolate presented intense symptoms of wilt 7 days earlier compared with the other 19 isolates (data not shown).

3.2. Bacterial transformation, plasmid stability and selection of transformants isolates

Xam CMF53 transformation by electroporation was efficient with the plasmids pVSP61 and pRZT3-GFP and therefore, isolates transformed with both plasmids were selected for further tests of bacterial growth, virulence, and plasmid stability in comparison with the wild type (non transformed isolate).

The bacterial isolates, transformed with plasmid pRZT3-GFP showed a reduce growth when compared to wild type bacteria (Fig. 1A) In contrast isolates transformed with the plasmid pVSP61 did not show any growth alteration (Fig.1B). The growth rate was different for isolates transformed with pRZT3-GFP and equal for isolates transformed with pVSP61 (Fig. 2)

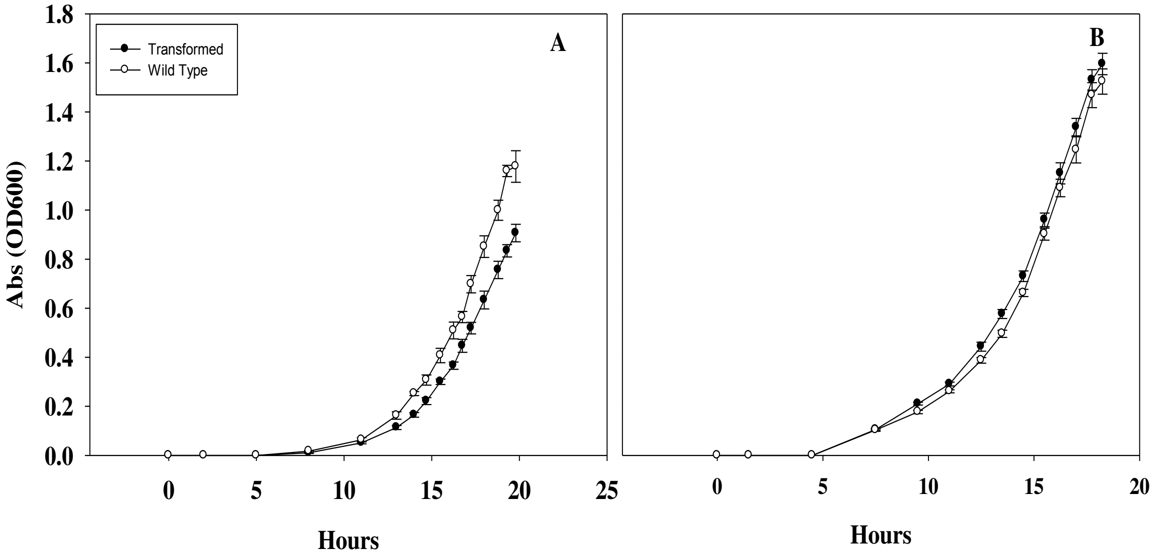

Figure 1. Bacterial growth curve assays of transformed and non transformed *Xam* CMF53 isolates. Bacteria transformed with the plasmid pRZT3-GFP (A) and pVSP61 (B). Bars represent the standard error of the means.

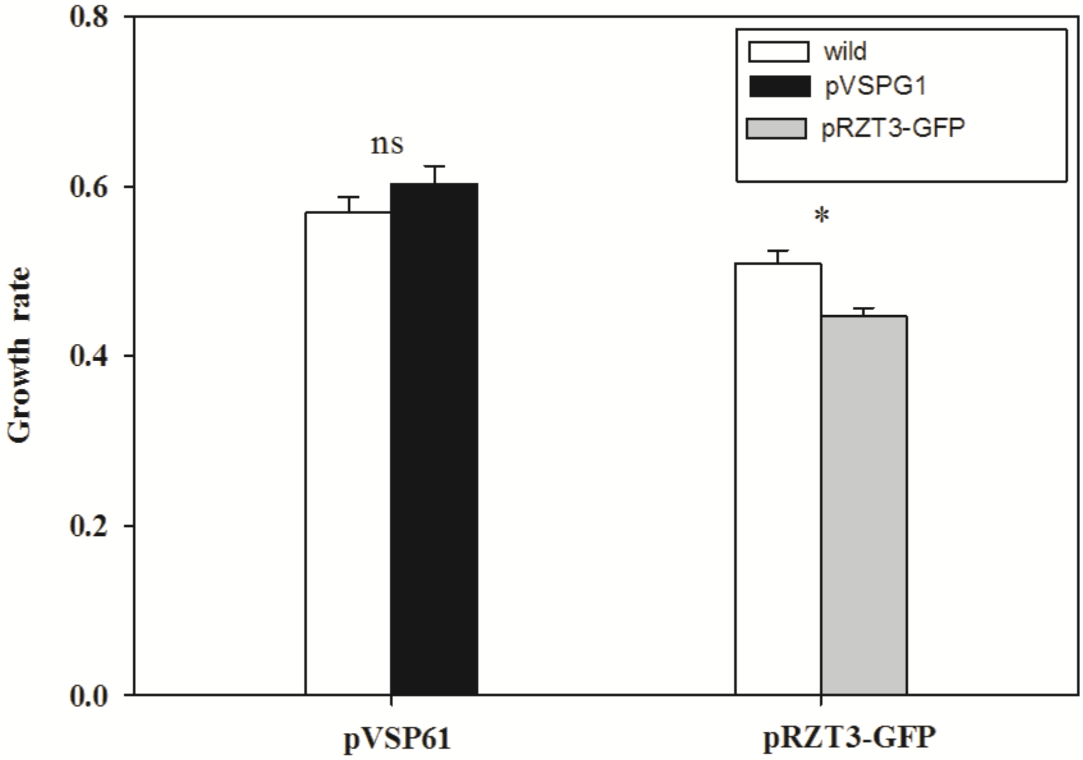

Figure 2. Growth rate of bacterial isolates transformed with plasmid pVSP61 and pRZT3-GFP. Means followed by an asterisk (*), for each sampling time, are significantly different ($P \leq 0.05$) by *t* test. Bars represent the standard error of the means

In order to rule out the effect of plasmid transformation on bacterial virulence, pathogenicity test were carried out on susceptible cassava plants. Both plasmids did not alter bacterial virulence (Figs. 3A and 3B).

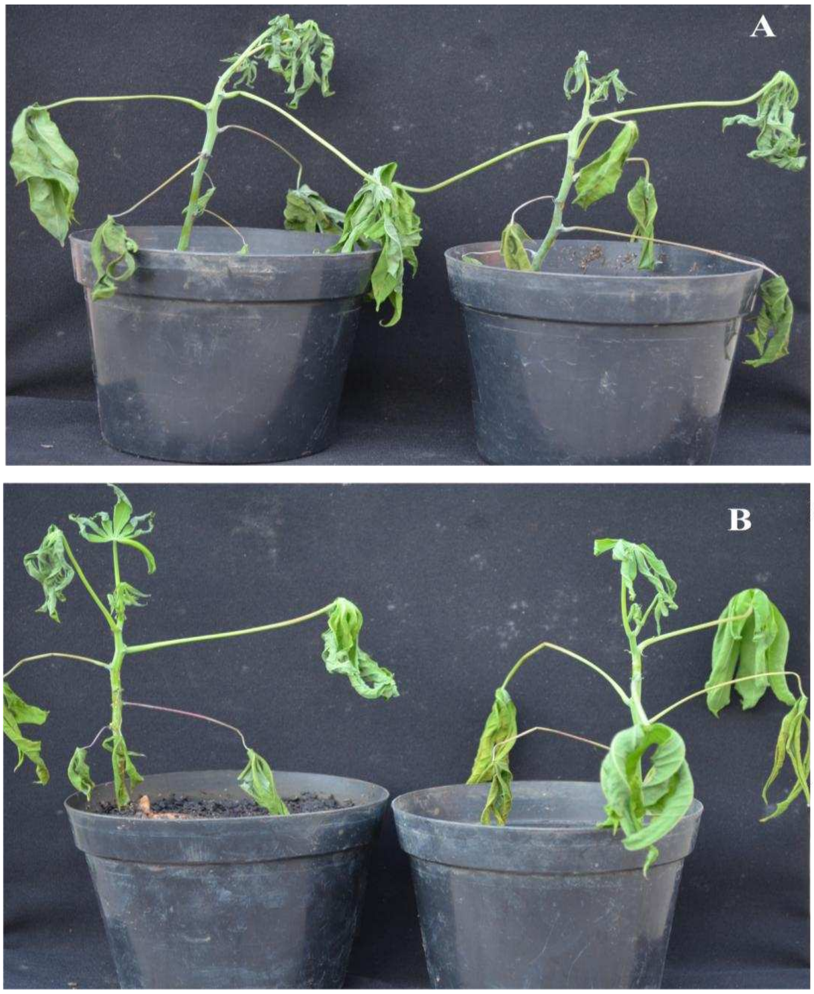

Figure 3. Pathogenicity tests of wild type and transformed bacterial isolates. Cassava plants (Cacau cultivar) 7 days after toothpick inoculation with the tested *Xam*. Bacteria transformed with plasmid pVSP61 (A, left) and the wild bacteria (A, rigth) and plasmid pRZT3-GFP (B, left) and wild bacteria (B, rigth).

To check plasmid stability *in planta*, two successive inoculations on susceptible cassava plants were performed with the transformed isolates, and plasmid maintenance evaluated. Not surprisingly, both plasmids were stable in the bacterial cells during *in planta* growth, up to 14 days. (Fig. 4A and 4B).

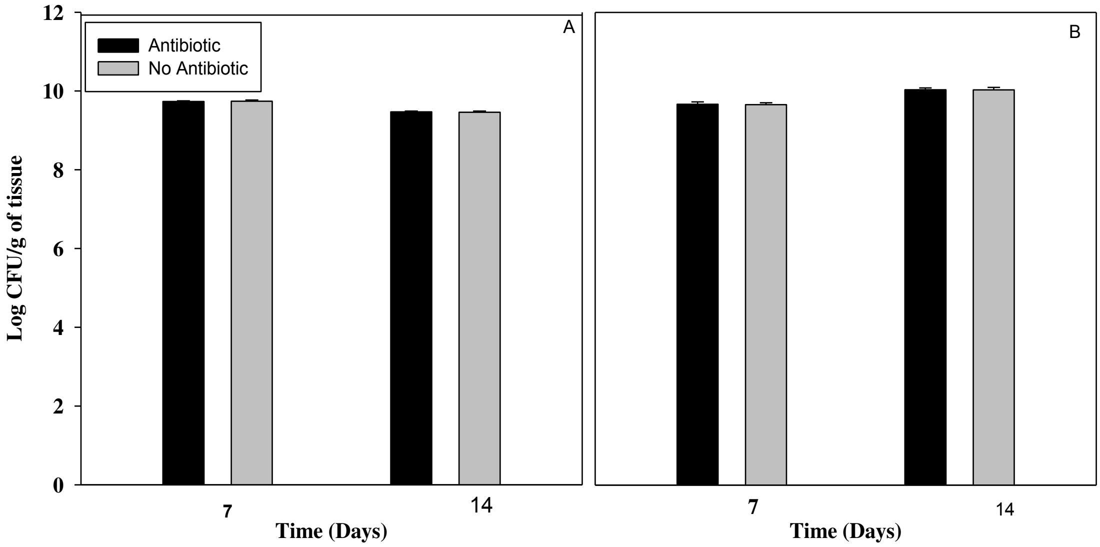

Figure. 4. *In planta* plasmid stability test. Bacterial counting on medium with (black bars) and without (gray bars) antibiotic. Bacterial isolates transformed with plasmid pVSP61 (A) and pRZT3-GFP (B). Bars represent the standard error of the means.

Figure 5. Plants of Fécula Branca cultivar 10 days after inoculation with *Xam*. No wilting symptoms could be observed.

