

## CONTROL OF *Lasiodiplodia theobromae*, THE CAUSAL AGENT OF DIEBACK OF SAPOTE MAMEY [*Pouteria sapota* (Jacq.) H. E. Moore and Stearn] GRAFTS IN MÉXICO

## CONTROL DE *Lasiodiplodia theobromae*, AGENTE CAUSAL DE LA MUERTE DESCENDENTE DE INJERTOS DE ZAPOTE MAMEY [*Pouteria sapota* (Jacq.) H. E. Moore y Stearn] EN MÉXICO

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### SUMMARY

Dieback of sapote mamey [*Pouteria sapota* (Jacq.) H. E. Moore and Stearn] grafts caused by *Lasiodiplodia theobromae* significantly reduces the success of grafting in Mexican nurseries. Semi-commercial tests were conducted in a sapote mamey nursery during two grafting seasons to evaluate the effectiveness of a physical treatment (washing) and three chemical treatments (fungicide dip) on scions. Washing reduced disease incidence up to 31 %, whereas chemical treatment reduced it up to 62 %. The combination of washing and thiabendazole dip resulted in an incidence decrease of 81 %. Twelve other fungicides were screened *in vitro* to evaluate mycelial growth inhibition of *L. theobromae*. The pre-formulated mix of cyprodinil + fludioxinil was the most effective fungicide with EC<sub>50</sub> value of 0.0043 µg mL<sup>-1</sup>, followed by pyraclostrobin + boscalid, prochloraz and tebuconazole with EC<sub>50</sub> values < 0.1 µg mL<sup>-1</sup>, and iprodione and thiabendazole with EC<sub>50</sub> values < 1.0 µg mL<sup>-1</sup>. This study provides novel information about strategies for controlling *L. theobromae* infection during the grafting process of sapote mamey and it also showed the effectiveness of several fungicides under *in vitro* conditions for controlling this plant pathogenic fungus.

**Index words:** *Pouteria sapota*, *Lasiodiplodia theobromae*, fungicides, nursery, propagation.

### RESUMEN

La muerte descendente de varetas injertadas de zapote mamey [*Pouteria sapota* (Jacq.) H. E. Moore y Stearn] causada por *Lasiodiplodia theobromae* reduce significativamente el prendimiento de injertos en viveros en México. Se realizaron pruebas semi-comerciales en un vivero de zapote mamey durante dos épocas de injerto para evaluar la efectividad de tratamientos físicos (lavado) y químicos (inmersión en fungicida) en varetas de este frutal. El lavado disminuyó la incidencia de la enfermedad hasta 31 %, mientras que, el tratamiento químico la redujo hasta en 62 %. La combinación del lavado con thiabendazole abatió la incidencia hasta 81 %. Otros doce fungicidas se evaluaron *in vitro* para inhibir el crecimiento micelial de *L. theobromae*. La mezcla pre-formulada de cyprodinil + fludioxinil fue el fungicida más efectivo con valores de EC<sub>50</sub> = 0.0043 µg mL<sup>-1</sup>, seguido por pyraclostrobin + boscalid, prochloraz y tebuconazole con EC<sub>50</sub> < 0.1 µg mL<sup>-1</sup>, e iprodione y thiabendazole con valores EC<sub>50</sub> < 1.0 µg mL<sup>-1</sup>. Este estudio provee información novedosa sobre estrategias para controlar a *L. theobromae* durante el proceso de injerto de zapote mamey, además de mostrar la efectividad *in vitro* de varios fungicidas para el control de este hongo fitopatógeno.

