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Induced Host Resistance by Non-pathogenic *Fusarium* Endophyte as a Potential Defense Mechanism in Fusarium Wilt Management of Banana

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ABSTRACT

In this study, the role of a fungal endophyte identified as *Fusarium* spp. UPM31P1 in suppressing Fusarium wilt development in susceptible banana plantlets via induced host resistance was established. The endophyte, isolated from roots of wild banana, elicited the production of host enzymes related to induced resistance upon reintroduction into the commercial cultivar Pisang Berangan "Intan". A significant increase in all levels of enzymes and biochemical markers assayed (peroxidase, polyphenoloxidase, phenylalanine ammonia lyase, phenol content and lignothioglycolic acid) was observed as compared to levels in plantlets from control. In comparison between diseased plantlets pre-treated and non-treated with endophyte UPM31P1, enzymatic levels were also significantly higher in plantlets pre-treated with endophytes. As a result, the endophyte pre-treated plantlets recorded lower percentages of disease incidence and disease severity, suggesting the possible role of induced host resistance triggered by the endophytic UPM31P1 as a mechanism for Fusarium wilt suppression.

Keywords: biocontrol agents, disease suppression, *Fusarium oxysporum* f. sp. *cubense* race 4, plant defense mechanism
Abbreviations: ANOVA, analysis of variance; **DI**, disease incidence; **DS**, disease severity; **FocR4**, *Fusarium oxysporum* f.sp. *cubense* race 4; **HSD**, honestly significant difference; **LTGA**, lignothioglycolic acid; **NA**, nutrient agar; **PAL**, phenylalanine ammonia lyase; **PDA**, potato dextrose agar; **PO**, peroxidase; **PPO**, polyphenoloxidase; **PVP**, polyvinyl pyrrolidone; **SAB**, sodium acetate buffer; **SBB**, sodium borate buffer; **SDW**, sterile distilled water; **SPB**, sodium phosphate buffer; **VCG**, vegetative compatibility group

INTRODUCTION

Endophytes are microorganisms that exist inside a host plant, often asymptomatically and can be isolated from the crown, stem and leaf tissues (Wilson 1995; Clay and Schardl 2002). The potential of endophytes as biocontrol agents are mainly attributed to their production of toxic alkaloids (Bacon *et al*. 1977; Bush *et al*. 1997), inhibitory metabolites (Findlay *et al*. 1997; Strobel *et al*. 1999), antibiotics (Harrison *et al*. 1991; Brady and Clardy 2000) and antioxidants (Strobel *et al*. 2002); which have been shown to confer resistance to pests and pathogens (Kimmons *et al*. 1990; Siegel and Latch 1991; Stovall and Clay 1991; Gwinn and Gavin 1992; Mahmood *et al*. 1993). In addition, endophytes also usually form beneficial association with the host plant, resulting in improved vegetative growth (Clay 1989; Rice *et al*. 1990; Ting *et al*. 2008), and tolerance to stress factors (Lewis *et al*. 1997; Cheplick *et al*. 2000; Malinowski and Belesky 2000).

Another beneficial endophyte-host association commonly observed is the significant increase in the production of induced host defense enzymes such as phytoalexins (van Peer *et al*. 1991) and pathogenesis-related proteins (Zdor and Anderson 1992). Increase in levels of peroxidases (PO) (Peng and Kuc 1992), polyphenoloxidases (PPO) (Klessig and Malamy 1994), phenylalanine ammonia lyase (PAL) (Bhattacharyya and Ward 1988; Klessig and Malamy 1994), total soluble phenols (Metraux and Raskin, 1993) and lingothioglycolic acid (LTGA) (Vance *et al*. 1980; Yates *et al*. 1997) indicate the expression of induced host resistance as a defense mechanism.

