Full Length Research Paper

Evidence of salicylic acid regulatory mechanisms of disease resistance against banana vascular wilt *Fusarium oxysporium* f.sp. cubense in *Arabidopsis thaliana*

Raju Radhajeyalakshmi [1,2]*, Yiji Xia[1] and Dhilip Shah[1]

[1]Donald Danforth Plant Science Center 975 N. Warson Road.St. Louis, MO 63132, USA.
[2]Department of Plant Pathology Center for Plant Protection Studies Tamil Nadu Agricultural University Coimbatore-641 003, TN, India.

Symptomatological studies on Arabidopsis- *Fusarium oxysporium f.sp. cubense* (*Foc*) interaction has led to the identification of signaling pathways required for plant resistance to *Foc*, as well as key regulators of innate immunity against this type of vascular wilt pathogens. From the *in planta* symptom expressions of *Foc* on Arabidopsis, there is a clear indication of involvement of salicylic acid (SA) and jasmonic acid (JA) for disease resistance. Mutations occur in synthesis of salicylic acid and Jasmonic acid, which modulate disease resistance with the evident of severe veinal necrosis on leaves and petiole. The typical symptom of leaf rosetting was the clear indication of the active participation of SA biosynthesis for *Foc* resistance in nahG, npr-1 plants. This analysis revealed that salicylic acid, ethylene (ET) and jasmonic acid (JA) pathways influence the *Foc* disease outcome in Arabidopsis. All the three signaling pathways interact in a positive way in the activation of Arabidopsis resistance to *Foc*. Hence, there must be co-ordinate regulation of both SA and JA for *Foc* resistance in Arabidopsis. Constitutive expressions of some transcriptional regulators of these pathways are sufficient to confer enhanced resistance to *Foc* and it might be an oligogenic trait.

Key words: *Fusarium oxysporium f.sp.cubense, Arabidopsis thaliana,* disease resistance.

INTRODUCTION

Fusarium wilt of banana, commonly referred to as Panama Disease, is caused by *Fusarium oxysporum* Schlechtend.: Fr. *f. sp. Cubense* (E.F. Sm.) W.C. Snyder and H.N. Hans. *Fusarium* wilt is the preferred name for what was first called Panama disease because it became prominent in that Central American country early last century. Cavendish plantations has been affected with annual losses over 75 millions USD with effects on family income of thousands of workers and farmers (Masdek et al., 2003; Nasdir, 2003). The global distribution of the disease has an important anthropogenic component; as the infected rhizomes are frequently free of symptoms, is

not unusual that *Foc* were introduced into new areas with conventional plantation material (Ploetz and Pegg, 2000). *Foc* is thought to have originated in Asia, and then spread during the 20th century to become a major problem throughout most banana production regions of the world. An important exception is the South Pacific, where *Fusarium* wilt is a new disease and not yet widespread (Davis et al., 2000; Smith et al., 2002). The fungus infects banana plants through the roots and invades the plant's water conducting tissues. Once *Foc* is introduced into banana gardens, it remains in the soil making it impossible to grow susceptible bananas in the same location for up to several decades.

As *Foc* disrupts the plants' water conducting vessels, leaves become yellow (progressing from older to younger leaves) and wilted. This is also a sign of drought stress that is reduced when water supply is good. Distinctive symptoms appear inside the pseudo stem: brown, red or yellow lines are visible in vertical section which appears as rings in cross-section. These are the infected water conducting vessels. Smaller brown streaks or flecks appear in the corm, at ground level. Later, all leaves turn yellow and die and internal rotting becomes extensive. Splits may also appear in the pseudo stem. Infected plants usually do not produce fruit.

Foc grows better in warmer condition, global warming might positively influence its incidence. *Foc* can persist in affected fields for an extended period of time on plant stubbles as macro conidia or even survive on soils as dormant chlamydospores in the absence of a suitable host plant. Therefore, there is much interest in determining the molecular and genetic basis of plant innate immunity against this type of pathogens.

