[BIO01] Managing Fusarium wilt of bananas with endophytic microorganisms

Adeline Ting Su Yien[1], Sariah Meon[1], Jugah Kadir[1], Son Radu[2], Gurmit Singh[3]

[1]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang UPM, Selangor, Malaysia
[2]Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, 43400 Serdang UPM, Selangor, Malaysia
[3]Research Department, United Plantations Bhd., Jenderata Estate, Teluk Intan, Perak, Malaysia

Introduction

Fusarium wilt of banana is a common disease in many banana growing countries worldwide. Attempts to control this disease using cultural practices, chemical application and breeding for resistant varieties, has met with little success. The pathogen, *Fusarium oxysporum* f. sp. *cubense* (Foc) spends part of its life cycle in the host tissues, thus 'escaping' the effect of control applications. The potential use of endophytic microorganisms as biocontrol agents (BCAs) is then investigated. Endophytes are natural internal tissue colonizers, and are presumed to be more effective BCAs, competing with FocR4 for nutrients and similar niche for colonization and establishment.

This study was undertaken to screen and select endophytic isolates recovered from roots of wild bananas, as potential BCAs in managing Fusarium wilt in bananas.

Materials and Methods

Isolation of endophytes from roots of wild bananas

Endophytes were isolated based on the sterilization and isolation techniques modified from Gardner *et al.* (1982) and Kuldau and Yates (2000). This technique involved immersion of root samples in a series of Chlorox® (NaOCl) and ethanol, and direct plating of surface sterilized tissues on agar plates (Fusarium Selective Media for *Fusarium* spp., Nutrient Agar for bacterial endophytes and Potato Dextrose Agar for non*Fusarium* spp.).

In vitro screening for antagonistic activity

All endophytes were tested for their antagonistic activity against *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4), as this race caused the most devastating effects on banana plantations in Malaysia. The antagonistic activity of the endophytes against FocR4 was determined using the Dual Culture Test, and their inhibition efficacy was calculated based on Percentage Inhibition of Radial Growth (PIRG) (Rihakova *et al*., 2002; Adeline et al., 2003). Poison food test and double plate test was also conducted to detect the production of nonvolatile and volatile inhibitory substances, respectively (Adeline *et al.,* 2003).

Evaluation on the effect of endophyte-host association on plant growth

This experiment was conducted in a Split Split Plot Design using isolates UPM31F4, UPM31P1, UPM14B1, UPM13B8 and UPM39B3. The effect of endophytic infection on the host plant was observed based on the development of external (foliar) and internal symptoms, and plant vegetative growth (plant height, pseudostem diameter, total number of leaves). Assays of biochemical markers like peroxidase (PO) (Hammerschmidt *et al*., 1982), polyphenoloxidase (PPO) (Hammerschmidt *et al.,* 1982), phenylalanine ammonia lyase (PAL) (Klessig and Malamy, 1994), total soluble phenol (Swain and Hillis, 1959) and lignothioglycolic acid (LTGA) (Hammerschmidt *et al.,* 1984), were also conducted to determine the presence of induced host resistance triggered by endophytic infection. All data analyzed according to ANOVA and means compared with DMRT ($P<0.05$) (Duncan Multiple Range Test) using the SAS Programme version6.2.

Evaluation on the efficacy of UPM31P1 and UPM39B3 as biocontrol agents in suppressing Fusarium development

Seedlings were pretreated with UPM31P1 and UPM39B3 at a rate of 10^6 c.f.u. (colony forming units) mL^{-1} and 10^9 c.f.u. mL^{-1}, respectively, a week prior to challenge with FocR4 (10^6 c.f.u. mL^{-1}). Control efficacy was assessed based on the development of disease severity of the foliar symptoms. Presence of induced host resistance as a defense mechanism against FocR4 was determined in the changes of enzymatic activities of PO, PPO, PAL, total soluble phenol and LTGA content. In addition, the vegetative growth of the seedlings were also evaluated to assess the effect of endophytes on plant overall growth in both Fusarium infected and uninfected seedlings. This experiment was conducted in a Split Split Plot Design, and the data analyzed using ANOVA and means compared with DMRT ($P<0.05$) (Duncan Multiple Range Test) using the SAS Programme version6.2.

