International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2736-1756, Vol. 16 (7), pp. 001-007, July, 2022. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Isolation and characterization of antagonistic bacteria against fusarium wilt and induction of defense related enzymes in banana

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Accepted 20 June, 2022

Fusarium wilt caused by *Fusarium oxysporum* is considered a fatal disease to bananas. Chemical control of Fusarium wilt in bananas has received little efficiency. Alternatively, biological control is a viable strategy against soil-borne diseases. The objectives of this research were to isolate antagonistic bacteria and investigate the possible mechanisms against Fusarium wilt. An antagonistic *Bacillus* strain, termed KY-21 against *F. oxysporum* f. sp. *Cubense* was isolated from the soil of banana's rhizosphere by dual culture. The strain was identified as *Bacillus subtilis* according to the characteristics of its morphology and by homology analysis of its 16S rDNA sequence. Mycelium growth of the pathogen was seriously inhibited after treatment with the fermentation filtrate of KY-21. Observation revealed that the tips of the hypha were deformed into spherical structures that were remarkably constricted. The strain KY-21 displayed a good ability to colonize and can transfer into banana tissues. The activities of polyphenol oxidase (PPO) and peroxidase (POD) in bananas showed an increase after inoculated with KY-21, compared to control. *In vivo* biocontrol assays showed a significant reduction in wilt index and vascular discoloration. These results indicated that the antagonistic mechanisms against Fusarium wilt were involved in the inhibition of mycelial growth and the improvement in activity of defense related enzymes.

Key words: *Fusarium oxysporum* f. sp. *Cubense*, antagonistic bacteria, screen, colonization, enzyme activity.

INTRODUCTION

Banana is one of the most important fruits in tropical and subtropical regions. However, the banana industry is being threatened by Fusarium wilt (Moore et al., 2001). Fusarium wilt in bananas is a serious soil-borne disease caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*). In the physiological races of *Foc*, race 4 (*Foc* 4) is the most harmful for farming, it can infect almost all banana cultivars. The pathogenic fungus enters through root wound and proliferates within the vascular tissue, leading to the typical wilt symptoms. Eventually, the whole plant wilts and dies as the disease progresses.

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Foc can be disseminated by farming activities, which increase the difficulty of controlling the disease. Planting resistant banana cultivars can provide an effective and economical method for controlling the disease, but these resistant banana cultivars do not always possess good market quality (Smith et al., 2006). Chemical control of Fusarium wilt in bananas has received little efficiency due to the production of thick-walled, long-living chlamydospores. Moreover, chemical fungicides have potential hazards to the environment (Shi et al., 1991).

As an environmentally-sound alternative for these control measures, biological control offers an attractive method against soil-borne diseases (Berg et al., 2001). *Bacillus* species have several advantages as biological control agents because they can produce endospores, which are tolerant to heat and desiccation. Some

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secondary metabolites of *Bacillus* have broad-spectrum activities against pathogens. *Bacillus* species have been extensively used against many soil-borne plant pathogens. *B. substilis*, *Bacillus cereus*, *Bacillus polymyxa*, *Bacillus megaterium* and *Bacillus pumillus* have been identified as biocontrol agents to reduce disease caused by a variety of soil-borne plant pathogens, including Rhizoctonia (Yu et al., 2002) and Fusarium (Schisler et al., 2002).

In spite of the identification of many biocontrol agents, it is difficult to maintain consistent and effective control of plant diseases under field conditions. An effective biocontrol agent must be adapted to a certain environment with various environmental factors, including pathogens, host and soil. Some rhizobacteria are proven biocontrol agents against soil-borne fungal pathogens (Trotel-Aziz er et al., 2008; Cavaglieri et al., 2004). These bacteria have good colonization abilities in the rhizosphere from which they were isolated. Because they can preferentially occupy the living space of the rhizosphere, the growth of soilborne fungal pathogens, which infect crops from the root were inhibited. Although some biocontrol strains were isolated to control Fusarium diseases, effective strains that are active against *Foc* 4 have not been identified.

The cell wall plays an important role in a fungal pathogen's development. Its damage can hamper the pathogen's capacity to survive, and as a result, the pathogen could die. In this research, using the cell wall of *Foc* 4 as the only carbon source, a *Bacillus* against *Foc* was isolated from the rhizosphere of banana trees. The objectives of this research were to: (i) isolation and identification of the strain and describe its activity against *Foc*; (ii) evaluation the control efficacy against *Foc* under greenhouse conditions; (iii) discussion the colonization of antagonistic bacteria and the effects on activity of defense related enzymes in banana.

MATERIALS AND METHODS

Growth media and samples source

PCW medium: Using pathogen cell wall of *Foc* as sole carbon. *Foc* were grown on potato dextrose agar (PDA) medium at 28°C for 6 day. Then the mycelium were collected, washed thoroughly with distilled water, dried and powdered. The PCW medium was made with powdered *Foc* 4 cell wall (6 g L^{-1}), $FeSO_4.7H_2O$ (0.01 g L^{-1}), KH_2PO_4 (0.3 g L^{-1}), K_2HPO_4 (0.7 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}), $ZnSO_4.7H_2O$ (0.01 g L^{-1}) and agar (12.0 g L^{-1}).

Pathogen: *Foc* 4 was isolated from infected bananas in Haikou, China. *Foc* 4 was cultured on PDA and stored at 4°C.