In our study, we aim to establish the beneficial role of the fungal endophyte, *Fusarium* spp. UPM31P1, as a biocontrol agent in suppressing Fusarium wilt incidence in susceptible banana plantlets. This disease is caused by *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4) and is a serious disease affecting many commercial banana plantations worldwide. This fungal pathogen is prevalent in many countries, found in Australia, Africa, the Pacific Islands, Central and South America and in many Asian countries (Stover 1962). To date, there are no feasible control methods available. Control measures using cultural improvements (Su *et al.* 1986), and chemical applications (Beckman 1987; Larkin and Fravel 1998) were unable to control *Fusarium* outbreak or in destroying the infective propagules of *Fusarium*. Our attempt to utilise an endophyte as a biological control agent is prompted by the fact that the endophyte exist in host tissues, sharing a similar niche with the pathogenic FocR4. This strategy hopefully surpasses the limitations from applications by soil drenching (Quadt-Hallman and Kloepper 1996), root dipping (Baker *et al*. 1978) or via artificially "induced" suppressive soil (Ting *et al*. 2003) as these applications are influenced by the presence of competition from resident microbes (Bacon and Hinton 1996) and exposure to unfavorable soil conditions.

This paper reports our investigations on the biochemical changes caused by endophytic infection by *Fusarium* spp. UPM31P1 in healthy and FocR4-infected Pisang Berangan "Intan" plantlets. The potential role of this endophyte in conferring disease resistance by induced host resistance was examined and established in this study.

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MATERIALS AND METHODS

Isolate preparation

The fungal endophyte was previously isolated from roots of wild bananas (*Musa acuminata* ssp. *malaccensis*) according to methods described in Ting *et al.* (2008). Pure culture obtained was established on Potato Dextrose Agar (PDA, Difco) and the isolate was identified by DNA sequencing as *Fusarium* spp. (S.Radu). The fungal pathogen, *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4) (VCG01213/01216, Bentley *et al*. 1998), obtained in filter-paper culture form, was also cultured on PDA.

Inoculation of banana plantlets

Six-week old Pisang Berangan "Intan" plantlets (14 cm in height, three to four leaf stage), were treated with three different treatments; inoculation with endophyte UPM31P1, inoculation with FocR4 and application with sterile distilled water (SDW). Inoculation for both fungal isolates was performed by drenching the soil with 100 ml (10^7 cfu ml^{-1}) of inoculums. SDW was also applied at 100 ml $plantlet^{-1}$. The inoculated plantlets were placed in individual pots using the "double-potting" method. Plantlets were maintained in the glasshouse by watering twice daily with SDW. Observation was conducted for 35 days with sampling performed at a 7-day interval. During each sampling time, the roots were collected, washed and frozen overnight prior to enzymatic assay.

Biochemical assay

Assay for peroxidase (PO), polyphenoloxidase (PPO), phenylalanine ammonia lyase (PAL), total soluble phenols and lignothioglycolic acid (LTGA) was conducted to determine the influence of endophytes in triggering host induced resistance. The assay for PO was initiated by grinding 1 g of the frozen root sample in 1 ml of pre-chilled 0.05 M sodium acetate buffer (SAB) (pH 5) amended with 5 mg of polyvinyl pyrrolidone (PVP40) (Reuveni 1998). The slurry produced was centrifuged (14,000 rpm, 20 min, 4°C) and the supernatant transferred into new tubes for reaction. Changes in PO activity were determined by adding 200 µl of the extract to 3 ml of reaction substrate (80 ml Sodium Phosphate Buffer (SPB) (pH 6), 1 ml of hydrogen peroxide (H_2O_2) (30% v/v) and 20 ml of guaicol) and incubated at room temperature. Sterile distilled water was used instead of extracts for preparation of blanks. The absorbance value was determined at 470 nm using a spectrophotometer (Model Pharmacia LKB Novaspec® II). The total amount of PO produced (units g^{-1} tissue) was calculated using the formula from Kokkinakis and Brooks (1979):

$$\text{Units g}^{-1}\text{ tissue} = \frac{\text{absorbance at 470 nm} \times \text{dilution factor}}{\text{Amount of tissue assayed, g}} \times 1000$$

The procedure for PO assay was also adopted for the assay of PPO, with the changes in PPO activity determined by mixing 0.1 ml of the extract with the reaction substrate containing 3 ml of 5×10^{-4} M chlorogenic acid in 5×10^{-2} M SPB (pH 6) (Robinson and Dry 1992). Blanks were also prepared by substituting the extracts with SDW. The absorbance was read at 410 nm and expressed as total PPO produced (units g^{-1} tissue) using the previous formula by Kokkinakis and Brooks (1979).