3.3. Cultivars resistance

Prior to biochemical and molecular analysis of the interaction, pathogenicity tests were carried out to assay the response of both cultivars, Cacau and Fécula Branca to *Xam* infection. As expected the results confirmed Fécula Branca as partial resistant and Cacau as susceptible to *Xam* infection. Fécula Branca plants did not show any disease symptoms at 10 days after inoculation (Fig. 5). Conversely, all Cacau plants had wilted 7 days after inoculation (Fig.3A and 3B). As expected, bacterial population in the tissues of both cultivars did correlate with the observed symptoms (Fig. 6). In the susceptible cultivar the bacterial growth was constant. In the resistant cultivar at 0, 24 and 72 hai the bacteria had the maximum growth and decrease drastically after this time.

3.3. Biochemical analysis

In general, POX activity was higher on inoculated plants of both cultivars. POX activity was significant different between inoculated and non inoculated Fécula Branca plants at 24 and 72 hai, where a peak could be observed at 24 hai (Fig. 7B). Conversely, it did differ statistically between health and diseased plants in all other sampling times. POX activity on Cacau plants showed significant differences activity between treatments only at 72 hai (Fig. 7A). GLU activity on Fécula Branca cultivar was significantly different between treatments at 24 hai (Fig. 7D). Similarly to POX activity on Cacau plants, GLU activity was significantly different between inoculated and non inoculated plants only at 72 hai (Fig 7C). PPO activity was significantly different between the plants at 24 and 72 hai for resistant cultivar (Fig. 7F). In susceptible cultivar PPO activity was similar to the POX one, differing statically at 72 hai between plants (Fig. 7E). CHI activity was higher in inoculated resistant plants at 24 and 72 hai when compared to non inoculated plants (Fig. 8B). In the resistant and susceptible cultivars, CHI activity was significantly lower on inoculated plants when compared to the non inoculated ones at 168 and 120 hai, respectively. In addition, CHI activity in the susceptible cultivar in all other time points did not differ significantly between plants (Fig. 8A). PAL activity on inoculated plants of both cultivars was higher at 24 hai in comparison to non inoculated plants (Fig. 8C and 8D). CAT activity was higher at 24, 72, 120 and 168 hai in inoculated plants of the resistant cultivar (Fig. 8F). On the other hand, CAT activity on the susceptible cultivar did not differ any time point between treatments (Fig. 8E).

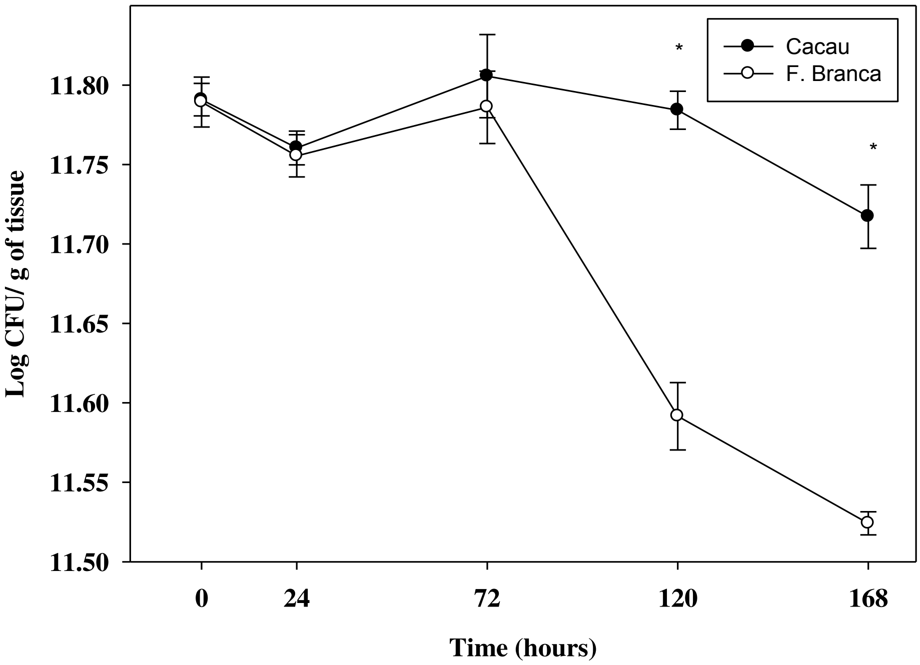

Figure 6. Bacterial growth curve in stems of cassava plants from 'Cacau' and 'Fécula Branca inoculated with *Xanthomonas axonopodis* pv. *manihotis*. Treatments followed by an asterisk (*), for each sampling time, are significantly different ($P \leq 0.05$) by *t* test. Bars represent the standard error of the means.

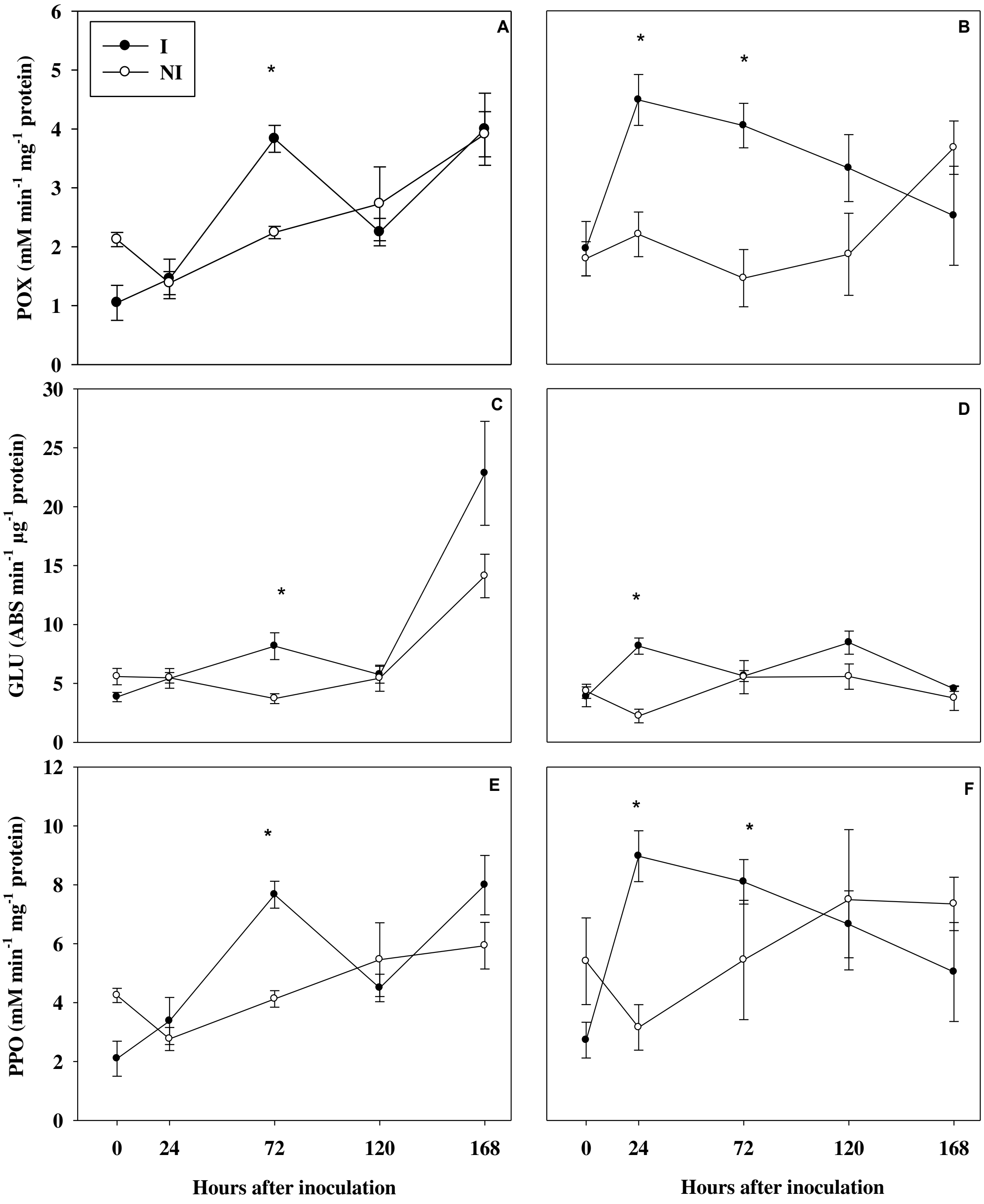

Figure 7. Enzyme activity measurement of I and NI plants of Cacau and Fécula Branca cultivars of Peroxidase (POX; A and B); Glucanase (GLU; C and D) and Polyphenoloxidase (PPO; E and F) in stems of cassava cultivars ‘Cacau’ (A, C and E) and ‘Fécula Branca’ (B, D, and F) non inoculated (NI) or inoculated (I) with *Xam* CMF53. Means from NI and I treatments followed by an asterisk (*), for each time point, are significantly different by *t* test ($P \leq 0.05$). Bars represent the standard error of the means.

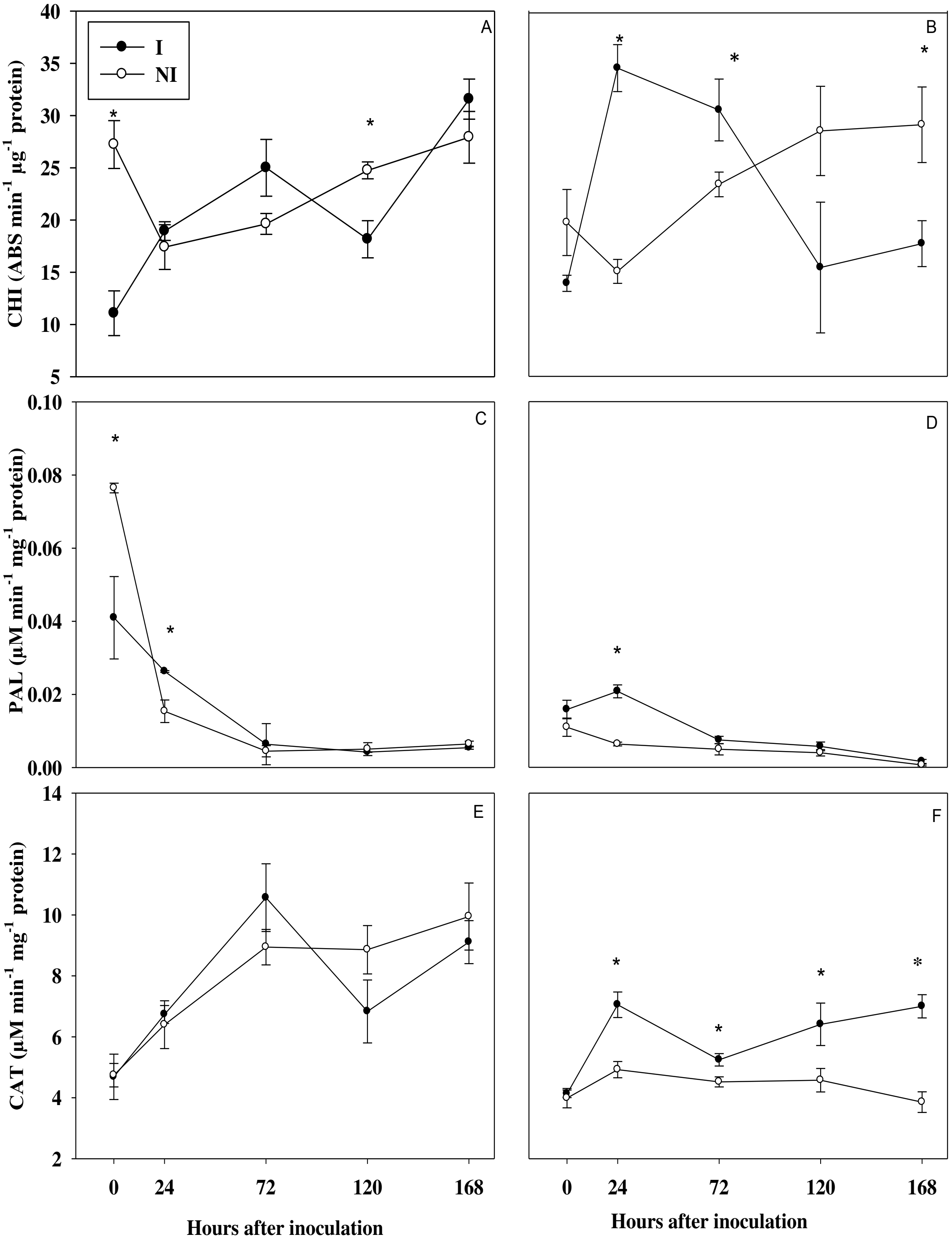

Figure 8. Enzyme activity measurement of I and NI of Cacau and Fécula Branca cultivars of Chitinase (CHI; A and B); Phenylalanine ammonialyase (PAL; C and D) and Catalase (CAT; E and F) in stems of cassava cultivars 'Cacau' (A,C and E) and 'Fécula Branca' (B, D, and F) non inoculated (NI) or inoculated (I) with *Xam* CMF53. Means from NI and I treatments followed by an asterisk (*), for each time point, are significantly different by *t* test($P \leq 0.05$). Bars represent the standard error of the means.