Results

Isolation of endophytes from roots of wild bananas

A total of 341 isolates were isolated from roots of wild bananas, with bacterial endophytes dominating the endophyte population with 213 isolates, followed by 124 fungal endophytes, and 4 isolates of Actinomyces. *Fusarium* spp. were the most common fungal endophytes (70 isolates), while other species like *Aspergillus* spp., *Penicillium* spp., *Cylindrocarpon* spp. and *Trichoderma* spp. were also isolated.

In vitro screening for antagonistic activity

Results showed that only a few bacterial endophytes (33%, 70 isolates) were antagonistic towards FocR4 in contrast to all isolates of fungal endophytes (100%, 70 isolates). Mechanism of antagonism was attributed to production of volatile and nonvolatile inhibitory substances. Three bacterial isolates (UPM14B1, UPM13B8 and UPM39B3) and two fungal isolates (UPM31P1 and UPM31F4) which recorded the highest PIRG values were selected for subsequent testing (Table 1).

TABLE 1 Endophytic isolates and their respective PIRG (Percentage Inhibition of Radial Growth) values derived from Dual culture test

Isolates	PIRG values
UPM 31F4	58%
UPM31P1	65%
UPM14B1	52%
UPM13B8	52%
UPM39B3	63%

Evaluation on the effect of endophyte-host association on plant growth

Seedlings infected with UPM14B1, UPM13B8, UPM39B3, UPM31F4 and UPM31P1, did not express any wilt symptoms. Plants remained symptomless till the end of the experiment. Endophytic infection also did not inhibit plant growth, instead treatment with bacterial endophytes (UPM14B1, UPM13B8, UPM39B3) recorded higher mean increase in pseudostem diameter, seedling height and root volume in comparison to untreated seedlings (Table 2). All endophytes were able to induce host resistance, with UPM31P1 and UPM39B3 as the most effective fungal and bacterial endophyte, respectively. Both recorded high PO, PPO and PAL activities, as well as total soluble phenol and LTGA content, as compared to untreated seedlings and treatment with other endophytes (Table 3).

TABLE 2 Effect of endophyte-host association on plant vegetative growth (mean values from 8 replicates)

Isolates	diameter (cm)	height (cm)	root volume (cm^3)
UPM31F4	0.97b	2.15ab	8.71a
UPM31P1	1.04ab	1.94b	9.67a
UPM14B1	0.99b	2.62ab	14.30a
UPM13B8	0.98b	2.35ab	12.58a
UPM39B3	1.12a	3.15a	15.35a
Control	0.98b	2.53ab	10.15a

means with the same letters not significantly different at $P<0.05$ (DMRT)

TABLE 3 Mean enzymatic activities and total phenol and lignin content in root tissues of seedlings treated with endophytes (mean of 8 replicates)

Isolates	PO[1]	PPO[1]	PAL[2]	PHENOL[3]	LTGA[4]
UPM31F4	0.87ab	0.31a	1.43a	0.62a	0.23a
UPM31P1	1.07ab	0.11d	1.67a	0.71a	0.48a
UPM14B1	1.26a	0.11c	1.38a	0.63a	0.43a
UPM13B8	0.85ab	0.11e	1.53a	0.48ab	0.511a
UPM39B3	0.61bc	0.12b	1.59a	0.46ab	0.25a
Control	0.28c	0.05f	0.85b	0.23b	0.10b

[1] △ in Abs min^{-1} g^{-1} fresh weight of tissues
[2] nanomole cinnamic acid produced min^{-1} g^{-1} fresh weight of tissues
[3] mg g^{-1} fresh weight of tissues
[4] LTGA content g^{-1} fresh weight of tissues
means with the same letters not significantly different at P<0.05 (DMRT)

Evaluation on the efficacy of UPM31P1 and UPM39B3 as biocontrol agents in suppressing Fusarium wilt development

All FocR4 infected seedlings displayed typical Fusarium wilt symptoms of foliar yellowing and discolouration in the corm. The most effective control was derived from single inoculation of UPM31P1 (T6) (20%), followed by combined treatment of UPM31P1 and UPM39B3 (T7) (33.5%), combined treatment with UPM13B8, UPM14B1 and UPM39B3 (T9) (46%), and single treatment with UPM39B3 (T8) (49.5%). Untreated seedlings (T10) recorded 74% disease severity (Figure 1). More than 50% of the seedlings from T10 collapsed and died over a period of 49 days due to extensive pseudostem wilt, while less than 5% of the seedlings from T6, T7, T8 and T9 died despite having yellowed and wilted leaves.