The assay for PAL was performed by grinding 1 g of frozen tissue sample in 5 ml of 0.1 M sodium borate buffer (SBB) (pH 8.8 with 5 mM 2-mercaptoethanol). The slurry produced was centrifuged (15,000 rpm, 10 min) and 1 ml of the resulting supernatant was incorporated into a reaction mixture containing 300 µM SBB and 30 µM L-phenylalanine prior to incubation for 1 h at 40°C. The absorbance value was measured at 290 nm against a blank substrate made up of 300 µM SBB and 30 µM L-phenylalanine with SDW. The total PAL assayed was expressed as nanomole cinnamic acid produced min^{-1} g^{-1} fresh weight of tissues (Podile and Laxmi 1998).

Total soluble phenols from root tissues were extracted by grinding the frozen root tissues (1 g) in two changes of 4 ml of methanol. The slurry was centrifuged (5000 rpm, 5 min), and the supernatant (1 ml) was added into 0.5 ml of Folin-Ciocalteau Reagent (diluted in SDW at 1:1 ratio). The mixture was thoroughly mixed, incubated at room temperature for 3 min, and added with 0.5 ml of 1 M sodium bicarbonate (Na_2CO_3). The mixture was further incubated at room temperature for 1 h and the absorbance value subsequently determined at 725 nm. The total phenol content was estimated from a standard curve generated separately using various concentrations of chlorogenic acid, and the amount of phenol assayed is expressed as mg of phenol g^{-1} fresh weight of tissues (Swain and Hillis 1959).

The assay for LTGA was performed by firstly immersing the tissue samples into 5 ml of absolute methanol for 48 h with four changes of methanol to softened the tissues. The tissues were then transferred into fresh test tubes containing a mixture of 0.5 ml thioglycolic acid and 0.5 ml 2 N hydrochloric acid (HCl). The capped tubes were heated (95°C, 4 h) and subsequently cooled and centrifuged at 12,000 rpm for 5 min. Five ml of the resulting supernatant was transferred and resuspended in 5 ml of SDW, and centrifuged again (12,000 rpm, 5 min). The resulting supernatant (5 ml) was transferred to a new tube containing 5 ml of 0.5 N sodium hydroxide (NaOH) for an overnight incubation. After incubation, 2 ml of SDW was added and the mixture centrifuged. Ten ml of the resulting supernatant was then transferred into a new tube containing 1 ml of concentrated HCl. This mixture was incubated for 4 h at 4°C to allow precipitation of LTGA. After precipitation, the mixture was centrifuged at 5000 rpm for 15 min and the pellet collected, washed twice with 2 ml 0.1 N HCl, and finally dissolved in 0.5 N NaOH to a final volume of 2.5 ml. The absorbance value of the sample was then read at 280 nm, calculated and expressed as LTGA g^{-1} tissues (Dean and Kuc 1987).

This experiment was conducted in a Randomized Complete Block Design. Each treatment has eight replicates. All data recorded from the assays were analyzed using ANOVA, with means compared using Tukey's Studentized Range Test ($HSD_{(0.05)}$).

Biocontrol assessment

Four treatments were applied for this asessment, TA: UPM31P1, TB: SDW, TC: UPM31P1+FocR4 and TD: FocR4. Inoculation was performed by applying (soil-drenching) 100 ml of inoculum (10^7 cfu ml^{-1}) suspension (or SDW) $plantlet^{-1}$. Seven days after endophyte inoculation, plantlets assigned for FocR4-challenged inoculation (TC and TD) were inoculated with FocR4 (10^7 cfu ml^{-1}, 100 ml $plantlet^{-1}$). The banana plantlets were maintained in the glasshouse in individual pots containing 1 kg of sterilized soil mixture (3: 2: 1 w/w ratio of topsoil: peat: sand) using the "double-potting" technique, and watered twice daily with SDW.