**Palabras clave:** *Pouteria sapota*, *Lasiodiplodia theobromae*, fungicidas, vivero, propagación.

### INTRODUCTION

*Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. (syn. *Botryodiplodia theobromae* Pat.) the anamorphic stage of *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx, is widely distributed in tropical and subtropical regions and has been associated with approximately 500 hosts. It induces diseases such as dieback, root rot, fruit rot, blights, gummosis, stem necrosis, leaf spot and witches' broom disease (Punithalingam, 1980). The major industrial crops and fruit trees in which it has been found causing pre-harvest (dieback and gummosis) and/or post-harvest (fruit rot) diseases are: avocado (*Persea americana* Mill.) (Darvas and Kotze, 1987), soursop (*Annona muricata* L.) (Ponte, 1985), cocoa (*Theobroma cacao* L.) (Mbenoun *et al.*, 2008), citrus (*Citrus* spp.) (Adisa and Obinyereokwu, 1988), coconut (*Cocos nucifera* L.) (Correia and Costa, 2005), guava (*Psidium guajava* L.) (Cardoso *et al.*, 2002), mango (*Mangifera indica* L.) (Meah *et al.*, 1991), papaya (*Carica papaya* L.) (Wang *et al.*, 2007), grapevine (*Vitis vinifera* L.) (Bester *et al.*, 2007) and sapote mamey (*Pouteria sapota* (Jacq.) H. E. Moore and Stearn) (Bautista *et al.*, 2002; Gómez *et al.*, 2009; Vásquez *et al.*, 2009). This fungus has also been associated with dieback and necrosis at the grafting site of cashew (*Anacardium occidentale* L.) (Freire *et al.*, 2002), guava (Cardoso *et al.*, 2002), citrus (Davis *et al.*, 1987), grapevine (Aroca *et al.*, 2008) and sapote mamey (Tovar *et al.*, 2012).

Incidence of *L. theobromae* induced dieback of sapote mamey grafts during grafting is high (> 70 %) in Mexico (Tovar *et al.*, 2012), however, there is no information regarding physical or chemical treatment of vegetative material used in grafting of this fruit.

Fungicides have become the most important means of controlling fungal pathogens. Several *in vitro* tests have

determined the sensitivity of *L. theobromae* to fungicides (Bester *et al.*, 2007; Da Silva *et al.*, 2012), however, there are no reports of *in vitro* tests for the control of *L. theobromae* isolates obtained from sapote mamey.

Based on this information, this study evaluated the potential effect of fungicide dip (chemical treatment) and washing of scions (prophylactic physical treatment) to prevent graft infection by *L. theobromae* during vegetative propagation of sapote mamey and to determine the *in vitro* effectiveness of a range of fungicides to inhibit mycelial growth of *L. theobromae*.

## MATERIALS AND METHODS

### Nursery tests

Chemical and physical prophylactic treatments were conducted during two grafting seasons (Spring and Fall 2009) in a commercial nursery of sapote mamey located in Alpoeyca, Guerrero, México, using naturally infected scions. Rootstocks were grown from seeds of freshly harvested fruits. Conventional practices of nutrition and irrigation were applied until plants reached 80 cm in height and a minimum of 2 cm in diameter over a period of 10 to 12 months. The experimental shoots (straight, 25 cm long, without physical damage and with several dormant buds and mature leaves) were collected from the tree “El Mexicano”, a 20-years-old selection. The scions collected during March and October 2009, were manually defoliated. A total of 256 sapote mamey scions were grafted by the veneer grafting technique as recommended by Villegas and Mora (2008).

Scions were washed by prophylactic physical treatment with a natural fiber and water with the aim to remove dead tissue (pubescence) and organic residues. Water excess was removed with blotting paper; washed scions were allowed to air dry for 15 min and then grafted.

The prophylactic chemical treatment was dipping scions in the fungicide suspension for 15 min. The following fungicides were tested: thiabendazole (600 mg L<sup>-1</sup>), mancozeb (800 mg L<sup>-1</sup>), and thiabendazole + mancozeb (600 mg L<sup>-1</sup> + 800 mg L<sup>-1</sup>). Excess fungicide suspension was removed with blotting paper, and treated scions were allowed to air dry for 15 min before grafting.

Washing prior to application of each of the three fungicide dips was performed to determine the interaction between both groups of treatments. Control scions were neither washed nor dipped into the fungicide suspension. The experimental design consisted of four randomized blocks. Each block contained eight treatments. Each treatment was

applied on 4 scions. In the whole experiment, each treatment was applied to 16 scions.