3.3.2. Determination of the concentration of malonic aldehyde (MDA) and hydrogen peroxide (H_2O_2)

MDA accumulation on tissue of both cultivars, inoculated and noninoculated, did not show correlation with disease resistance. In the resistant cultivar at 0, 24 and 72 hai MDA concentration was higher in non inoculated ones, when compared with the inoculated plant, and did not differ between plants in the other time points. (Fig. 9B). In the susceptible cultivar, MDA concentration was higher on inoculated plants only at 0 hai In the other times (24, 72, 120 and 168 hai) the values between treatments were not statistically significant (Fig. 10 A).

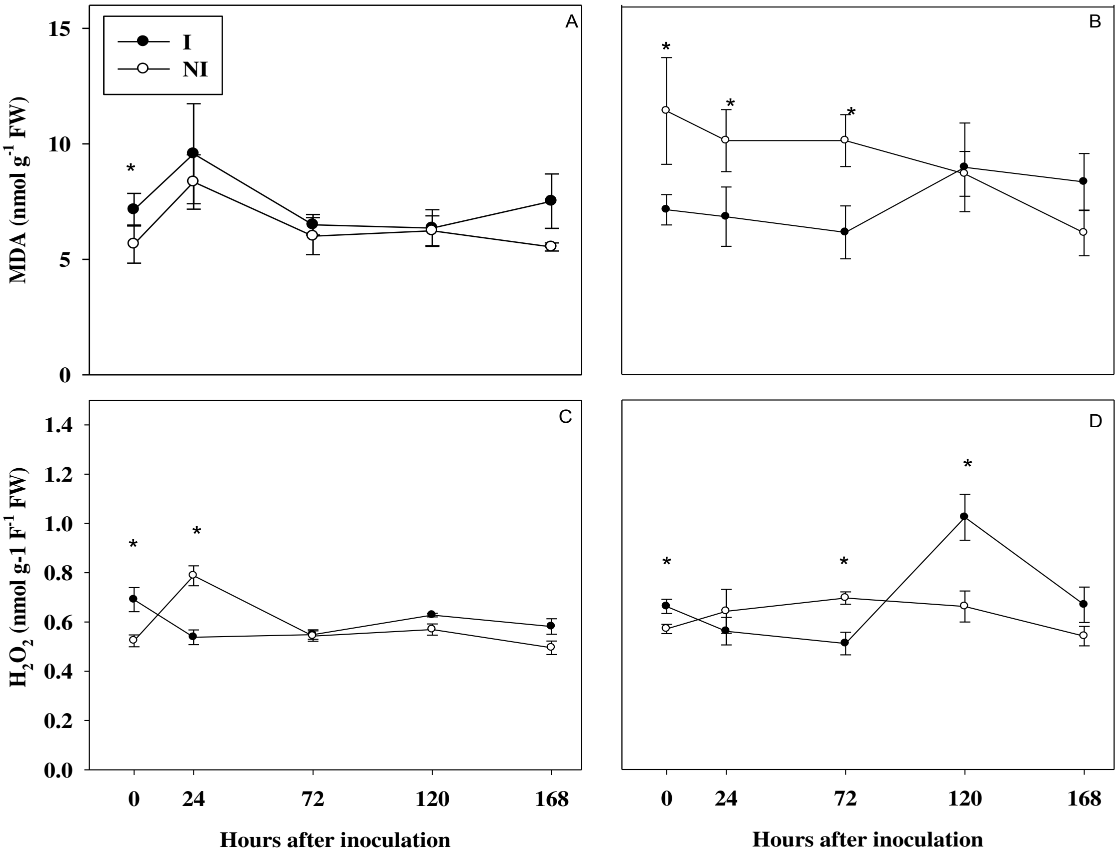

Figure 9. Concentration of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) between Cacau and Fécula Branca cultivars. MDA (A and B) and hydrogen peroxide (H_2O_2) (C and D) in stems of the cassava cultivars 'Cacau' (A and C) and 'Fécula Branca (B and D) none inoculated or inoculated with *Xam*. Means from NI and I treatments followed by an asterisk (*), for each sampling time, are significantly different ($P \leq 0.05$) by *t* test. Bars represent the standard error of the means. FW = fresh weight.

There was a significantly higher accumulation of H_2O_2 at 120 hai in resistant cultivar. On the contrary, accumulation was significantly lower at 72 hai in comparison with the non inoculated plants in the same cultivar (Fig. 9D). In the susceptible cultivar, (H_2O_2) accumulation did not show correlation with disease progress since it was significantly higher in non inoculated plants at 24 hai (Fig. 9C).

3.4. Gene expression analyses

In tissues of resistant cultivar, Pox expression showed a 5 fold increased at 24 hai. Conversely, Pox expression was increased only at 72 hai in the susceptible cultivar (Fig. 10A). For other time points, Pox expression was repressed relatively to the non inoculated control (Fig, 10A). Cat expression in resistant plants showed an increased at 24 and 72 hai followed by a repression at 120 hai (Fig 10B). In the susceptible cultivar Cat expression was unaltered at 24 hai followed by a repression at 72 and 120 hai (Fig. 10B). PR-5 expression was higher in the resistant cultivar at 24 and 72 hai. A 10-fold increase could be observed at 120 hai in the susceptible cultivar (Fig. 10C). Expression of the CC-NBS-LRR like gene was repressed in all studied time points for both cultivars (Fig. 10D). Glu gene expression increased at 24 hai in resistant cultivar, followed by a repression at 72 and 120 hai (Fig 10E). Expression was also increased in susceptible cultivar, but at a later time point (72 hai) followed by a repression (Fig. 10E). The Et gene expression showed a slight increase at 24 and 72 hai followed by a strong repression at 120 hai in the resistant cultivar (Fig. 10F). In susceptible cultivar the Et gene expression was slightly repressed at all time points (Fig 10F). Expression of Chi gene was strongly induced in the resistant cultivar at 24 and 72 hai .Chi expression increased 100-fold followed by a repression at 120 hai (Fig 10G). Chi was weakly expressed in the susceptible cultivar occurred at 24 and 72 hai., followed by a repression at 120 hai (Fig. 10G).

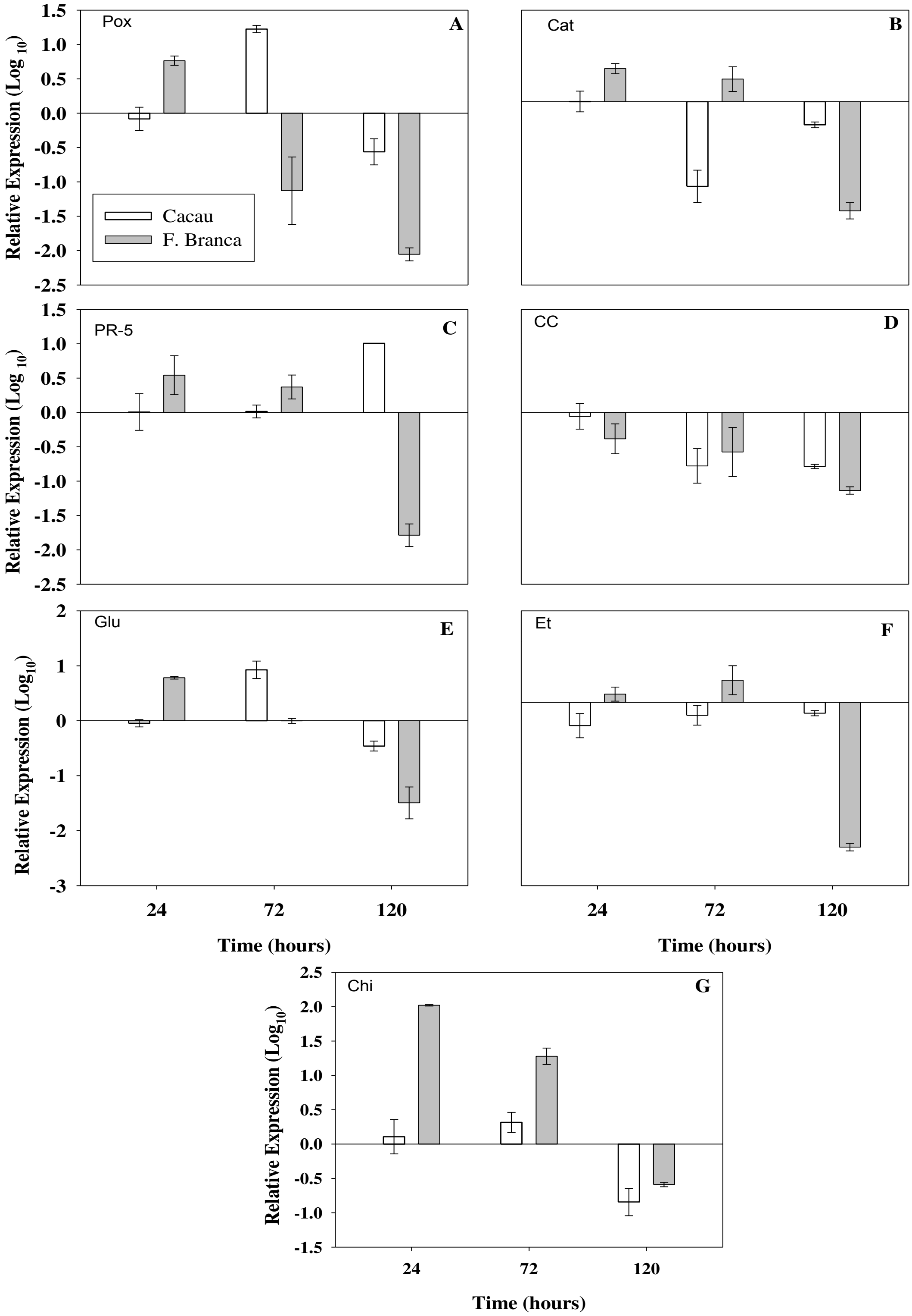

Figure 10. Gene expression analyses of inoculated and non inoculated Cacau and Fécula Branca cultivars. Expression of Peroxidase (A), Catalase (B), PR-5 (C) and CC-NBS-LRR like genes (D), Glucanase (E), Ethylene (F) and Chitinase (G) in stems of cassava plants ‘Cacau’ and ‘Fécula Branca’. Bars represent the standard deviation of the means.

3.5. Microscopic analysis

Xam-GFP CMF53 translocation on cassava stems was analyzed by CLSM at 0, 5, 8, and 10 dai. The GFP-tagged bacteria were efficiently detected at inoculation site (Fig. 11C). As expected, no GFP signal could be detected on tissues of plants either healthy or inoculated with the wild type bacteria (Figs. 11A and 11B). At 5 and 8 dai bacterial cells were visualized up to 4 cm above and below the inoculation site (Figs. 12 and 13). No bacterial cells could be detected at 6 and 8 cm both above and below the inoculation site. At 10 dai fluorescence bacteria was visualized up to 6 cm above and below the inoculation site (Fig. 14). No bacterial cells could be detected at other studies distances. As expected, no GFP signal could be detected on tissues of either healthy plants or inoculated with wild type bacteria.