The efficacy of the various treatments of endophytes in suppressing Fusarium wilt incidence was determined based on the percentages of disease incidence (DI) and disease severity (DS) (Vanderplank 1984). Disease incidence (DI) reflects the number of plantlets visibly diseased in relation to the total number of plantlets assessed, and is a suitable parameter for wilt disease whereby one lesion unit $plantlet^{-1}$ is considered fatal (Campbell and Madden 1990; Cubeta *et al*. 2004). Disease severity (DS) refers to the volume of plantlet tissue that is diseased relative to the total volume of the plantlet. It is expressed as the percentage of plantlet with symptoms of disease at a particular recording time, and is dependent on the extent of yellowing on leaves. In this study, the scale used is as follows: 0-healthy plantlet, 1-lowest leaf with yellowish streaks, 2-less than 50% of the total number of leaves with yellowish streaks, 3-more than 50% of the total number of leaves with yellowish streaks, 4-100% of total number of leaves with yellowish streaks, 5-collapsed or dead plants.

The untransformed percentages of disease incidence (DI) and disease severity (DS) were calculated as follows (Cubeta *et al*. 2004):

$$\text{DI} = \frac{\text{number of diseased plantlets}}{\text{total number of plantlets assessed}} \times 100\%$$

$$\text{DS} = \frac{\text{number of diseased plantlets in each scale}}{\text{total number of plantlets assessed}} \times 100\%$$

This experiment was performed in a Split-Plot Design to separate FocR4-infected plantlets from non-infected plantlets. Each treatment has eight replicates for each sampling time at 0, 7, 14,

21, 28, 35, and 49 days after FocR4-challenged inoculation. For every sampling time, percentages of DI and DS were observed and the enzymatic assay performed as described previously. Data collected were analyzed using ANOVA, with means compared using Tukey's Studentized Range Test ($HSD_{(0.05)}$).

RESULTS AND DISCUSSION

Influence of endophyte UPM31P1 in inducing host defense enzymes

Endophytic infection by the fungal endophyte UPM31P1 triggered the production of inducible compounds in the root tissues. The levels for all compounds assayed (PO, PPO, PAL, phenol and LTGA) were significantly higher in these plantlets compared to plantlets in control (SDW) (**Fig. 1**). The asymptomatic infection by UPM31P1 induced the highest PO, PPO, PAL, phenol and LTGA levels in root tissues, with 95.02 units g^{-1} tissues, 95.13 units g^{-1} tissues, 0.40 nanomole cinnamic acid min^{-1} g^{-1} tissues, 1.03 mg phenol g^{-1} tissues and 0.14 LTGA g^{-1} tissues, respectively (**Fig. 1**). The inducible compounds produced are related to the biosynthesis and oxidation of phenols to produce lignin, which is a common response to early establishment of host induced resistance (Hammerschmidt *et al*. 1984; Podile and Laxmi 1998). This provides an alternative for disease control as the primary infection by endophytes can pre-condition the host plant to respond rapidly to secondary infection by the pathogen, thus is a good mechanism to suppress wilt incidence (Goodman *et al.* 1986). This suggested the role of UPM31P1 as a biological elicitor to enhance production of inducible compounds related to induce host resistance as a defense mechanism.

The level of enzymes assayed in control plantets (SDW) were the lowest (**Fig. 1**), indicating that without the presence of endophyte (UPM31P1) or pathogen (FocR4), plantlets produced these enzymes at low levels and it is only upon infection, endophytic or pathogenic, that results in the increase of the enzymatic levels. This further confirms the role of endophyte UPM31P1 as a biological elicitor responsible for induced host resistance. Contrary, plantlets infected with FocR4 produced significantly lower levels of inducible compounds compared to UPM31P1-infected plantlets for all enzymes assayed (**Fig. 1**). However, they remained higher than enzyme levels assayed from plantlets in control (SDW) as FocR4 can also induce host resistance due to the fusaric acid produced. Nevertheless, banana plantlets seemed to respond better to infection by endophytes as higher levels of inducible compounds were assayed.