All grafts were covered with clear plastic bags to avoid dehydration. Grafted plants were maintained under 75 – 80 % shade cloth, and inspected every week for disease development. The effect of washing and fungicide dip was assessed 30 d after grafting. Diseased grafted plants for each treatment were taken to the laboratory to confirm the presence of *L. theobromae*. Incidence of diseased grafts for each treatment was calculated with the following equation:  $I_i = \sum ni / Ni$ ; where,  $I_i$  = incidence of diseased grafts at the moment  $I$ ;  $ni$  = number of diseased grafts at the moment  $I$ ;  $Ni$  = total population of grafted plants for each treatment.

### *In vitro* test

*Lasiodiplodia theobromae* isolate (GenBank Accession No. JQ245975) used in *in vitro* screening tests was obtained from a commercial nursery of sapote mamey grafted plants that showed necrosis in the graft union and dieback of scions (Tovar *et al.*, 2012). This isolate is maintained at the culture collection of the herbarium “CMPH” (Accession No. CB007) at Colegio de Postgraduados, Campus Montecillo, Texcoco, Estado de México, México.

Seven systemic and five contact fungicides, representing ten different chemical groups, were selected for the *in vitro* mycelial growth inhibition tests (Table 1).

Fungicide stock solutions (1000 µg mL<sup>-1</sup>) were made using sterile distilled water. Aliquots of stock solutions were incorporated into autoclaved potato-dextrose-agar (PDA) (BD Bioxon®, Becton Dickinson, USA) when the medium was at 45 to 50 °C to provide concentrations of 0.1, 1, 10 and 100 µg mL<sup>-1</sup>. The pre-formulated mix of cyprodinil + fludioxonil was tested at 0.001, 0.005, 0.01, 0.05, and 0.1 µg mL<sup>-1</sup>. PDA without fungicide was used as control. Approximately 20 mL of fungicide-amended medium was poured into Petri dishes. Mycelial plugs (5 mm in diameter) obtained from the margins of actively growing culture (4 days-old) of *L. theobromae* on PDA were transferred to the fungicide-amended plates. Four replicates were used per fungicide concentration. Plates were incubated at 28 °C and the diameter of radial mycelial growth of each colony was measured perpendicularly twice every 24 h for 3 d. The complete experiment was repeated twice.

### Statistical analysis

Incidence data of the nursery trials were transformed to arcsine values before the analysis to normalize the variances. Data were subjected to analysis of variance (ANOVA) using SAS (Statistical Analysis System, version 9.1, SAS Institute

**Table 1. Fungicides tested for *in vitro* mycelial growth inhibition of *Lasiodiplodia theobromae*, isolated from sapote mamey (*Pouteria sapota*) grafts with symptoms of dieback.**

Chemical group	Active ingredient (a.i)	Action	a. i. (%)	Formulation
Anilinopyrimidines	Pyrimethanil	S <sup>†</sup>	54.6	SC
Anilinopyrimidines/ Phenylpyrroles	Cyprodinil + Fludioxinil	S	37.5/25	WG
Benzimidazoles	Thiabendazole	S	60	WP
Chloronitriles	Chlorothalonil	C	75	WP
Dicarboximides	Iprodione	C	50	WP
Dithiocarbamates	Mancozeb	C	80	WP
DMI- Imidazoles	Prochloraz	S	42.1	EC
DMI- Triazoles	Tebuconazole	S	25	EW
Inorganic (Copper)	Copper hydroxide	C	37.5	SC
Phthalamides	Captan	C	50	WP
QoI- Methoxycarbamates/ Pyridinecarboxamides	Pyraclostrobin + Boscalid	S	25.2/12.8	WG
QoI- Oximinoacetates	Kresoxim-methyl	S	50	WG

<sup>†</sup>S = systemic fungicide; SC = suspension concentrate; WG = water dispersible granule; WP = wettable powder; C = contact fungicide; DMI = demethylation inhibitors; EC = emulsifiable concentrate; EW = emulsion oil in water; QoI = quinone outside inhibitors.

Cary, NC). Treatment means were compared using the least significant difference (LSD) test ( $P \leq 0.05$ ). Data from each grafting season were analyzed separately.