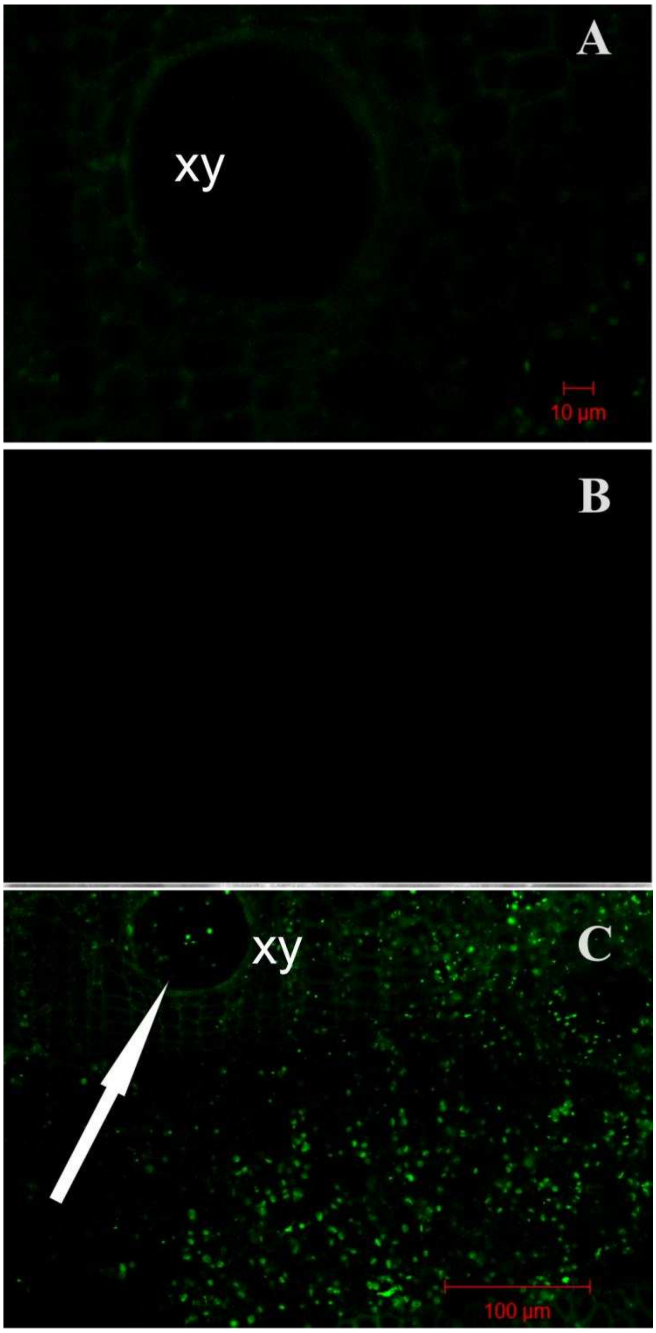

Figure 11. Detection of GFP-tagged *Xanthomonas axonopodis* pv. *manihotis* on cassava inoculated plants 0 days after inoculation (dai). Cassava seedlings (45 day old) were puncture inoculated with the bacterial and was examined by confocal laser scanning microscopy. Images were taken from transversally cut: non inoculated plant (A), wild type bacteria (B), and GFP-tagged bacteria (C). xy stands to xylem tissue. The arrows indicate the bacteria inside the xylem vessels.

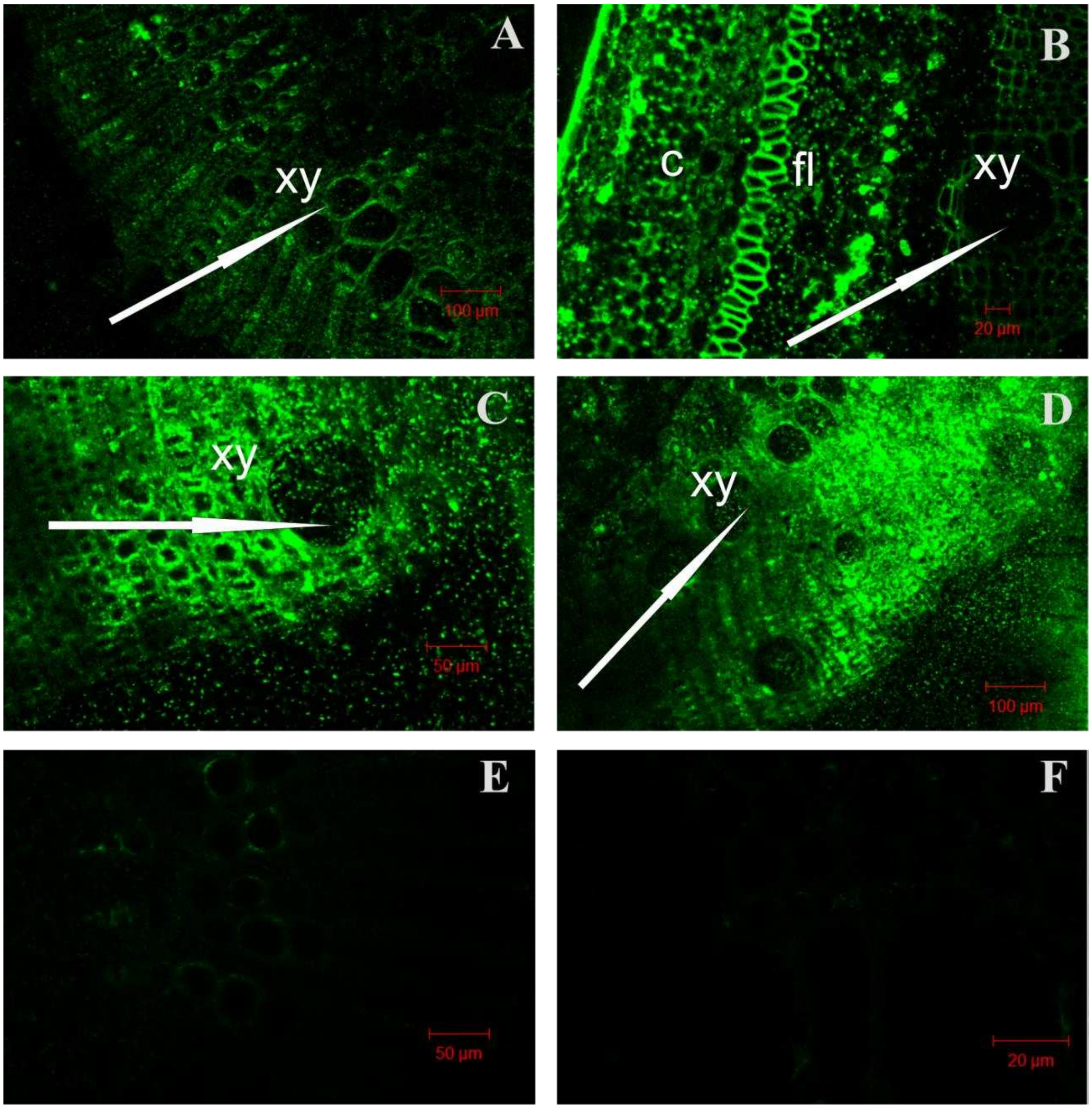

Figure 12. Vascular translocation of GFP-tagged *Xanthomonas axonopodis* pv. *manihotis* on cassava stems 5 days after inoculation. Cassava seedlings (45 day old) were puncture inoculated with the bacterial and was examined by confocal laser scanning microscopy. Images were taken from transversally cut: 2 cm below the inoculation site (A), 2 cm above the inoculation site (B), 4 cm below the inoculation site (C), 4 cm above the inoculation site (D), non inoculated plant (E) and wild type bacteria (F). xy stands to xylem tissue, c stands to cortex and fl stands to phloem. The arrows indicate the bacteria inside the xylem vessels.

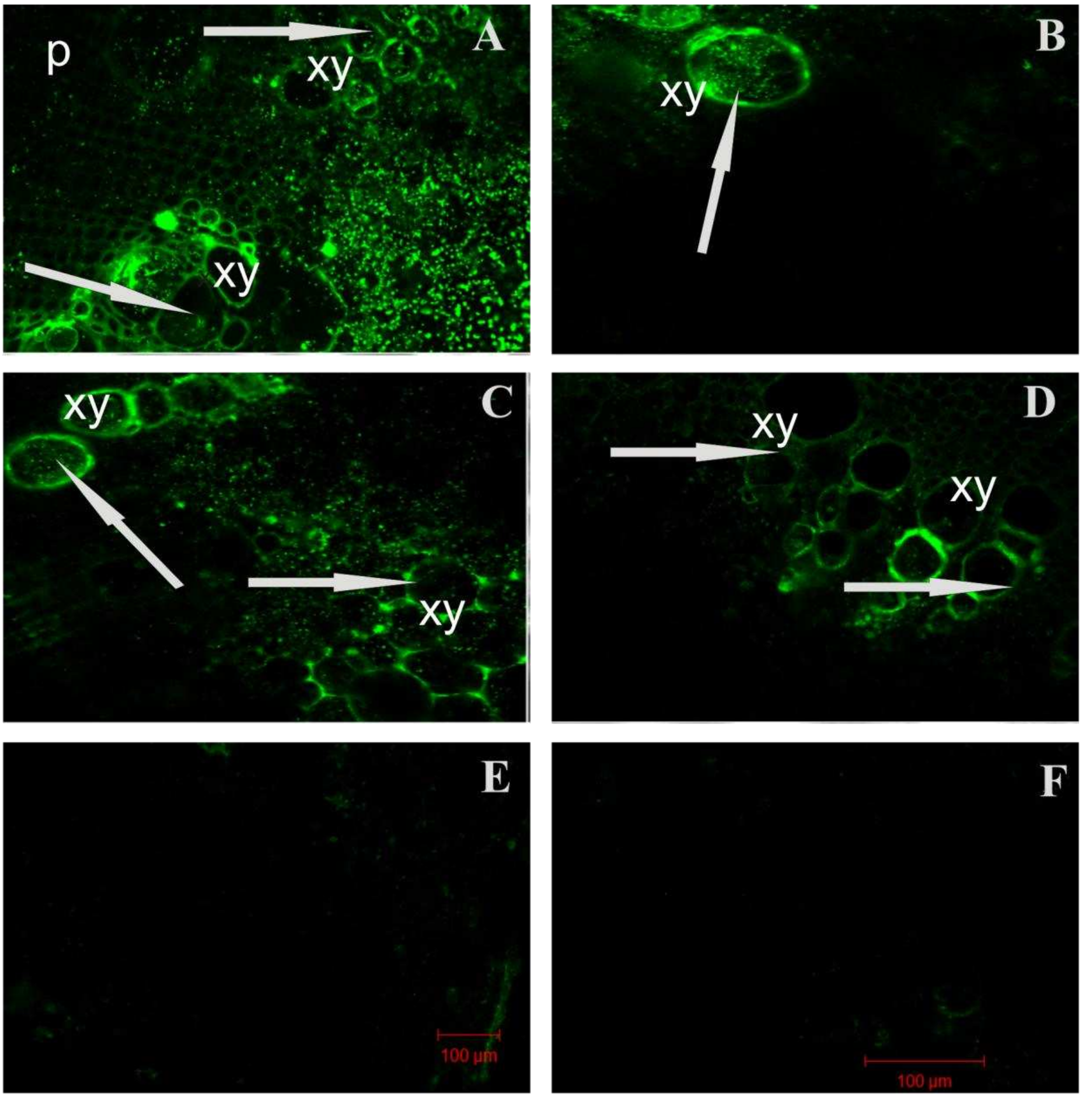

Figure 13. Vascular translocation of GFP-tagged *Xanthomonas axonopodis* pv. *manihotis* on cassava stems 5 days after inoculation. Cassava seedlings (45 day old) were puncture inoculated with the bacterial and was examined by confocal laser scanning microscopy. Images were taken from transversally cut: 2 cm below the inoculation site (A), 2 cm above the inoculation site (B), 4 cm below the inoculation site (C), 4 cm above the inoculation site (D), none inoculated plant (E) and wild type bacteria (F). p stands to pith and xy to xylem tissue. The arrows indicate the bacteria inside the xylem vessels.