Foc can invade the weed roots in banana plantations as saprophyte or as a weak parasite of the tissues of senescent roots in decomposition remaining in soil by long periods. There are reports of the isolations of *Foc* from roots of the weeds *Euphorbia heterophylla* L. (Euphorbiaceae), *Tridax procumbens* L. (Poaceae), *Chloris inflata* (Link.) and *Cyanthillium cinereum* L. (Asteraceae) (Waite y Dunlap, 1953); *Cyperus iria* L., *Cyperus rotundus* L., *Gnaphalium purpureum* L., *Fimbristylis koidzuminana* Ohwi (Su et al., 1986) and from decolorated roots without wilting of the species *Paspalum fasciculatum* Sw., *Panicum purpurascens* (Roddi.), *Ixophorus unisetus* Schl., and *Commelina* spp (Podovan, 2003). Persistance of *Foc* disease can be attributed to two principal factors: resistance appears to be genetically complex and thus is a difficult trait to confer by breeding. The role of the signaling pathways mediated by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) in the Arabidopsis innate immune response is well established (Glazebrook, 2005).

Hence, in this study, we investigate the underlying molecular mechanisms of plant resistance to *Foc*, when infect the dicot *Arabidopsis thaliana*. The report is pertaining to the pathogenicity of the various *Foc* strains and corresponding response of Arabidopsis mutants to *Foc*.

MATERIALS AND METHODS

Inoculation of *Fusarium oxysporium f.sp.cubense* and disease scoring

We maintained a frozen stock of each *Fusarium oxysporium f.sp.cubense* isolate in 50% glycerol at -80°C. *Fusarium* was thawed and grown on Czapek-Dox (CzD) plates (CzD broth with 1.5% Bacto agar, Becton Dickinson, Sparks, MD) at 22-28°C. Liquid cultures were inoculated from plates. The following are the *Foc* isolates:

FGSC # 8359
Genotype:*F.oxysporium f.sp.cubense*
Alleles : Australia
Stock Number : 01219
Ref : Phytopathology : 87:915-923

FGSC # 8361
Genotype:*F.oxysporium f.sp.cubense*
Alleles : Australia
Stock Number : 8611
Ref : Phytopathology : 87:915-923

FGSC # 8363
Genotype:*F.oxysporium f.sp.cubense*
Alleles : Malawi
Stock Number : MAL 11
Ref : Phytopathology : 87:915-923

FGSC # 8368
Genotype:*F.oxysporium f.sp.cubense*
Alleles : Taiwan
Stock Number : F9130
Ref : Phytopathology : 87:915-923

Arabidopsis lines, growth conditions

Seeds of Arabidopsis ecotypes, including Col1 and Salk insertion lines, eds5, npr1, jin1, C-24, nahg, ler were provided by Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH). Seeds were disinfected in 10% household bleach for 15 min and subsequently washed three times with excess sterile water. Plants were kept at 22°C in a greenhouse with supplemental fluorescent lighting to maintain a 12 h day length. Four plants for each mutant were maintained for *foc* infection.

Fusarium bud cells were cultured in 700 ml CzD broth in a 2 L Erlenmeyer flask by shaking at 250 rpm at 22-28°C for 5 days. To harvest bud cells, the culture was filtered with Steri-Pad gauze pads (Johnson and Johnson Medical, Arlington, TX), settled by centrifugation at 8000 rpm for 15 min, washed with water, and finally resuspended in water. The soft bud-cell pellet was washed by resuspending bud cells in water and settling by centrifugation three consecutive times. Bud-cell culture density was measured using a hemocytometer, and bud cells were diluted with sterile water. Unless stated otherwise, the inoculum density was 13106 bud cells ml^{-1}. For soil inoculation, 5 ml of bud-cell suspension was applied below the surface of the soil with a pipette. Infested plantings were incubated in a growth chamber (Percival Scientific, Perry, IA) with a 12 h day, under a light density of 120 mEm^{-2} sec^{-1} and at 26°C and 70% relative humidity. Czapek Dox (CzD) broth without bud cells

has been used as mock for inoculation.

Disease was evaluated using the disease index (DI). To measure stunting, we determined the distance from the stem to the distal end of the midrib (leaf length). The three longest leaves of each rosette were included in each measurement. Disease symptoms of *Fusarium* wilt DI score, a representative plant exhibits the symptoms of that score: 0, the plant is dead; 1, older leaves are dead and younger leaves are severely stunted; 2, older leaves are chlorotic, yellow, or dead and younger leaves are stunted; 3, older leaves have vascular chlorosis and the rosette appears compact because leaves are stunted; 4, leaf petioles are stunted; and 5, plants are indistinguishable from mock inoculated plants (Diener and Ausubel, 2005).