Inhibition of mycelial growth for each fungicide concentration of the *in vitro* tests was calculated as a percentage with respect to the control treatment (un-amended PDA). Percentages of mycelial growth inhibition were converted to Probits and plotted against  $\log_{10}$  values of the fungicide concentration. Probit regression analysis was used to calculate the effective concentration values that inhibited mycelial growth by 50 %.  $EC_{50}$  values were processed by an ANOVA performed with the General Linear Model (GLM) of SAS and treatment means were separated using the least significant difference (LSD) value at  $P \leq 0.05$ .

## RESULTS AND DISCUSSION

### Nursery tests

Washing significantly reduced dieback incidence of grafts during the spring season but not during the fall, as compared to the control (Table 2). In spring, disease incidence decreased from 87.50 % of the control to 56.25 % of the washed scions (a reduction of 31 %), while in fall the corresponding incidence reduction only was of 18 %. This result was probably related to a higher concentration of inoculum during the fall season, as it was shown by Vásquez *et al.* (2009) who found that the highest concentration of conidia in the air was captured in volumetric traps during the fall seasons from 2007 to 2009 in Alpoeyca, Guerrero.

According to Tovar *et al.* (2012), *L. theobromae* inoculum is located in the cortex and trichomes of sapote ma-

mey scions; thus, it is presumed that treatment by washing removed the fungal inoculum present on the scions. Hot water treatment is effective in reducing or eradicating the inoculum source of fungal pathogens that cause diseases in grapevine nurseries (Edwards *et al.*, 2004; Halleen *et al.*, 2007).

No significant difference was detected among chemical treatments. However, all chemical treatments significantly reduce disease incidence with values ranging from 37 to 62 % compared to the control during both grafting seasons, respectively (Table 2 and Figure 1). Retief *et al.* (2006) and Gramaje *et al.* (2009) showed that dipping of material in fungicide suspension eliminates the inoculum source from the surface and/or decreases superficial fungal growth during the graft union process in grapevine nurseries. No phytotoxicity symptoms were observed with any of the fungicides tested.

The combination of physical and chemical treatments reduced the disease incidence up to 81 %, compared with the untreated control scions during both seasons (Table 2). These results coincide with reports by Halleen *et al.* (2007) in grapevine propagating material, in which the reduction of disease incidence was more consistent in the propagating material that received the combination of physical and chemical treatments. Our results support the statement that an integrated management program is most effective in reducing the incidence of diseases caused by fungal pathogens that penetrate during the grafting process (Fourie and Halleen, 2005, 2006).

We observed mycelial growth and conidia of *L. theobromae* on the stem cortex, and presumably, it only penetrated

**Table 2.** Mean incidence of dieback of sapote mamey grafts, after physical and chemical prophylactic treatments during two grafting seasons in 2009, in Guerrero, México.

Treatments	Incidence (%)	
	Spring	Fall
Thiabendazole (T)	25.00 cd <sup>†</sup>	37.50 c
Mancozeb (M)	31.25 c	43.75 c
Thiabendazole/mancozeb (T + M)	43.75 bc	56.25 bc
Washing (W)	56.25 b	75.00 ab
W + T	6.25 d	12.50 d
W + M	6.25 d	37.50 c
W + (T + M)	31.25 c	50.00 c
Control (without treatment)	87.50 a	93.75 a

<sup>†</sup>Means with the same letter in a column are not significantly different (LSD, 0.05).

through deep and extensive wounds, such as the ones made to scions and rootstocks during the grafting process. However, the inoculum was effectively removed by washing and by treating with the fungicide dip, especially with thiabendazole. These findings suggest that the combination of washing and chemical treatments should be recommended to prevent infections by *L. theobromae* in grafts and increase the success of young plants established in sapote mamey orchards.

**Figura 1.** Effect of washing and chemical treatments on sapote mamey scions, 30 d after grafting. a) Dead untreated scion; b) Healthy treated scion.