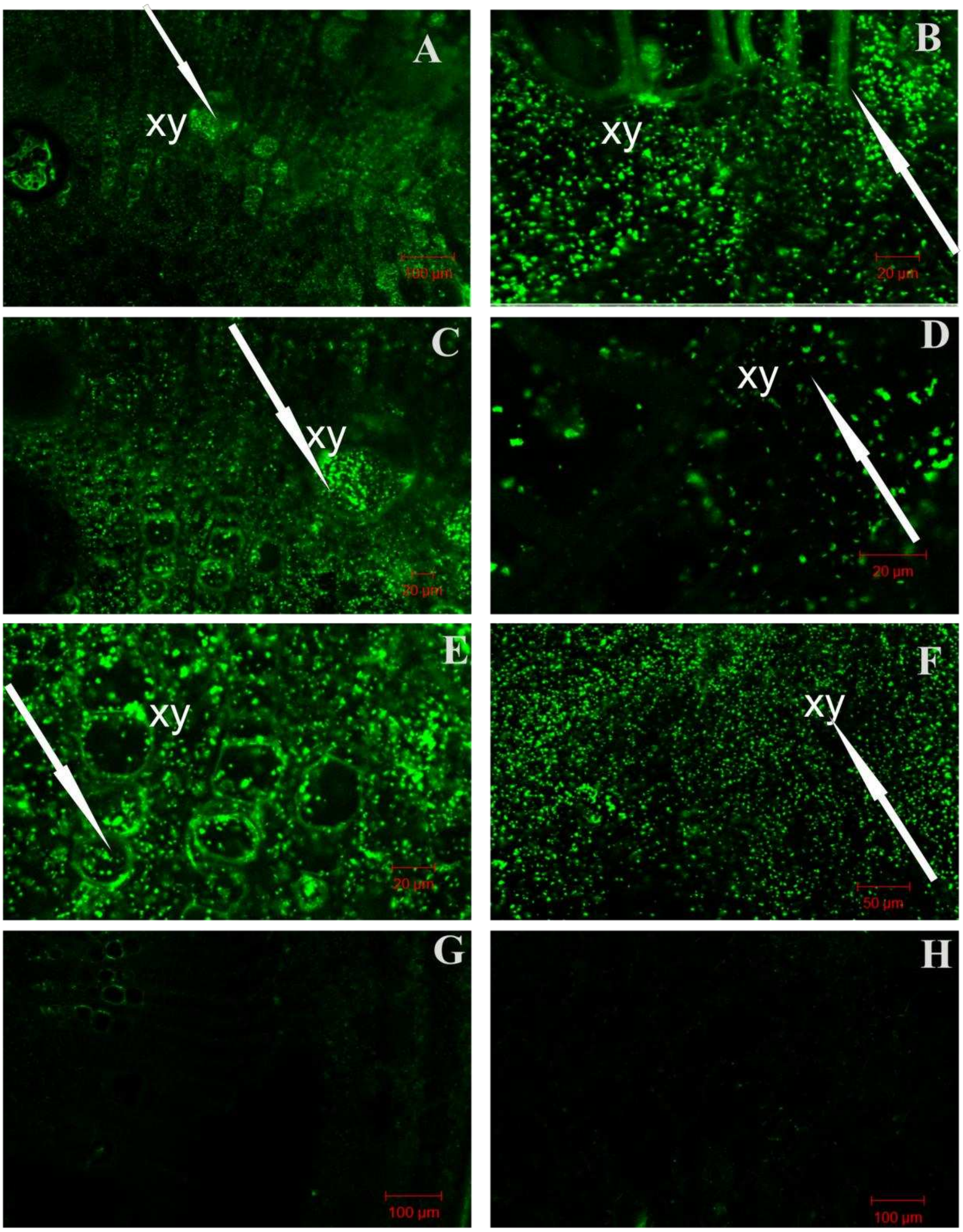

Figure 14. Vascular translocation of GFP-tagged *Xanthomonas axonopodis* pv. *manihotis* on cassava stems 5 days after inoculation.. Images were taken from transversally cut: 2 cm below the inoculation site (A), 2 cm above the inoculation site (B), 4 cm below the inoculation site (C), 4 cm above the inoculation site (D), 6 cm below the inoculation site (E), 6 cm above the inoculation site (F) none inoculated plant (G) and wild type bacteria (H). xy stands to xylem tissue. The arrows indicate the bacteria inside the xylem vessels.

4. DISCUSSION

In this study we characterized the cassava defense responses against *Xanthomonas axonopodis* pv. *manihotis* looking at the biochemical and molecular alterations occurring at the plant-bacteria interface. To accomplish this, we carried out studies on the enzymatic activity of the plant defense related proteins POX, PPO, CAT, CHI, GLU and PAL as well as the accumulation of MDA and H_2O_2. In addition, we analyzed the relative expression levels of a selected subset of defense genes by RT-qPCR analyses. Although a number of studies on cassava-*Xam* interaction have been reported on some countries, little is known about *Xam* cassava interaction, mainly in the Brazilian conditions. To date, the basis of the Fécula branca resistance to CBB is unknown.

The absence of CBB wilting symptoms in Fécula Branca plants in our experiments, corroborated the resistance in the field, and reinforces the strength of our experiments in reproducing field conditions. Vidigal Filho *et al.* (2000) screened several cassava cultivars for resistance to bacterial blight and found that Fécula Branca resistance was superior in comparison with the other tested cultivars, under field conditions. On the contrary, the Cacau cultivar has been shown to be highly susceptible to CBB (Fialho *et al.*, 2009) Supporting the data provided by these reports, the present study brings novel evidence that the increased expression of some defense related proteins and genes in cassava stems may be linked to the resistance of to CBB (Pereira *et al.*, 2000).

POX activity increased on both cultivars during infection by *Xam*. However, the increase occurred earlier in Fécula Branca than in Cacau plants. While the higher activity occurred at 24 hours in the resistant cultivar this response occurred only at 72 hai in the susceptible one. Moreover, the activity was maintained high up to 72 hai on Fécula Branca while activity on Cacau was high only at 72 hai. Following the same trend of the biochemical assay, Pox expression by RT-qPCR resistant cultivar also occurred earlier than in susceptible one. However, at 72 hai the expression decreased on Fécula Branca plants. Plant peroxidases have been extensively studied and many functions have been described, e.g., scavenging of peroxide, participation in lignification, hormone signaling and plant defense (Hiraga *et al.*, 2001). Ngadze *et al.* (2012) also related the increase of POX activity enhance resistance on potato tubers infected by *Pectobacterium* and *Dickeya*. High POX activity in tomato plants infected by *Pseudomonas syringae* pv. *tomato* (*Pst*) treated with a resistance inducer correlated reduced disease severity (Andrade *et al.*, 2013). POX activity was higher on leaves of resistant cassava plants infected by *Xanthomonas cassavae* when compared to leaves of the susceptible cultivar (Pereira *et al.*, 2000)

PPO enzyme followed the same POX activity pattern. This activity increased on both cultivars during infection by *Xam*. However, the increased was detected earlier on the resistant cultivar showing a high expression at 24 and 72 hai. Differential expression was only detected at 72 hai on the susceptible cultivar. PPOs are enzymes responsible for the formation of quinones, which have antimicrobial activity (Mayer and Staples, 2002), and thus is considered an important enzyme for plant defense against pathogens. Transgenic tomato plants overexpressing a gene enconding PPO led to enhanced bacterial disease resistance (Li and Steffens, 2002). On resistance of potato tubers infected by soft rot causing bacteria has been related the increase of PPO activity (Ngadze *et al.*, 2012). Tomato plants treated with resistance inducers also showed high PPO levels when inoculated with *Pst* (Andrade *et al.*, 2013).

PAL activity was higher at 24 hai in both cultivars, but the response was more robust in resistant cultivar. The response was always higher on the resistant cultivar than in the susceptible one. PAL is the first enzyme in the phenylpropanoid pathway and its increased activity has been associated with increases in lignin synthesis and other plant defense compounds (Whetten and Sederoff, 1995). The first reaction of the phenylpropanoid biosynthetic pathway is the deamination of phenylalanine to cinnamic acid by PAL which ultimately culminates in the synthesis of antimicrobial compounds. A number of reports show the induction of PAL by various biotic and abiotic stresses (Kale and Choudhary, 2001).Pereira *et al.* (1999) reported that resistant and susceptible cassava cultivars presented increased on PAL activity; however the activity in resistant cultivar was higher than in the susceptible one when infected by *Xam*. Wheat plants infested by the aphid *Sitobion avenae* showed a more elevated expression of PAL on resistant than on susceptible cultivars at earlier stages of infestation; however the resistant cultivar presented a more robust activity (Han *et al.*, 2009).Similarly, PAL activity was higher on a resistant chickpea cultivar infected by *Fusarium oxysporum* f. sp. *ciceri* when compared with a susceptible one, despite that expression increased on susceptible cultivar could also be observed (Raju *et al.*, 2008).

GLU and CHI are pathogeneses related proteins (PR), PR-2 and PR-3 respectively. GLU activity increased in both cultivars infected by *Xam*. However, the increase was earlier for Fécula Branca than for Cacau plants. While the major response occurred at 24 hours in the resistant cultivar, it occurred only at 72 hai in the susceptible one. The RT-qPCR results corroborated this observation. GLU expression showed a peak at 24 hai and 72 hai in the resistant and susceptible cultivars, respectively. GLU is a enzyme that breaks down glucans. Glucans are produced by fungi and bacteria, which participate in the formation of the cell wall, the main structural feature of β - glucans is to assist in maintaining the rigidity and integrity of the cell wall. This role against fungal infection is well known; however, the function of this hydrolytic enzyme in plant resistance to infection by bacterial pathogens is still incipient (Cavalcanti *et al.*, 2006). Resistant melon plants infected by *Sphaeroteca fusca* showed faster GLU induction when compared to plants that were susceptible (Rivera *et al.*, 2002). An increased of GLU activity was also related on tomato plants infected by the bacterial pathogens *Pst* and *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) treated with resistance inducers, remaining higher on treated plants (Cavalcanti *et al.*, 2006; Andrade *et al.*, 2013).

CHI expression was higher in resistant cultivar in both biochemical and molecular assays at 24 and 72 hai. On the biochemical assays the susceptible plants inoculated with *Xam* did not show any differential activity when compared with the non inoculated plants. Chi expression in the susceptible cultivar was only moderately induced at 24 and 72 hai (3-fold); however the expression was much lower than in the resistant cultivar (20-fold). CHIs are hydrolytic enzymes similar to glucanase. This enzyme catalyze the hydrolysis of chitin, a linear homopolymer of p-1, 4-linked N-acetylglucosamine residues (Collinge *et al.*, 1993). Due the high expression of this enzyme in the resistant cultivar, our data point out a very important role for CHI in the resistance of Fécula Branca against *Xam* infection. This result is of great interest since the role of chitinase during bacterial diseases, as well as glucanases, is not well understood. Despite showing antibacterial activity, due primarily to their lisozimic action on the cell wall (Stintzi *et al.*, 1993), there are few studies on the action of chitinase on bacterial colonization in plants (Cavalcanti *el at.*, 2006). *Colletotrichum falcatum* the causal agent of red rot disease in sugar cane plants induced CHI expression on plants. Resistant cultivars showed much higher expression when compared with the susceptible one (Malathi *et al.*, 2012). Tobacco plants overexpressing a CHI protein showed to be more resistance not only to the bacteria *Pseudomonas syringae* pv. *tabacci* but also to *Rhizoctonia solani* infection and abiotic stress (Dana *et al.*, 2006). Rice plants silenced in CHI genes presented enhanced susceptibility to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* and two fungal pathogens *Rhizoctonia solani* and *Magnaporthe oryzae* pv.*oryzae* (Snelling, 2010). Tomato plants treated with resistance inducers and inoculated with *Xcv* also showed high values of CHI expression and disease resistance (Cavalcanti *et al.*, 2006). Lopez et al. (2005) reported that CHI expression was up-regulated on a resistant cultivar when inoculated with *Xam* in the first hours after inoculation. Lopez result corroborated our results about the CHI role against the *Xam* infection resistance cassava plants, since this work presented similar results.

CAT activity in the susceptible cultivar was not induced during infection. However, in resistant cultivar CAT activity was higher on inoculated plants at 24, 72, 120 and 168 hai. Cat gene expression analyses corroborated these results. In susceptible cultivar CAT activity was suppressed on inoculated plants, while in resistant ones the expression was induced at 24 and 72 hai. CAT is an enzyme commonly found in all aerobic organisms that functions against the plants oxidative stress though rapid degradation of H_2O_2. High CAT activity on a wheat resistant cultivar infected by *Pyricularia oryzae* correlated with disease resistance and protection against reactive oxygen species (ROS). Resistant plants presented lower disease severity and cellular damage when compared to susceptible cultivar (Debona *et al.*, 2012). *Sorghum* plants treated with silicon (a resistance potentiating agent) and infected with *Colletotrichum sublineolum* presented high CAT activity and low cell damage when compared with the control plants (Resende *et al.*, 2012).