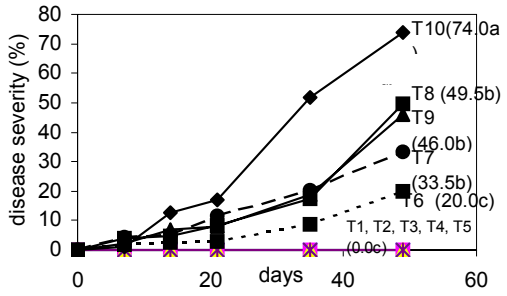

FIGURE 1 Disease severity curve for seedlings treated with various endophytic isolates (single or combined treatment) for 49 days after FocR4 challenge

Disease suppression was attributed to induced host resistance elicited by the presence of endophytes in the host system. In all treatments with low disease severity (T6, T7, T9 and T8), high enzymatic activities were recorded especially in T6 and T7, whereby DS% is the lowest. Treatments with endophytes also improved seedling growth, even in FocR4 infected seedlings. Mean of seedling height, pseudostem diameter, and the enzymatic activities of each treatment is in Table 4 and 5 respectively.

TABLE 4 Vegetative growth of seedlings treated with various endophytes (mean increase in values from 8 replicates)

treatments	diameter (cm)	height (cm)	root volume (cm^3)	no. of leaves
T1	0.23a	3.25a	0.84b	5.15ab
T2	0.22a	2.48ab	0.59cd	6.05bc
T3	0.21a	3.08ab	0.64c	5.88b
T4	0.21a	2.36ab	0.50e	6.2ab
T5	0.25a	3.11ab	0.90a	6.43a
T6	0.22a	2.46abc	0.44e	5.98bc
T7	0.18a	2.51abc	0.57d	5.43de
T8	0.19a	2.51abc	0.30g	5.48de
T9	0.19a	2.25bc	0.37f	5.75cd
T10	0.18a	1.70c	0.31fg	5.25e

T1:UPM31P1,T2:UPM31P1+UPM39B3, T3:UPM39B3, T4:UPM14B1+UPM13B8+UPM39B3, T5:CONTROL, T6:UPM31P1+**FocR4,**T7:UPM31P1+UPM39B3+**FocR4,**T8:UPM39B3+**FocR4,**T9:UPM14B1+UPM13B8+ UPM39B3+**FocR4,**T10:**FocR4**
means with the same letters not significantly different at P<0.05 (DMRT)

TABLE 5 Mean enzymatic activities and total phenol and lignin content in root tissues of seedlings treated with endophytes (mean of 8 replicates)

Isolates	PO[1]	PPO[1]	PAL[2]	PHENOL[3]	LTGA[4]
T1	2.13a	0.14cd	0.3abc	1.21cde	2.43b
T2	2.12a	0.15c	0.30ab	1.24bc	1.54f
T3	1.89d	0.11f	0.34abc	1.23bcd	1.97d
T4	1.91cd	0.12e	0.37ab	1.18cde	2.33c
T5	1.71e	0.15c	0.24c	1.04f	1.97d
T6	2.09ab	0.29a	0.35ab	1.14e	2.44b
T7	2.00abc	0.23b	0.27bc	1.15de	2.63a
T8	1.92cd	0.12cde	0.36ab	1.22bcde	2.25c
T9	1.89d	0.11ef	0.39a	1.33a	1.69e
T10	1.91cd	0.09f	0.37ab	1.29ab	1.77e