RESULTS

Symptoms of *Foc* on Arabidopsis have led to the identification of signaling pathways, as well as key regulators of innate immunity against this type of vascular wilt pathogens. The investigations have been initiated with isolates of *Foc* from the culture collections of FGSC # 8359 (Australian isolate), FGSC # 8361 (Australia), FGSC # 8363 (MALI), FGSC # 8368 (Taiwan) and their disease producing ability in Arabidopsis mutants and ecotypes, Col1 and Salk insertion lines, eds5, npr1, jin1, c-24, nahg, ler Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH). According to Diener and Ausubel (2005), the disease ratings was measured (0 = dead, 1 = severe stunting of young leaves and senescence of older leaves, 2 = chlorosis and premature senescence, 3 = more stunting and mild chlorosis in older leaves, 4 = rosette leaf stunting, 5 = unaffected).

The strains FOC#8359, FOC#8361 exhibited slow growth after 12 h with less branching and minimum number of spores. The mycelium is ceonocytic, septate with micro as well macro conidia. Whereas strains FOC#8363 and FOC#8368 was able to produce well grown ceonocytic, separate branched hyphae with micro and macro conidia. Excessive branching was noticed as typical growth patterns in the above said strains under *in vitro* conditions. The signal transduction network controlling Arabidopsis resistance to *Foc* has been explored by analyzing the pathogen susceptibility in different defective mutants *viz.,* ET (eds-5)-enhanced disease susceptibility 5, which is a salicylic acid induction deficient mutant and a negative regulator of defense response involved in ethylene biosynthesis, JA (jin-1)-jasmonate insensitive 1, which is response to wounding, ABA, chitin, JA signaling pathways, NahG-bacterial Salicylic Acid hydrolase gene suppressing Salicyic Acid (SA) accumulation in plants and npr1-Inactivation of PR-1 gene expression mutant.

Some of the Arabidopsis ecotypes ler and C-24 have also been tested for the resistance to *Foc.* Among the strains, FGSC#8359 (Australia) producing typical symptoms viz. petiole, stem necrosis, resetting of young leaves and chlorosis on all the mutants of Arabidopsis (Table 1). The symptoms indicate that *Foc* was shown to induce acquired resistance (SAR) and pathogenesis-related proteins (PRs) in Arabidopsis.

DISCUSSION

From the *in planta* symptom expressions of *Foc* on Arabidopsis, there is a clear indication of involvement of salicylic acid (SA) and Jasmonic acid (JA) for disease resistance (Figure 1). When the mutations occur in synthesis of salicylic acid and Jasmonic acid, it modulates disease resistance with the evident of severe veinal necrosis on leaves and petiole. The typical symptom of leaf rosetting was the clear indication of the active participation of salicylic acid biosynthesis for *Foc* resistance in nahG and npr-1 plants. Leaf resetting is a phenomenon by which pathogen-induced senescence allow cells to dedifferentiate and subsequently trans-differentiate to switch function (Grafi et al., 2011).This analysis revealed that salicylic acid (SA), ethylene (ET) and Jasmonic acid (JA) pathways influence the *Foc* disease outcome in Arabidopsis with respect to pathogen-induced structural modifications. All the three signaling pathways interact in a positive way in the activation of Arabidopsis resistance to *Foc.* Hence, there must be co-ordinate regulation of both SA and JA for *Foc* resistance in Arabidopsis.

Constitutive expressions of some transcriptional regulators of these pathways are sufficient to confer enhanced resistance to *Foc* and it might be an oligogenic trait (Marta Berrocal-Lobo and Antonio Molina, 2007). Moreover, the C-24 ecotype is having a single dominant locus (VET1); specific to root pathogen resistant mechanism located in xylem and may be identified for disease resistance QTL mapping against *Foc.* Typical aboveground symptoms of *Verticillium* infection on *Brassica napus* and *Arabidopsis thaliana* are stunted growth, vein clearing, and leaf chlorosis as observed by Michaele Reusche et al. (2012). Vein clearing is caused by pathogen-induced trans-differentiation of chloroplast, containing bundle sheath cells to functional xylem elements. Re-initiation of cambial activity and trans-differentiation of xylem parenchyma cells results in xylem hyperplasia within the vasculature of Arabidopsis leaves, hypocotyls and roots. Hyperplasia is generally defined as an induced increase in cell number and has been reported as a symptom of host plant infection by fungal plant pathogens (Malinowski et al., 2012). Salicylic acid-dependent and Jasmonic acid -ethylene dependent pathways induce the expression of different PR genes and also confer resistance to different pathogens (Lorenzo and Solano, 2005). Pieterse et al. (1998) identified a convergence point between different pathways in NPR1, which is required for both Salicylic Acid (SA)-dependent systemic acquired resistance (SAR) and jasmonic acid (JA)-Ethylene (ET) dependent induced systemic resistance (ISR).