The cellular damage caused by the oxidative stress related to infection and plant defense responses can be estimated by the products of the peroxidation of membrane lipids. The MDA produced during lipid peroxidation is an indicator of cellular damage that results in an increase in electrolytic leakage (Debona *et al*., 2012). In our work, MDA concentration and thus lipid peroxidation did not show any response to bacterial infection. This result is somewhat expected in the case of a xylem limited pathogen, since xylem vessels are composed of dead cells and are not expected to show electrolytic leakage. On leaves of wheat and sorghum infected by *Pyricularia oryzae* and *Colletotrichum sublineolum* respectively, the increase of MDA concentration has been related with more disease severity (Debona *et al*., 2012; Resende *et al*., 2012).

Here, H_2O_2 concentration on susceptible cultivar was stable with low variation during the infection process. However, in resistant plants, H_2O_2 production during *Xam* infection showed a peak at 120 hai. This is an important observation, since the oxidative burst and generation of ROS are characteristic of the state of plant defense (Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003). H_2O_2 can act as molecular signal exerting role in the activation of defense responses without compromising the integrity of the cells (Lamb and Dixon, 1997). It may be that H_2O_2 plays an important role in resistant plants by activating signaling pathways and defense responses. Thus, ones might speculate that the H_2O_2 peak can be one of the mechanisms by which plant resistance stimulates plant defense machinery of cassava infected by *Xam*. The fact that the bacterial growth decreased at 120 hai, at the same time of the of H_2O_2 accumulation corroborate this hypothesis.

PR-5 is a pathogenesis related protein like CHI and GLU. PR-5 expression was weakly induced in all time points for the resistant cultivar. Expression on susceptible cultivar was induced only at 120 hai (8-fold). Although the response was higher, it was not constant and only detected at this time point .These proteins are called thaumatin-like proteins (TLPs), because exhibit amino acid and structural similarity to an intensely sweet tasting protein, thaumatin, from fruits of the West African shrub, *Thaumatococcus daniellii*. The proposed mechanism of their action involves plasma membrane permeablization of the target fungal cell (Roberts and Selitrennikoff, 1990; Vigers *et al.*, 1992) and signal transduction (Yun *et al.*, 1998). Some PR-5 proteins have strong antifungal activity (Vigers *et al.*, 1992; Abad *et al.*, 1996; Velazhahan *et al.*, 2002), and disease progress was delayed in transgenic plants expressing heterologous TLPs (Liu *et al.*, 1994; Zhu *et al.*, 1996; Chen *et al.*, 1999). However, the role of these proteins in plant defense to bacterial infections has been little studied. Plum plants infected by *Monilinia fructicola* presented a high and early expression of PR-5 on resistant cultivar than in the susceptible one. Additionally, transgenic *Arabidopsis* plants expressing this gene, showed enhanced resistance against *Alternaria brassicicola* and activated other defense pathways, including phytoalexin production (El-Kereamy *et al.*, 2011). Transgenic potato overexpressing an osmotin (PR-5 like protein) caused a delay on *Phytophthora infestans* symptons (Liu *et al.*, 1994). Lopez *et al.* (2005) reported that osmotin expression on a resistant cassava cultivar infected by *Xam* was up-regulated. However the higher expression occurred at 7 and 15 days after inoculation. This result can explain the weakly expression of PR-5 on our experiments which studied earlier time points after infection.

The ET marker gene expression on the resistant cultivar was moderately induced at 24 and 72 hai, while the expression was repressed at all time points in susceptible cultivar. The gaseous hormone ethylene is known to regulate multiple physiological and developmental processes in plants, such as seedling emergence, leaf and flower senescence, ripening, and organ abscission. It is also a signaling molecule important the response of plants to abiotic and biotic stresses (Abeles *et al.*, 1992). Ethylene treatment has been shown to increased some enzymes activity, such as GLU, CHI, POX, PPO (Cavalcanti *et al.*, 2006; Andrade *et al.*, 2013). However, the role of ethylene in the plant response to biotic stresses has long remained controversial due to conflicting results, there is compelling genetic evidence that ethylene signaling can contribute either to disease resistance or disease susceptibility, depending on the plant–pathogen interaction (Hirsch *et al.*, 2002). Some reports showed the increase in disease symptoms on tomato plants infected by bacteria caused by ethylene, e.g, *Ralstonia solanaceraum* (Hirsch *et al.*, 2002), *Xanthomonas campestris* pv.*vesicatoria* and *Pseudomonas syringae* pv. *tomato* (Lund *et al.*, 1998). Others author reported that elevated ethylene production with resistance of plants. Carrot slices inoculated with *Botrytis cinerea* presented more susceptibly where the ethylene production was inhibited (Hoffman *et al.*, 1988). Tobacco plants insensitive to ethylene response presented enhance susceptibly to non pathogenic organisms (Knoester *et al.*, 1998). In our case, because of the weakly expression of ethylene marker gene it is difficult to infer about the relevance of ethylene in cassava resistance against *Xam*.

CC-NBS-LRR like gene expression did not show correlation with cassava resistance against *Xam* in first the hours after inoculation. However, its expression was repressed at all time points on both studied cultivars. NBS-LRR proteins are the most represented group of resistance genes. This large family is encoded by hundreds of diverse genes and can be subdivided into two functionally distinct subfamilies; TIR-domain-containing (TNL) and CC-domain-containing (CNL). Their role in pathogen has been one of the most studied research areas in the past decade. They are capable of either recognizing the pathogen directly or indirectly monitoring the status of plant defense related proteins that are targeted by pathogen effectors (Mchale *et al.*, 2006). Lopez *et al.* (2005) reported the up-regulated expression of this gene 7 days after inoculation with *Xam in* cassava plants. In our study, all gene expression analyses were performed up to 5 days after inoculation; this may explain the observed repression at the studies time point.

The results of the present study indicate that the defense markers studied here, induced mainly in the first hours of infection on the resistant cultivar, as well as, the H_2O_2 concentration at 120 hai, may limit bacterial growth and disease development, thereby contributing to a greater resistance against bacterial blight. Likely, these results can help us to understand the cassava defense mechanisms and can contribute to the development of tolerant and resistant cultivars against CBB. Considering that resistant cultivars is most effective control strategy utilized by cassava producers, plants overexpressing these genes, mainly chitinases, may help in the control this disease in the field and may decrease the losses caused by this pathogen.

Although many bacterial pathosystems have already been microscopically studied, for example, *Dickeya* sp. on potato plants (Czajkowski *et al.*, 2012) *Clavibacter michiganensis* subsp. *michiganensis* on tomato plants (Chalupowicz *et al.*, 2012) and *Xyllela fastidiosa* on grape plants (Newman *et al*., 2003), *Xam* translocation on cassava plants had not been explored prior to this work. This work is the first report of *Xam* vascular translocation on cassava plants, using CLSM. Knowledge about the vascular translocation of pathogen in their hosts, likely, will help in the development of better control strategies.

To accomplish this, we developed a GFP-tagged strain of *Xam*. GFP possesses excellent features as a reporter protein and it has been broadly used for studying bacterial population in the soil and in the rhizosphere, pathogen colonization of the plant tissues and for tracing particular proteins in the cell (Rosochacki and Matejczyk, 2001). The plasmid pRZT3-GFP (Bloemberg *et al*., 2000) used in this study, was efficient in the transformation of *Xam*. Plasmid stability and pathogenicity of the transformants were confirmed. In spite of the fact that the transformants presented slightly lower growth when compared to wild type isolate, its pathogenicity and plasmid stability was not affected. Therefore, GFP expression did not significantly affected the biological features of *Xam*.

Bacterial movement inside the plant was evaluated up to dai. As expected for a xylem pathogen, during this time, bacteria translocated about 6 cm upward. However, unexpectedly bacteria translocated about 6 cm downward occurred. *Xam* inoculation on cassava stem reproduced the typical wilting symptoms. Water movement in xylem vessels is expected to drag the bacteria rapidly to distant plant parts (Rand, 1983; Czajkowski *et al.*, 2012). Movement of sap in xylem vessels is dependent upon evaporation of water from the surface of mesophyll cells (transpiration) in leaves, thereby pulling water up from the soil via the root system. Therefore, the directional movement of water is usually from roots to leaves

However, downward vascular translocation is somewhat unexpected. In theory, there are three mechanisms by which bacteria are transported downward in xylem vessel: via xylem degradation followed by the colonization of xylem elements (Nelson and Dickey, 1970; Czajkowski *et al.*, 2012), reverse water transport during periods without light (Tattar and Tattar, 1999; Czajkowski *et al.*, 2012) and upstream via pilus-driven twitching motility (Bové and Garnier, 2003; Meng *et al.*, 2005; Czajkowski *et al.*, 2012). In case of this work the cause was not investigated. This work checked on translocation only up to 10 dai and bacteria translocation to roots as well as leaves was not investigated. Future studies following bacteria translocation in whole plant can bring novel insights to *Xam*-cassava interaction, for example, if the bacteria can infect the progenies or soil. On potato plants, *Dickeya* sp. inoculated on stems was able to translocate to roots, soil and progenies (Czajkowski *et al.*, 2009). Therefore, it is reasonable to assume that *Xam* may translocate to distant above and underground tissues. It will be interesting to study *Xam* translocation on susceptible and mainly on resistant cassava plants. The use of the GFP-tagged *Xam* isolate will certainly advance our knowledge in biological aspects of the interaction, helping us to understand other aspect of the resistance and to know the *Xam* behavior on both cultivars.

5. CONCLUSIONS

The defense responses were more accentuated on the resistant cassava than in the susceptible one. Enzymes activities and defense gene expression was more robust on Fécula Branca than in Cacau.

The chitinase activity and gene expression was strongly induced on resistant plants, and may have important role in resistance against *Xam*.

In general, the resistance of cassava plants to Xam infection seems to be highly on the expression of several defense related proteins such as POX, PPO, GLU and mainly CHI.

The bacterial movement on cassava vessels of cultivar Cacau occurred either up or down and reached 6 cm until 10 days after inoculation.

6. REFERENCES

ABAD, L.R.; D'URZO, M.P.; LIU, D.; NARASIMHAN, M.L.; REUVENI, M.; ZHU, J.K.; NIU, X.; SINGH, N.K.; HASEGAWA, P.M.; BRESSAN, R.A. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. **Plant Science**, v.118, n.1, p.11-23, 1996.

ABELES, F.B.; MORGAN, P.W.; SALTVEIT JR, M.E. **Ethylene in plant biology**. San Diego: Academic press, 1992. 346 p.

ADIE, B.; CHICO, J.M.; RUBIO-SOMOZA, I.; SOLANO, R. Modulation of plant defenses by ethylene. **Journal of Plant Growth Regulation**, v.26, n.2, p.160-177, 2007.

AN, D.; YANG, J.; ZHANG, P. Transcriptome profiling of low temperature-treated cassava apical shoots showed dynamic responses of tropical plant to cold stress. **BMC genomics**, v.13, n.1, p.64, 2012.

ANDRADE, C.C.L.; RESENDE, R.S.; RODRIGUES, F.A.; SILVEIRA, P.R.; RIOS, J.A.; OLIVEIRA, J.R.; MARIANO, R.L.R. Indutores de resistência no controle da pinta bacteriana do tomateiro e na atividade de enzimas de defesa. **Tropical Plant Pathology**, v.38, p.28-34, 2013.

BACCARI, C.; LINDOW, S.E. Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape cultivars. **Phytopathology**, v.101, n.1, p.77-84, 2011.

BLOEMBERG, G.V.; WIJFJES, A.H.M.; LAMERS, G.E.M.; STUURMAN, N.; LUGTENBERG, B.J.J. Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. **Molecular Plant-Microbe Interactions**, v.13, n.11, p.1170-1176, 2000.