Biocontrol efficacy-glasshouse trial

Results revealed that pre-treatment of plantlets with UPM31P1 was able to suppress wilt incidence in susceptible Berangan "Intan" plantlets. Pre-treatment with UPM31P1 (TC) resulted in only 53% DI and 20% DS compared to 100% DI and 74% DS observed in plantlets with FocR4 only (TD) (**Fig. 2**). The lower percentages of DI% and DS% recorded for plantlets pre-treated with UPM31P1 (TC) compared to plantlets with FocR4 only (TD) suggested that the presence of endophyte in the host plant prior to FocR4 introduction, render some form of protective effect towards the plantlets. As a result, the disease development especially the development of symptoms were delayed and appeared slower compared to plantlets without endophyte pre-treatment (TD: FocR4).

Results for enzymatic assay for plantets in TA, TB, TC and TD revealed that plantlets pre-treated with UPM31P1 prior to FocR4-challenged inoculation (TC) and without FocR4 (TA), have relatively higher levels of PO, PPO, and PAL (**Fig. 3**). Here again, the beneficial role of UPM31P1 in triggering host induced enzymes were established. The primary stimulation benefited the plantlets when challenged with FocR4 as the host defense mechanism was produced more rapidly to increase tolerance of plantlets to FocR4

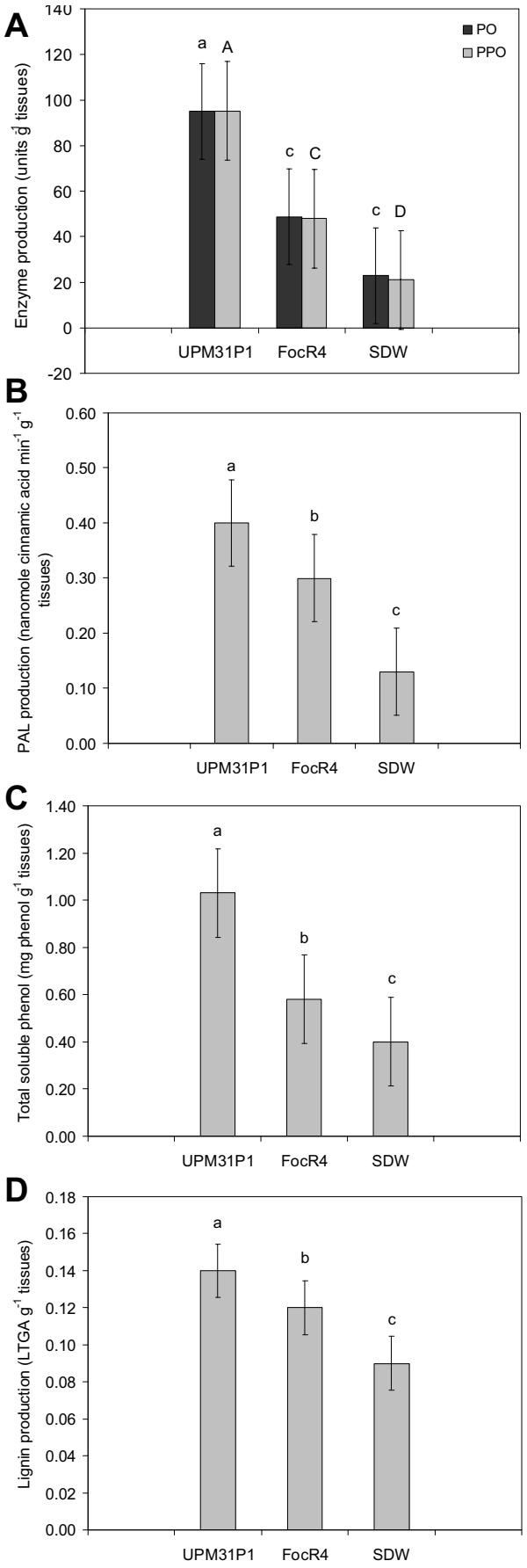

Fig. 1 Production of inducible compounds assayed from banana plantlets treated with endophyte UPM31P1 compared to plantlets infected with FocR4 and applied with SDW (control). (**A**) Production of PO and PPO (**B**) PAL (**C**) total soluble phenols and (**D**) LTGA content in the root tissues. Values are cumulative means throughout the experimental period. Means with the same letters and captions are not significantly different as determined by Tukey's Studentized Range Test ($HSD_{(0.05)}$). Vertical bars indicate standard errors of mean.