Table 1. Variability in symptom expressions by Arabidopsis mutants and ecotypes against *FOC* strains under *in vitro* conditions.

Arabidopsis Mutants	Description	FOC (FGSC#8359)	FOC (FGSC#8361)	FOC (FGSC#8363)	FOC (FGSC#8368)
Col-1-	Wild type	Chlorosis and rosetting of younger leaves	Chlorosis	Chlorosis	Chlorosis
eds-5	Enhanced disease susceptibility-5,salicylic acid induction deficient mutant and a negative regulator of defense response involved in ethylene biosynthesis	Veinal necrosis, collapsed petiole and stem, chlorosis on older leaves	Mild chlorosis on leaves	Not producing any symptoms	Not producing any symptoms
Npr-1	Inactivation of PR-1 gene expression mutant	Severe veinal necrosis, complete necrotization of petiole and stem, blocks in nutrient flow	Mild chlorosis on leaves	Not producing any symptoms	Not producing any symptoms
Jin-1	JA- jasmonate insensitive 1, which is response to wounding, ABA, Chitin, JA signaling pathways	Veinal necrosis, mild rosette leaf stunting	Not producing any symptoms	Not producing any symptoms	Not producing any symptoms
Nah g	Bacterial SA hydrolase gene suppressing SA accumulation in plants	Rosette leaf stunting of young leaves, veinal necrosis, complete collapse of petiole and stem	Not producing any symptoms	Not producing any symptoms	Not producing any symptoms
C-24	Ecotype	Veinal necrosis, complete necrotization of petiole	Not producing any symptoms	Not producing any symptoms	Not producing any symptoms
Ler	Ecotype	Veinal necrosis, complete necrotization of petiole	Not producing any symptoms	Not producing any symptoms	Not producing any symptoms

From the disease ratings, we would like to conclude that the strain FGSC#8359 (Australia) might be able to produce typical symptoms of panama wilt with vascular browning and necrotization of tissues in Arabidopsis lines (Figure 2) and furthering research activities in potentiating the Salicylic Acid (SA) regulatory mechanisms for developing disease resistance strategies in banana cultivars (Edgar et al., 2006). Zhu et al. (2012) revealed from the time-course RNA-seq analysis results upon *F. oxysporum* infection, the biogenesis and signaling signaling pathways of ET, SA and JA were coordinately activated with the ET-mediated signaling pathway activated earlier than the JA and SA mediated signaling pathways. A number of genes responsive to *F. oxysporum* infection identified have been previously shown to be part of the defense network in various plant-pathogen interactions, for instance, genes involved in jasmonic acid (JA), indole-3-ylmethy-glucosinolate (I3G) and camalexin biosynthesis pathways (Bednarek et al., 2009; Clay et al., 2009; Kidd et al., 2011; Pfalz et al., 2009)

The symptoms produced by FOC strain FGSC#8359 have coincidences with the findings of Batlle and Pérez (2003) who found that Cuban *Foc* isolates of race 1 and 2 indistinctly can or not produce volatiles aldehyde in direct relationship with pathogenesis (Moore et al., 1991). From these findings, we wish to conclude that the defense response of Arabidopsis lines against *Foc* strains is under the control of minor resistance genes, rather than a single dominant "R" gene (Hwang and Ko, 2004). Different climatic zones can determine the disease development and can classify the strains into "Tropical" and "Subtropical" strains (Ploetz et al., 1990).

Conflict of Interest

The author(s) have not declared any conflict of interest.