BOLWELL, G.P.; BINDSCHEDLER, L.V.; BLEE, K.A.; BUTT, V.S.; DAVIES, D.R.; GARDNER, S.L.; GERRISH, C.; MINIBAYEVA, F. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. **Journal of Experimental Botany**, v.53, n.372, p.1367-1376, 2002.

BOVÉ, J.M.; GARNIER, M. Phloem-and xylem-restricted plant pathogenic bacteria. **Plant Science**, v.164, n.3, p.423-438, 2003.

BRADFORD, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical biochemistry**, v.72, n.1, p.248-254, 1976.

CAKMAK, I.; HORST, W.J. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). **Physiologia Plantarum**, v.83, n.3, p.463-468, 1991.

CARDOSO, E.L.C.; H, G.A.; S, S.J. Aspectos econômicos e mercado. In: TROPICAL, E.M.E.F. (Ed.). **Aspectos socioeconômicos e agronômicos da mandioca**. Cruz das Almas, 2006. p.41-70.

CAVALCANTI, F.R.; RESENDE, M.L.V.; PEREIRA, R.B.; COSTA, J.C.B.; CARVALHO, C.P.S. Atividades de quitinase e beta-1, 3-glucanase após eliciação das defesas do tomateiro contra a mancha-bacteriana. **Pesquisa Agropecuária Brasileira**, v.41, n.12, p.1721-1730, 2006.

CHALFIE, M.; TU, Y.; EUSKIRCHEN, G.; WARD, W.W.; PRASHER, D.C. Green fluorescent protein as a marker for gene expression. **Science**, v.263, n.5148, p.802-805, 1994.

CHALUPOWICZ, L.; ZELLERMANN, E.M.; FLUEGEL, M.; DROR, O.; EICHENLAUB, R.; GARTEMANN, K.H.; SAVIDOR, A.; SESSA, G.; IRAKI, N.; BARASH, I. Colonization and movement of GFP-labeled *Clavibacter michiganensis* subsp. *michiganensis* during tomato infection. **Phytopathology**, v.102, n.1, p.23-31, 2012.

CHANCE, B.; MAEHLY, A.C. Assay of catalases and peroxidases. **Methods in enzymology**, v.2, p.764-775, 1955.

CHEN, M.S. Inducible direct plant defense against insect herbivores: a review. **Insect science**, v.15, n.2, p.101-114, 2008.

CHEN, W.P.; CHEN, P.D.; LIU, D.J.; KYNAST, R.; FRIEBE, B.; VELAZHAHAN, R.; MUTHUKRISHNAN, S.; GILL, B. Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene. **Theoretical and Applied Genetics**, v.99, n.5, p.755-760, 1999.

COLLINGE, D.B.; KRAGH, K.M.; MIKKELSEN, J.D.; NIELSEN, K.K.; RASMUSSEN, U.; VAD, K. Plant chitinases. **The Plant Journal**, v.3, n.1, p.31-40, 1993.

CONSTABEL, C.P.; RYAN, C.A. A survey of wound-and methyl jasmonate-induced leaf polyphenol oxidase in crop plants. **Phytochemistry**, v.47, n.4, p.507-511, 1998.

CZAJKOWSKI, R.; DE BOER, W.J.; VAN VEEN, J.A.; VAN DER WOLF, J.M. Characterization of bacterial isolates from rotting potato tuber tissue showing antagonism to *Dickeya* sp. biovar 3 in vitro and in planta. **Plant Pathology**, v.61, n.1, p.169-182, 2012.

DANA, M.M.; PINTOR-TORO, J.A.; CUBERO, B. Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. **Plant physiology**, v.142, n.2, p.722-730, 2006.

DANGL, J.L.; JONES, J.D.G. Plant pathogens and integrated defence responses to infection. **nature**, v.411, n.6839, p.826-833, 2001.

DANHORN, T.; FUQUA, C. Biofilm formation by plant-associated bacteria. **Annual Review of Microbiology**, v.61, p.401-422, 2007.

DE WIT, P.J.G.M. How plants recognize pathogens and defend themselves. **Cellular and Molecular Life Sciences**, v.64, n.21, p.2726-2732, 2007.

DEBONA, D.; RODRIGUES, F.A.; RIOS, J.A.; NASCIMENTO, K.J.T. Biochemical changes in the leaves of wheat plants infected by *Pyricularia oryzae*. **Phytopathology**, v.102, p.1121-1129, 2012.

DOW, J.M.; CROSSMAN, L.; FINDLAY, K.; HE, Y.-Q.; FENG, J.-X.; TANG, J.-L. Biofilm dispersal in *Xanthomonas campestris* is controlled by cell–cell signaling and is required for full virulence to plants. **Proceedings of the National Academy of Sciences**, v.100, n.19, p.10995-11000, 2003.

EL-KEREAMY, A.; EL-SHARKAWY, I.; RAMAMOORTHY, R.; TAHERI, A.; ERRAMPALLI, D.; KUMAR, P.; JAYASANKAR, S. *Prunus domestica* pathogenesis-related protein-5 activates the defense response pathway and enhances the resistance to fungal infection. **PLoS One**, v.6, n.3, p.1-10, 2011.

FAO. **FAOSTAT. Disponível em: http://faostat.fao.org/site/339/default.aspx**. Acesso em: 25/01/2014 2014.

FIALHO, J.D.F.; VIEIRA, E.A.; SILVA, M.S.; PAULA-MORAES, S.V.D.; FUKUDA, W.M.G.; SANTOS FILHO, M.O.S.D.; SILVA, K.N. Desempenho de variedades de mandioca de mesa no Distrito Federal. **Revista Brasileira de Agrociência**, v.15, n.1-4, p.31-35, 2009.

FLEURI, L.F.; SATO, H.H. Beta-1, 3 Glucanases e quitinases: aplicação na lise de leveduras e inibição de fungos. **Ciência e Agrotecnologia**, v.32, n.4, p.1224-1231, 2008.

FUKUDA, C. **Aspectos socioeconômicos e agronômicos da Mandioca**. Cruz das Almas: 2006.

GAY, C.; GEBICKI, J.M. A critical evaluation of the effect of sorbitol on the ferric–xylenol orange hydroperoxide assay. **Analytical biochemistry**, v.284, n.2, p.217-220, 2000.

HAMMOND-KOSACK, K.E.; PARKER, J.E. Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding. **Current opinion in biotechnology**, v.14, n.2, p.177-193, 2003.

HAN, Y.; WANG, Y.; BI, J.L.; YANG, X.K.; HUANG, Y.; ZHAO, X.; HU, Y.; CAI, K.N. Constitutive and induced activities of defense-related enzymes in aphid-resistance and aphid-susceptible cultivars of wheat. **Journal of Chemical Ecology**, v.35, p.176-182, 2009.

HARMAN, G.E.; HAYES, C.K.; LORITO, M.; BROADWAY, R.M.; DI PIETRO, A.; PETERBAUER, C.; TRONSMO, A. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. **Phytopathology**, v.83, n.3, p.313-318, 1993.

HAVIR, E.A.; MCHALE, N.A. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. **Plant physiology**, v.84, n.2, p.450-455, 1987.

HERRERA, C.M.; KOUTSOUDIS, M.D.; WANG, X.; VON BODMAN, S.B. *Pantoea stewartii* subsp. *stewartii* exhibits surface motility, which is a critical aspect of Stewart's wilt disease development on maize. **Molecular Plant-Microbe Interactions**, v.21, n.10, p.1359-1370, 2008.

HIRAGA, S.; SASAKI, K.; ITO, H.; OHASHI, Y.; MATSUI, H. A large family of class III plant peroxidases. **Plant and Cell Physiology**, v.42, n.5, p.462-468, 2001.

HIRSCH, J.; DESLANDES, L.; FENG, D.X.; BALAGUÉ, C.; MARCO, Y. Delayed symptom development in *ein2-1*, an *Arabidopsis* ethylene-insensitive mutant, in response to bacterial wilt caused by *Ralstonia solanacearum*. **Phytopathology**, v.92, n.10, p.1142-1148, 2002.

HOFFMAN, R.; ROEBROECK, E.; HEALE, J.B. Effects of ethylene biosynthesis in carrot root slices on 6-methoxymellein accumulation and resistance to *Botrytis cinerea*. **Physiologia Plantarum**, v.73, n.1, p.71-76, 1988.

IBGE. **Levantamento Sistemático da Produção Agrícola. Disponível em: http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/lspa/default_publ_completa.shtm**. Acesso em: 25/01/2014 2014.

IORIO, E.; TOROSANTUCCI, A.; BROMURO, C.; CHIANI, P.; FERRETTI, A.; GIANNINI, M.; CASSONE, A.; PODO, F. *Candida albicans* cell wall comprises a branched β-d-(1→ 6)-glucan with β-d-(1→ 3)-side chains. **Carbohydrate research**, v.343, n.6, p.1050-1061, 2008.

JONES, J.D.G.; DANGL, J.L. The plant immune system. **nature**, v.444, n.7117, p.323-329, 2006.

JOOSTEN, M.H.A.J.; HENDRICKX, L.J.M.; DE WIT, P.J.G.M. Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of *Cladosporium fulvum* (syn. *Fulvia fulva*). **Netherlands Journal of Plant Pathology**, v.96, n.2, p.103-112, 1990.

KADO, C.I.; HESKETT, M.G. Selective media for isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas and Xanthomonas. **Phytopathology**, v.60, p.969-976, 1970.

KALE, M.C.; CHOUDHARY, A.D. Induction of phenylalanine ammonia- lyase in groundnut cutltivars in response to biotic and abiotic stress. **Indian Phytopathology**, v.54, n.3, p.288-292, 2001.

KANG, B.-C.; YEAM, I.; JAHN, M.M. Genetics of plant virus resistance. **Annual Review of Phytopathology**, v.43, p.581-621, 2005.

KANG, Y.; LIU, H.; GENIN, S.; SCHELL, M.A.; DENNY, T.P. *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. **Molecular microbiology**, v.46, n.2, p.427-437, 2002.

KAR, M.; MISHRA, D. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. **Plant physiology**, v.57, n.2, p.315-319, 1976.

KNOESTER, M.; VAN LOON, L.C.; VAN DEN HEUVEL, J.; HENNIG, J.; BOL, J.F.; LINTHORST, H.J.M. Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. **Proceedings of the National Academy of Sciences**, v.95, n.4, p.1933-1937, 1998.

KOCZAN, J.M.; MCGRATH, M.J.; ZHAO, Y.; SUNDIN, G.W. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. **Phytopathology**, v.99, n.11, p.1237-1244, 2009.

KOUTSOUDIS, M.D.; TSALTAS, D.; MINOGUE, T.D.; VON BODMAN, S.B. Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. **Proceedings of the National Academy of Sciences**, v.103, n.15, p.5983-5988, 2006.

KUMAR, N.N.; DEOBAGKAR, D.N. Multifactional glucanases. **Biotechnology advances**, v.14, n.1, p.1-15, 1996.

KUO, M.C.; KAO, C.H. Aluminum effects on lipid peroxidation and antioxidative enzyme activities in rice leaves. **Biologia plantarum**, v.46, n.1, p.149-152, 2003.

LAMB, C.; DIXON, R.A. The oxidative burst in plant disease resistance. **Annual review of plant biology**, v.48, n.1, p.251-275, 1997.

LEVER, M. A new reaction for colorimetric determination of carbohydrates. **Analytical biochemistry**, v.47, n.1, p.273-279, 1972.

LI, L.; STEFFENS, J.C. Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. **Planta**, v.215, n.2, p.239-247, 2002.

LI, Y.-Z.; PAN, Y.-H.; SUN, C.-B.; DONG, H.-T.; LUO, X.-L.; WANG, Z.-Q.; TANG, J.-L.; CHEN, B. An ordered EST catalogue and gene expression profiles of cassava (*Manihot esculenta*) at key growth stages. **Plant molecular biology**, v.74, n.6, p.573-590, 2010.

LIMA, A.L.S.; DAMATTA, F.M.; PINHEIRO, H.A.; TOTOLA, M.R.; LOUREIRO, M.E. Photochemical responses and oxidative stress in two clones of *Coffea canephora* under water deficit conditions. **Environmental and Experimental Botany**, v.47, n.3, p.239-247, 2002.