[1] △ in Abs min^{-1} g^{-1} fresh weight of tissues
[2] nanomole cinnamic acid produced min^{-1} g^{-1} fresh weight of tissues
[3] mg g^{-1} fresh weight of tissues
[4] LTGA content g^{-1} fresh weight of tissues
T1:UPM31P1,T2:UPM31P1+UPM39B3,T3:UPM39B3, T4:UPM14B1+UPM13B8+UPM39B3,T5:CONTROL, T6:UPM31P1+**FocR4,**T7:UPM31P1+UPM39B3+**FocR4,** T8:UPM39B3+**FocR4,**T9:UPM14B1+UPM13B8+ UPM39B3+**FocR4,**T10:**FocR4**
means with the same letters not significantly different at P<0.05 (DMRT)

Repeated experiment using only UPM31P1 and combined treatment of UPM31P1 with UPM39B3, and a larger number of replicate, produced similar observations. Therefore, UPM31P1 and UPM39B3 were selected for field-testing in a 'hot-spot' area in United Plantations Bhd., Teluk Intan. Results on their efficacy, as BCAs to manage Fusarium wilt in bananas in the field is not presented here as the experiment is on going.

Discussions

Results presented in his study revealed the potential of utilizing endophytes recovered from roots of wild banana as BCAs in managing Fusarium wilt of banana in this country. From a pool of highly diversified endophytic microorganisms, only 2 isolates (UPM31P1 and UPM39B3) eventually emerged as potential BCAs. These two isolates have high antagonistic activity against FocR4 (*in vitro* screening), either by production of volatile or nonvolatile inhibitory substances. In addition, both UPM31P1 and UPM39B3 were able to induced host resistance, which benefited the plant as it rendered the plant tolerable to Fusarium wilt, prolonging the plant survivability and delaying disease progression. Presence of endophytes in the host plant system was also able to improve plant vegetative growth even when infected with Fusarium wilt. This suggested that pretreatment of seedlings with UPM31P1 and UPM39B3 (as single or combined treatment) boosted plant growth, strengthening host plant vigour to improve resistance to FocR4 infection. The isolates UPM31P1 and UPM39B3 have high commercial potential. Preliminary work has shown that both UPM31P1 and UPM39B3 were amenable to mass production on solid and liquid substrate. The application of the endophytes in substrate form for field testing has this far shown encouraging result, highlighting their potential as biopesticide in biocontrol management of Fusarium wilt in the near future.

Acknowledgements

The authors extend their gratitude and thanks to the Ministry of Science, Technology and Innovation Malaysia (MOSTI), for the National Science Fellowship (NSF) and IRPA grant awarded. And to United Plantations Bhd., Teluk Intan for their assistance in supplying planting materials field-testing facilities.

References

Adeline, T.S.Y., Sariah, M., Jugah, K and Anuar, A.R. 2003. Effect of artificially induced suppressive soil on Fusarium wilt. INFOMUSA, 12(1), 29-33.

Gardner, J.M., Feldman, A.W. and Zablotowicz, R.M. 1982. Identity and behaviour of xylem-residing bacteria in rough lemon of Florida citrus trees. *Applied Environmental Microbiology*, 43: 1335-1342.

Hammerschimdt, R., Nuckles, E.M. and Kuc, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance to *Colletotrichum lagenarium. Physiological Plant Pathology,* 20: 73-82.

Hammerschmidt, R., Lamport, D.T.A. and Muldon, E.P. 1984. Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerum. Physiological Plant Pathology,* 20:43-47.

Klessig, D.F. and Malamy, J. 1994. The salicylic acid signal in plants. *Plant Molecular Biology*, 26:1439-1458.

Kuldau, G.A. and Yates, I.E. 2000. Evidence of Fusarium endophytes in cultivated and wild plants. In *Microbial endophytes*. (Eds. Bacon, C.W. and White, J.F.Jr.) New York: Marcel Dekker, Inc. pp 85-117.

Rihakova, Z., Filip, V., Plockova, M., Smidrkal, J. and Cervenkova, R. 2002. Inhibition of *Aspergillus niger* DMF0801 by monoacylglycerols prepared from coconut oil. *Czech Journal of Food Science*, 20: 48-52.

Swain, T. and Hillis, W.E. 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture* 10:63-68.