#### *In vitro* test

**Mycelial growth assay.** The pre-formulated mix of cyprodinil + fludioxinil was the most effective fungicide in reducing mycelial growth with  $EC_{50}$  value =  $0.0043 \mu\text{g mL}^{-1}$

(Table 3). Similarity, Wang *et al.* (2007) found that the mix of cyprodinil + fludioxinil inhibited mycelial growth of *L. theobromae* isolates from papaya to  $EC_{50} = 0.0073 \mu\text{g mL}^{-1}$ .

The  $EC_{50}$  values of pyraclostrobin + boscalid, prochloraz, and tebuconazole ranged from 0.014 to  $0.048 \mu\text{g mL}^{-1}$ , followed by iprodione and thiabendazole ( $EC_{50} = 0.335$  to  $0.430 \mu\text{g mL}^{-1}$ ). Wang *et al.* (2007) found prochloraz, iprodione, and tebuconazole as the most effective fungicides to inhibit mycelial growth of *L. theobromae* isolates from papaya with  $EC_{50} < 1 \mu\text{g mL}^{-1}$ . In contrast, Da Silva *et al.* (2012) indicated that thiabendazole, prochloraz, and tebuconazole were effective with  $EC_{50}$  values  $> 1 \mu\text{g mL}^{-1}$ ; however, their isolates were obtained from papaya orchards subjected to several fungicide applications. Bester *et al.* (2007) reported that prochloraz and tebuconazole at low  $EC_{50}$  ( $< 0.6 \mu\text{g mL}^{-1}$ ) inhibited mycelial growth of *L. theobromae* isolates from grapevines, while kresoxim-methyl, iprodione and pyrimethanil were ineffective. Our results coincide with this report, except for iprodione, which, in this study, was observed to be effective with an  $EC_{50}$  value =  $0.335 \mu\text{g mL}^{-1}$ .

In this *in vitro* test, systemic fungicides were effective at low concentrations because our *L. theobromae* isolate had not been previously exposed to selection pressure by fungicide application in sapote mamey nurseries or orchards. Most of the contact fungicides (captan, chlorothalonil, copper hydroxide, and mancozeb) inhibited mycelial growth with  $EC_{50}$  values  $> 7 \mu\text{g mL}^{-1}$  as reported by Khanzada *et al.* (2005) and Gud and Raut (2008) in tests with isolates from mango.

We selected contact and systemic fungicides from different chemical groups, because the fungicide application programs that prevent pathogen populations from developing fungicide resistance are based on the rotation and/or combination of two action types (Denman *et al.*, 2004).



**Table 3.** EC<sub>50</sub> values for *in vitro* inhibition of mycelial growth of *L. theobromae* in PDA culture, by fungicides representing different chemical groups.

Fungicide	EC <sub>50</sub> (µg mL <sup>-1</sup> )	
	Range	Mean
Captan	(9.2500 - 16.955)	12.340 a †
Cyprodinil + fludioxinil	(0.0035 - 0.0056)	0.0043 e
Chlorothalonil	(5.0625 - 12.033)	7.598 b
Copper hydroxide	(7.5325 - 12.285)	9.590 b
Iprodione	(0.2450 - 0.448)	0.335 d
Kresoxim-methyl	(2.4925 - 5.140)	3.413 c
Mancozeb	(6.0350 - 9.143)	7.448 b
Pyrimethanil	(1.4000 - 2.318)	1.800 cd
Prochloraz	(0.0050 - 0.035)	0.015 d
Pyraclostrobin + boscalid	(0.0019 - 0.045)	0.014 d
Tebuconazole	(0.0175 - 0.090)	0.048 d
Thiabendazole	(0.3275 - 0.558)	0.430 d

† Means with the same letter in a column are not significantly different (LSD, 0.05).

Cyprodinil + fludioxinil, pyraclostrobin + boscalid, prochloraz, tebuconazole and iprodione showed high effectiveness against *L. theobromae* and should be evaluated in additional trials in sapote mamey nurseries to confirm their efficacy in the field.

### CONCLUSIONS

The combination of washing with water and fungicide dip was effective to prevent infections by *L. theobromae* during the grafting process of sapote mamey. Cyprodinil + fludioxinil, pyraclostrobin + boscalid, prochloraz, tebuconazole and iprodione effectively inhibited mycelial growth of *L. theobromae* *in vitro*.

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