LINTHORST, H.J.M.; VAN LOON, L. Pathogenesis-related proteins of plants. **Critical reviews in plant sciences**, v.10, n.2, p.123-150, 1991.

LIU, D.; RAGHOTHAMA, K.G.; HASEGAWA, P.M.; BRESSAN, R.A. Osmotin overexpression in potato delays development of disease symptoms. **Proceedings of the National Academy of Sciences**, v.91, n.5, p.1888-1892, 1994.

LOPER, J.E.; LINDOW, S.E. A biological sensor for iron available to bacteria in their habitats on plant surfaces. **Applied and environmental microbiology**, v.60, n.6, p.1934-1941, 1994.

LOPEZ, C.; SOTO, M.; RESTREPO, S.; PIÉGU, B.; COOKE, R.; DELSENY, M.; TOHME, J.; VERDIER, V. Gene expression profile in response to Xanthomonas axonopodis pv. manihotis infection in cassava using a cDNA microarray. **Plant molecular biology**, v.57, n.3, p.393-410, 2005.

LOZANO, J.C. Cassava bacterial blight: a manageable disease. **Plant Dis**, v.70, p.1089-1093, 1986.

LOZANO, J.C.; SEQUEIRA, L. Bacterial blight of cassava in Colombia: Etiology. **Phytopathology**, v.64, p.74-82, 1974.

LUND, S.T.; STALL, R.E.; KLEE, H.J. Ethylene regulates the susceptible response to pathogen infection in tomato. **The Plant Cell**, v.10, n.3, p.371-382, 1998.

MACHEIX, J.J.; FLEURIET, A.; QUESSADA, M.P. Involvement of phenols and peroxidases in wound healing and grafting. **Molecular and physiological aspects of plant peroxidases/edited by H. Greppin, C. Penel, Th. Gaspar**, p.267-286, 1986.

MALATHI, P.; VISWANATHAN, R.; SUNDAR, A.R. Chitinases as markers for red hot resistance in sugar cane. **International Journal of Biochemical and Life Sciences**, v.3, p.43-46, 2012.

MANIATIS, T.; FRITSCH, E.F.; SAMBROOK, J. **Molecular cloning: a laboratory manual**. Cold Spring Harbor Laboratory Cold Spring Harbor, NY, v.545, 1982.

MAYER, A.M.; STAPLES, R.C. Laccase: new functions for an old enzyme. **Phytochemistry**, v.60, n.6, p.551-565, 2002.

MCHALE, L.; TAN, X.; KOEHL, P.; MICHELMORE, R.W. Plant NBS-LRR proteins: adaptable guards. **Genome Biology**, v.7, n.4, p.212, 2006.

MENG, Y.; LI, Y.; GALVANI, C.D.; HAO, G.; TURNER, J.N.; BURR, T.J.; HOCH, H. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. **Journal of Bacteriology**, v.187, n.16, p.5560-5567, 2005.

MILLER, W.G.; LEVEAU, J.H.; LINDOW, S.E. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. **Molecular Plant-Microbe Interactions**, v.13, n.11, p.1243-1250, 2000.

MIURA, L.; MONTEIRO, A.J.A. Mandioca (*Manihot esculenta* Crantz)–Controle de doenças. **VALE, FX R.; ZAMBOLIM, L. Controle de Doenças de Plantas: grandes Culturas. Viçosa: UFV**, v.2, p.791-814, 1997.

MYSORE, K.S.; RYU, C.-M. Nonhost resistance: how much do we know? **Trends in plant science**, v.9, n.2, p.97-104, 2004.

NELSON, P.E.; DICKEY, R.S. Histopathology of plants infected with vascular bacterial pathogens. **Annual Review of Phytopathology**, v.8, n.1, p.259-280, 1970.

NEWMAN, K.L.; ALMEIDA, R.P.P.; PURCELL, A.H.; LINDOW, S.E. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. **Applied and environmental microbiology**, v.69, n.12, p.7319-7327, 2003.

NGADZE, E.; ICISHAHAYO, D.; COUTINHO, T.A.; VAN DER WAALS, J.E. Role of polyphenol oxidase, peroxidase, phenylalanine ammonia lyase, chlorogenic acid, and total soluble phenols in resistance of potatoes to soft rot. **Plant Disease**, v.96, n.2, p.186-192, 2012.

OWITI, J.; GROSSMANN, J.; GEHRIG, P.; DESSIMOZ, C.; LALOI, C.; HANSEN, M.B.; GRUISSEM, W.; VANDERSCHUREN, H. iTRAQ-based analysis of changes in the cassava root proteome reveals pathways associated with post-harvest physiological deterioration. **The Plant Journal**, v.67, n.1, p.145-156, 2011.

PEREIRA, L.F.; GOODWIN, P.H.; ERICKSON, L. The role of a phenylalanine ammonia lyase gene during cassava bacterial blight and cassava bacterial necrosis. **Journal of plant research**, v.112, n.1, p.51-60, 1999.

PEREIRA, L.F.; GOODWIN, P.H.; ERICKSON, L. Peroxidase activity during susceptible and resistant interactions between cassava (*Manihot esculenta*) and *Xanthomonas axonopodis* pv. *manihotis* and *Xanthomonas cassavae*. **Journal of Phytopathology**, v.148, n.11-12, p.575-578, 2000.

PINTO, M.S.T.; RIBEIRO, J.M.; OLIVEIRA, E.A.G. Estudo de genes e proteínas de defesa de plantas. **Revista Brasileira de Biociências,**, v.9, p.241-248, 2010.

PITSON, S.M.; SEVIOUR, R.J.; MCDOUGALL, B.M. Noncellulolytic fungal β-glucanases: their physiology and regulation. **Enzyme and Microbial Technology**, v.15, n.3, p.178-192, 1993.

PRASHER, D.C.; ECKENRODE, V.K.; WARD, W.W.; PRENDERGAST, F.G.; CORMIER, M.J. Primary structure of the *Aequorea victoria* green-fluorescent protein. **Gene**, v.111, n.2, p.229-233, 1992.

RAHMAN, M.; PUNJA, Z.K. Biochemistry of ginseng root tissues affected by rusty root symptoms. **Plant Physiology and Biochemistry**, v.43, n.12, p.1103-1114, 2005.

RAJU, S.; JAYALAKSHMI, S.K.; SREERAMULU, K. Comparative study on the induction of defense related enzymes in two different cultivars of chickpea (*Cicer arietinum* L) genotypes by salicylic acid, spermine and *Fusarium oxysporum* f. sp. *ciceri*. **Australian Journal of Crop Science**, v.2, n.3, p.121-140, 2008.

RAMEY, B.E.; KOUTSOUDIS, M.; VON BODMAN, S.B.; FUQUA, C. Biofilm formation in plant–microbe associations. **Current opinion in microbiology**, v.7, n.6, p.602-609, 2004.

RAND, R.H. Fluid mechanics of green plants. **Annual review of fluid mechanics**, v.15, n.1, p.29-45, 1983.

RESENDE, R.S.; RODRIGUES, F.Á.; CAVATTE, P.C.; MARTINS, S.C.V.; MOREIRA, W.R.; CHAVES, A.R.M.; DAMATTA, F.M. Leaf gas exchange and oxidative stress in sorghum plants supplied with silicon and infected by Colletotrichum sublineolum. **Phytopathology**, v.102, n.9, p.892-898, 2012.

RIVERA, M.E.; CODINA, J.C.; OLEA, F.; DE VICENTE, A.; PÉREZ-GARCíA, A. Differential expression of β-1, 3-glucanase in susceptible and resistant melon cultivars in response to infection by *Sphaerotheca fusca*. **Physiological and molecular plant pathology**, v.61, n.5, p.257-265, 2002.

ROBERTS, W.K.; SELITRENNIKOFF, C.P. Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. **Journal of general microbiology**, v.136, n.9, p.1771-1778, 1990.

ROSOCHACKI, S.J.; MATEJCZYK, M. Green fluorescent protein as a molecular marker in microbiology. **Acta Microbiologica Polonica**, v.51, n.3, p.205-216, 2001.

RYAN, C.A. The systemin signaling pathway: differential activation of plant defensive genes. **Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology**, v.1477, n.1, p.112-121, 2000.

SCHMITTGEN, T.D.; LIVAK, K.J. Analyzing real-time PCR data by the comparative CT method. **Nature protocols**, v.3, n.6, p.1101-1108, 2008.

SNELLING, J. **Role of Oryza sativa chitinases in disease resistance**. 2010.f. - Colorado State University2010.

SOOSAAR, J.L.M.; BURCH-SMITH, T.M.; DINESH-KUMAR, S.P. Mechanisms of plant resistance to viruses. **Nature Reviews Microbiology**, v.3, n.10, p.789-798, 2005.

STINTZI, A.; HEITZ, T.; PRASAD, V.; WIEDEMANN-MERDINOGLU, S.; KAUFFMANN, S.; GEOFFROY, P.; LEGRAND, M.; FRITIG, B. Plant ‘pathogenesis-related’proteins and their role in defense against pathogens. **Biochimie**, v.75, n.8, p.687-706, 1993.

SUAREZ, A.; GÜTTLER, A.; STRÄTZ, M.; STAENDNER, L.H.; TIMMIS, K.N.; GUZMÁN, C.A. Green fluorescent protein-based reporter systems for genetic analysis of bacteria including monocopy applications. **Gene**, v.196, n.1, p.69-74, 1997.

TATTAR, T.A.; TATTAR, S.J. Evidence for the downward movement of materials injected into trees. **Journal of arboriculture**, v.25, p.325-332, 1999.

TOMBOLINI, R.; UNGE, A.; DAVEY, M.E.; BRUIJN, F.J.; JANSSON, J.K. Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. **FEMS Microbiology Ecology**, v.22, n.1, p.17-28, 1997.

VELAZHAHAN, R.; JAYARAJ, J.; MUTHUKRISHNAN, S.; LIANG, G.H. Purification and characterization of an antifungal thaumatin-like protein from sorghum (*Sorghum bicolor* L) leaves. **Journal of Plant Disease and Protection**, v.109, p.452-461, 2002.

VERDIER, V.; RESTREPO, S.; MOSQUERA, G.; JORGE, V.; LOPEZ, C. Recent progress in the characterization of molecular determinants in the *Xanthomonas axonopodis* pv. *manihotis*–cassava interaction. **Plant molecular biology**, v.56, n.4, p.573-584, 2004.

VIDIGAL FILHO, P.S.; PEQUENO, M.G.; SCAPIM, C.A.; VIDIGAL, M.C.G.; MAIA, R.R.; SAGRILO, E.; SIMON, G.A.; LIMA, R.S. Avaliação de cultivares de mandioca na região noroeste do Paraná. **Bragantia**, v.59, n.1, p.69-75, 2000.

VIGERS, A.J.; WIEDEMANN, S.; ROBERTS, W.K.; LEGRAND, M.; SELITRENNIKOFF, C.P.; FRITIG, B. Thaumatin-like pathogenesis-related proteins are antifungal. **Plant Science**, v.83, n.2, p.155-161, 1992.

VILPOUX, O. A cadeia de mandioca no Brasil. **Campo Grande: SEBRAE**, p.82, 2006.

WHETTEN, R.; SEDEROFF, R. Lignin biosynthesis. **The Plant Cell**, v.7, n.7, p.1001-1014, 1995.

YOUNG, J.M.; SADDLER, G.S.; TAKIKAWA, Y.; DE BOER, S.H.; VAUTERIN, L.; GARDAN, L.; GVOZDYAK, R.I.; STEAD, D.E. Names of plant pathogenic bacteria 1864-1995. **Review of Plant Pathology**, v.75, n.9, p.721-763, 1996.

YUN, D.-J.; IBEAS, J.I.; LEE, H.; COCA, M.A.; NARASIMHAN, M.L.; UESONO, Y.; HASEGAWA, P.M.; PARDO, J.M.; BRESSAN, R.A. Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. **Molecular cell**, v.1, n.6, p.807-817, 1998.

ZHU, B.; CHEN, T.H.H.; LI, P.H. Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. **Planta**, v.198, n.1, p.70-77, 1996.

ZUCKER, M. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. **Plant physiology**, v.40, n.5, p.779, 1965.