ACKNOWLEDGEMENTS

Mutant Lines-FOC
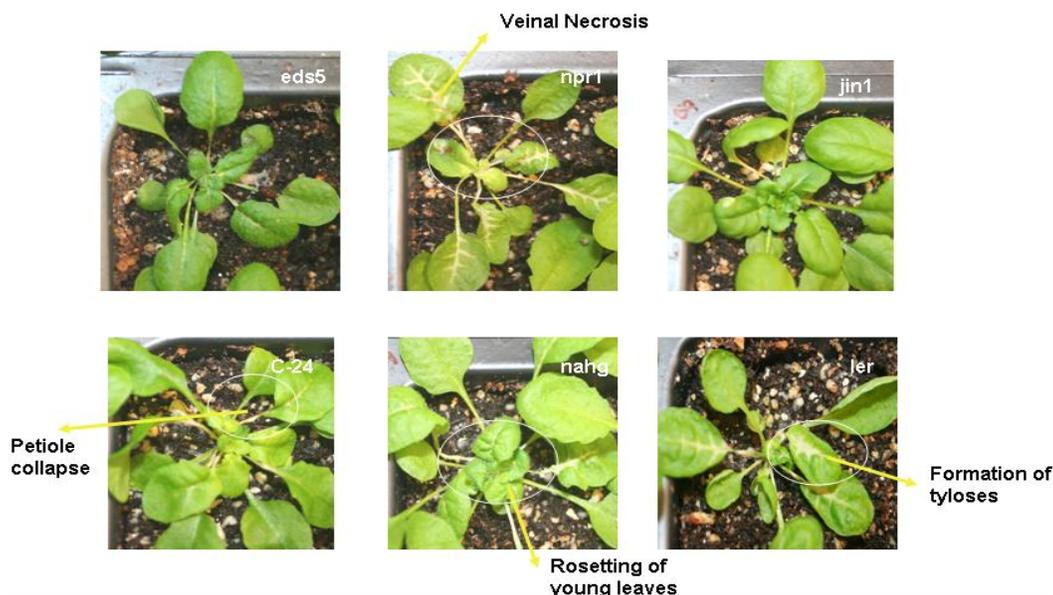
Figure 1. Arabidopsis lines expressing symptoms in response to *F. oxysporum f.sp.cubense* (*Foc*) strain FGSC#8359. Top: Left to right: eds5, npr1, jin1; Bottom: Left to right: C 24, nahg, ler.
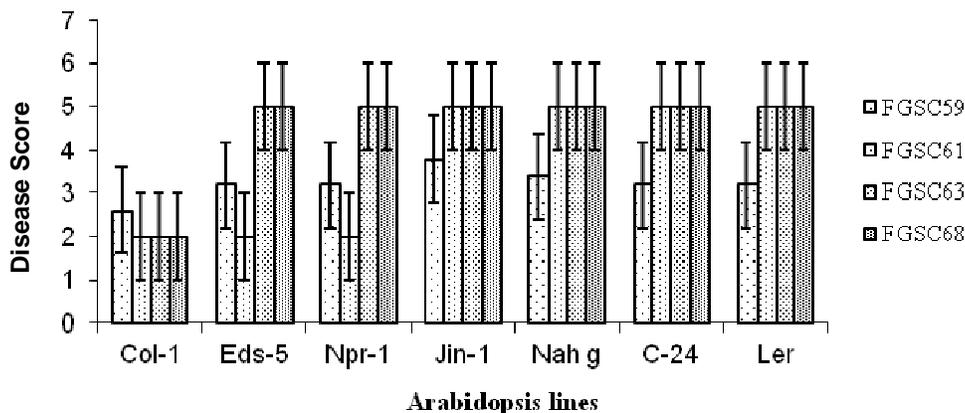
Figure 2. Measurement of Disease Index (DI) in Arabidopsis lines in response to *F.oxysporum f.sp.cubense* (*Foc*).

REFERENCES

Batlle A, Pérez L (2003). Population Biology of *Fusarium oxysporum f.sp. cubense* in Cuba. In: 2nd. International Symposium on *Fusarium* wilt on banana. PROMUSA-INIBAP/EMBRAPA. Salvador de Bahía, Brazil. pp. 22-26 .

Bednarek P et al (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science. 323:101-106.

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009). Glucosinolate metabolites required for an Arabidopsis innate immune response. Science 323:95-101.

Davis RI, Moore NY, Bently S, Gunua TG, Rahamma S (2000). Further records of *Fusarium oxysporum f.sp. cubense* from New Guinea. Australas. Plant Pathol. 29:224.

Diener AC, Ausubel FM (2005). Resistance to *Fusarium oxysporum* 1, a dominant Arabidopsis resistance gene, is not race specific. Genetics.171:305-321.