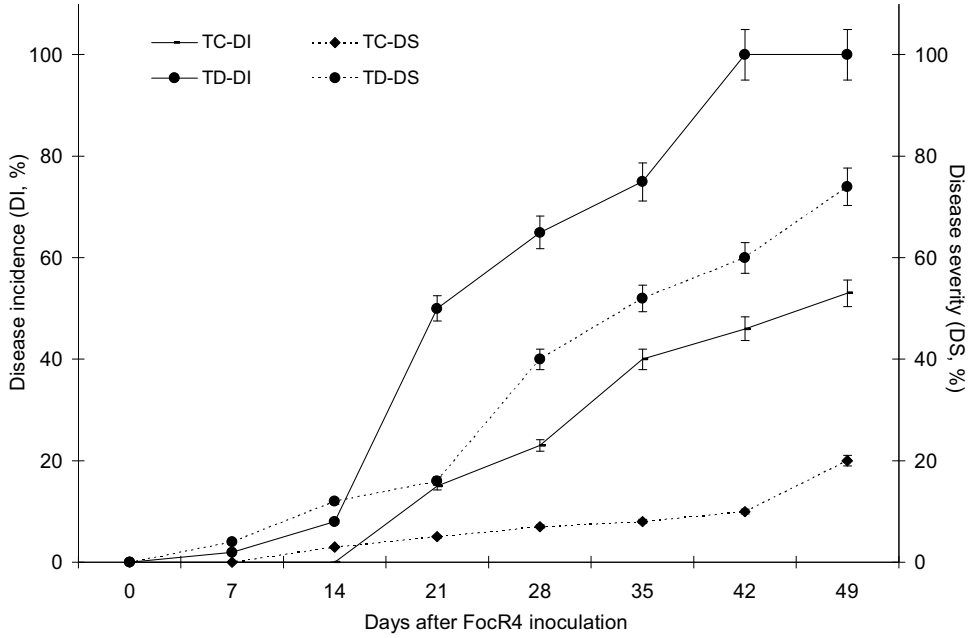

Fig. 2 Percentages of DI (-) and DS (---) recorded by plantlets pre-treated with endophyte UPM31P1 (TC) compared to plantlets without endophyte pre-treatment (TD). Values for each interval are means of eight replicates. Percentages for TA and TB remained at 0% throughout the experimental period therefore are not indicated in the line graph. Vertical bars indicate standard errors of mean.

infection. As a result, enzyme levels are generally higher in FocR4-infected plantlets (TC) compared to plantlets with just endophytes (TA). The enhanced production of PO, PPO, PAL are often reflective of the extent of defense mechanisms in the plantlets. PO is essential in generating antimicrobial toxins (Peng and Kuc 1992) and has roles in lignification of cell walls (Sato *et al.* 1993). Similarly, PPO and PAL are also associated with lignification (Goodman *et al.* 1986; Metraux and Raskin 1993). Thus, it can be concluded that the endophytic UPM31P1 enhance tolerance of plantlets towards FocR4 via lignification as its defense mechanism. In our study, plantlets in TC with relatively high levels of inducible compounds have been shown to have the least percentages of DI and DS compared to plantlets in TD (FocR4 only). As expected, the plantlets in TD clearly did not acquire host resistance prior to FocR4-challenged inoculation. The levels of inducible compounds were the lowest among all diseased plantlets (**Fig. 3**) and the absence of induced resistance resulted in 100% DI and 74% DS.

Hence, we linked the disease suppression achieved in plantlets with UPM31P1 pre-treatments (TC) was most likely attributed to the beneficial association between the endophyte and the host, that is via stimulation of host induced resistance. Results from the bioefficacy assessment confirmed the benefits of endophyte-host association and demonstrated their ability to confer tolerance to Fusarium wilt with the lower percentages in DS and DI achieved. The host resistance triggered by the endophytes via lignification was however not sustainable as the plantlets still succumbed to Fusarium wilt. Initial delay in symptom development and disease progression was not able to confer complete control over the FocR4 as DI and DS increased over time. This may explain as well the diminishing number of surviving plants in the field, even with the pre-treatment of endophytes at glasshouse and planting stage (Ting *et al.* 2009). This indicated that lignification alone is not effective (Goodman *et al.* 1986) or that it may occur too late to appear effective (Vance *et al.* 1980). Therefore, there is a need to correlate the defined time interval between induction and challenged-inoculation.

CONCLUSION

Our study showed that the fungal endophyte *Fusarium* spp. UPM31P1 has potential to confer resistance to Fusarium wilt via induced host resistance in the susceptible banana plantlets. This defense mechanism can be further studied and manipulated as an effective disease suppression mechanism stemming from the use of endophytes as biological control agents.

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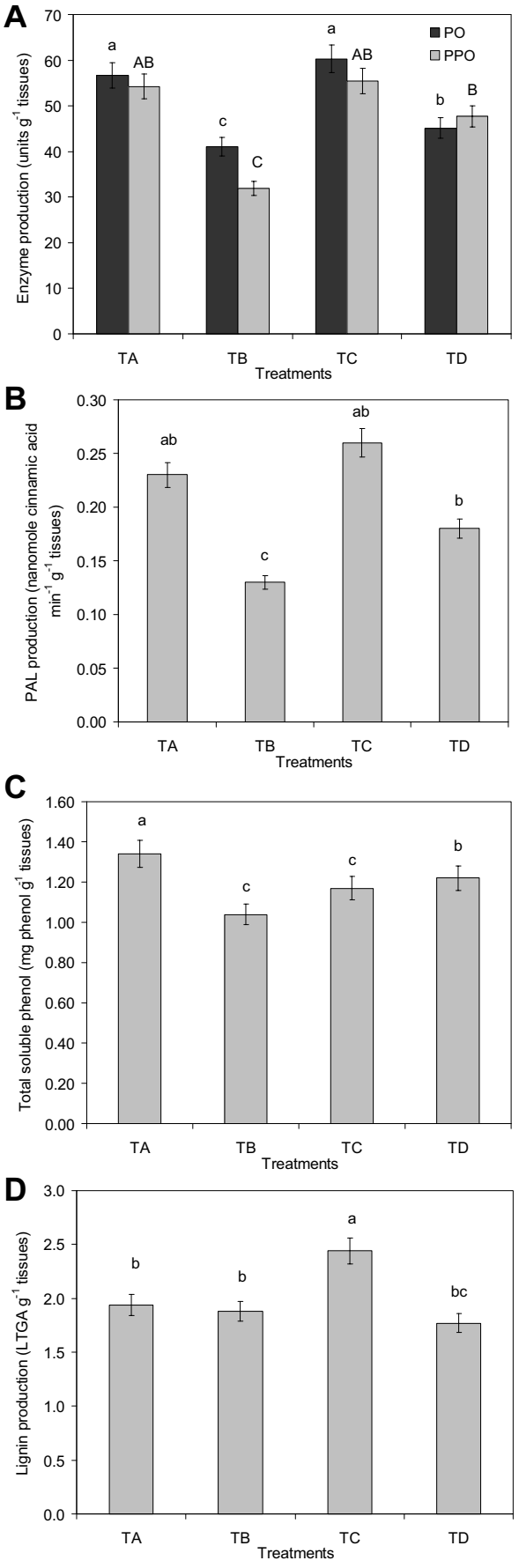

Fig. 3 Production of inducible compounds assayed from banana plantlets infected (TC, TD) and non-infected with FocR4 (TA, TB). Comparisons were also made between the treatments and control (SDW). (**A**) Production of PO and PPO (**B**) PAL (**C**) total soluble phenols and (**D**) LTGA content in the root tissues. Values are cumulative means throughout the experimental period. Means with the same letters and captions are not significantly different as determined by Tukey's Studentized Range Test ($HSD_{(0.05)}$). Vertical bars indicate standard errors of mean.

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