Edgar CI et al (2006). Salicylic acid mediates resistance to the vascular wilt pathogen *Fusarium oxysporum* in the model host *Arabidopsis thaliana*. Australas. Plant Pathol. 35:581-591.

Glazebrook J (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. 43:205-227.

Grafi G, Chalifa-Caspi V, Nagar T, Plaschkes I, Barak S, Ransbotyn V (2011). Plant response to stress meets dedifferentiation. Planta. 233:433-438.

Hwang S, Ko W (2004). Cavendish banana cultivars resistant to *Fusarium* wilt acquired through somaclonal variation in Taiwan. Plant Dis. 88:580-588.

Kidd BN et al (2011). Auxin signaling and transport promote susceptibility to the root-infecting fungal pathogen Fusarium oxysporum in Arabidopsis. Mol. Plant Microbe Interact. 24:733-748.

Lorenzo O, Solano R (2005). Molecular players regulating the jasmonate signaling network. Current Opinion in Plant Pathol. 8:532-540.

Malinowski R, Smith JA, Fleming AJ, Scholes JD, Rolfe SA (2012). Gall formation in clubroot-infected Arabidopsis results from an increase in existing meristematic activities of the host but is not essential for the completion of the pathogen life cycle. Plant J. 71:226-238.

Marta B-L, Antonio M (2007). Arabidopsis defense response against *Fusarium oxysporum*. Trends Plant Sci. 13(3):145-150

Masdek N, Mahmood M, Molina A, Hwang SC, Dimyati A, Tangaveli R, Omar I (2003). Global significance of *Fusarium* wilt: Asia. Abstracts of Papers 2nd. International Symposium on *Fusarium* wilt on banana. PROMUSA-INIBAP/EMBRAPA. Salvador de Bahía, Brazil. pp. 22-26.

Moore NY, Hargreaves PA, Pegg K, Irwin JAG (1991). Characterization of strains of *Fusarium oxysporum f. sp. cubense* by production of volatiles, Aust. J. Bot. 39:161-166.

Nasdir N (2003). *Fusarium* wilt race 4 in Indonesia. Research Institute for Fruits west Sumatra, Indonesia. Abstracts of Papers 2nd. International Symposium on *Fusarium* wilt on banana. PROMUSA-INIBAP/EMBRAPA.Salvador de Bahía, Brazil. pp. 22-26.

Pfalz M, Vogel H, Kroymann J (2009). The gene controlling the indole glucosinolate modifier1 quantitative trait locus alters indole glucosinolate structures and aphid resistance in Arabidopsis. Plant Cell. 21:985-999.

Pieterse CM, Van Wees SC, VanPelt JA, Knoester M, Laan R, Gerrits H, Weisbee PJ, Van Loon LC (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell 10:1571-1580.

Ploetz RC (1990). Population biology of *Fusarium oxysporum f. sp. cubense*. In: Fusarium wilt of banana. Ploetz, R.C. (Ed.) APS Press St Paul. pp. 63-76.

Ploetz RC, Pegg K (2000). *Fusarium* wilt. In: Diseases of Banana, Abaca and Enset. Jones, D.R. (Ed), CABI Publishing, Wallingford, UK. 143-159.

Podovan A, Henessey C, Walduck G (2003). Alternative hosts of *Fusarium oxysporum f.sp. cubense* tropical race 4 in northern Australia. In: 2nd. International Symposium on *Fusarium* wilt on banana. PROMUSAINIBAP/EMBRAPA. Salvador de Bahía, Brazil, pp. 22-26.

Smith LJ, Moore NY, Tree DJ, Bently S, Pattemore J (2002). Firs record of *Fusarium oxysporum f.sp cubense* from Yap, Federated States o Micronesia, Australas. Plan Pathol. pp. 31:101

Su HJ, Hwang SC, Ko WH (1986). Fusarial wilt of bananas in Taiwan. Plant Dis. 70:813-818.

Waite BH, Dunlap VC (1953). Preliminary host range studies with *Fusarium oxysporum f. cubense*. Plant. Dis. Rptr. 37:79-80.

Zhu QH et al (2012). Characterization of the defense transcriptome responsive to *Fusarium oxysporum*-infection in Arabidopsis using RNA-seq, Gene, http://dx.doi.org/10.1016/j.gene.2012.10.036.