Plant material: Tissue-cultured healthy banana plantlets (Musa sp., AAA, Cavendish group cv. Baxi) were transplanted into pots containing steam-sterilized nutrient soil.

Isolation of antifungal bacteria

Bacterial strains were isolated from the rhizosphere soil of healthy banana plants from the fields, which were infected by the pathogen of *Foc* 4. The sample of 10 g soil were suspended in 100 ml of sterile physiological water and shaken vigorously at 28°C for 30 min. Serial dilutions were plated on PCW isolation agar medium, and each dilution was incubated at 30°C until colonies were observed. Purified colonies were inoculated on NA plates and prepared for an antagonism test against *Foc* 4.

In vitro screening of antagonistic activity of isolates

Isolates were tested for antagonism against *Foc* 4 on PDA medium by the dual-culture plate method. One 0.5 cm diameter disc of pure culture was placed at the center of a Petri plate (10 cm), and bacterial isolates were inoculated near the edge of the PDA plate. Plates were cultured at 28°C for 5 day, and then the growth of antagonistic bacteria (length and width) and the inhibition zone were measured. Isolates that had the best antagonistic activity against *Foc* 4 were selected for further study. Each test was repeated three times, and results were recorded as the mean inhibition growth.

Inhibitory activity of sterilized liquid culture filtrates (SLCF) against mycelium growth

The inhibitory activity of SLCF was tested by the disc diffusion method. The KY-21 strain was inoculated in NA liquid medium and cultured at 30°C, 250 rpm for 3 day. The medium was then centrifuged at 8000 rpm for 10 min, and the cell free supernatant was sterilized by filtration through a 0.22 m Millipore membrane. An 8 mm agar plug with *Foc* 4 mycelium was inoculated in the center of a PDA plate and a sterile oxford cup (6 mm in diameter) was placed on the edge of the PDA medium impregnated with 200 l SLCF. The PDA plate was incubated at 28°C, and the inhibition of *Foc* 4 was observed. Marginal mycelia of the inhibition zone were picked out, and mycelial morphological changes were observed under optical microscopy (Nikon 80i). Assays were repeated three times.

Characterization of KY-21

The colony morphology of strain KY-21 was characterized by light microscopy (Nikon 80i). The following tests were performed according to standard microbiological procedures described by Dong and Cai (2001): oxidative and fermentative test; the Gram reaction; catalase, oxidase, urease and lipase (Tween 80 hydrolysis) activity assays; methyl red (MR), Voges-Proskauer (VP) test; formation of indole; production of 3-ketolactose; gelatin liquefaction test; tyrosine and starch hydrolysis test; 7% NaCl growth test; denitrification and nitrate reductase test; citrate utilization; and utilization of citric acid, glucose, xylose, arabinose and mannitol.

Phylogenetic analysis of the 16S rRNA gene

Purified colonies of KY-21 were cultured on NA plates. Polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene of KY-21 were finished by TaKaRa (Dalian, China). The sequence was compared with those available in the GenBank database and aligned using ClustalW (Ver.1.82). Phylogenetic analysis was performed using MEGA software packages (Version 2.0), and a neighbor-joining phylogenetic tree was constructed by bootstrap analysis with 1.000 replicates.

Table 1. Antagonistic activity of strains against *F. oxysporum* (mm).

Strains	Colony size of antagonistic Strains (length×width)[a]	Inhibition zone
KY -05	32x17	5 a
KY -11	28x19	3 b
KY -21	31x 11	10 c
KY -36	25x14	6 d

The colony size of antagonistic strains were determined on PDA plate after incubation for 5d at 28°C.Data are mean of three replications. Means followed by the same letter in a column are not significantly different (LSD P < 0.01).

Population dynamics of KY-21 in banana

KY-21 was inoculated on selective NA medium containing a series of concentrations of Rif (50 to 300 g ml^{-1}). Through continuous induction by Rif, KY-21 mutants resistant to Rif (300 g ml^{-1}) were acquired. The stable mutants that showed similar antagonistic capacities to the wild-type strains were selected for colonization studies.

KY-21 mutant colonies were cultured in NA liquid medium (30°C, 200 rpm) for 48 h, and then the cultures were diluted in NA liquid medium to a concentration of 10^5 colony forming units (CFU) ml^{-1}. The diluted cultures were used as inoculum and stored at 4°C.

Tissue cultured banana plantlets (cv. Baxi) were transplanted to plastic cups (15 x 15 x 10 cm) containing steam-sterilized soil and vermiculite (4:1,v:v). The plantlets were placed in a glasshouse and irrigated with nutrient solution once a week. Plantlets with six leaves were prepared for inoculation with a KY-21 mutant. A 10 ml of suspension (10^5 CFU ml^{-1}) of the KY-21 mutant was inoculated in each pot around the roots of the banana by the root-drenched method. For the control treatment, plants were inoculated with the same volume of sterile water. After inoculation, plants were managed as the methods described above. The experiment was replicated three times with 20 plants per treatment.

Every three days, until 30 days after inoculation, different parts of the banana were sampled for a colonization assay. The middle part of the roots, pseudostem and stems of the banana were cut and surface sterilized with 70% ethanol (60 s), 3% NaOCl (30 s), followed by thorough rinsing in sterile water. After drying with sterile paper, fresh weights (FWs) of each sections (approximately 0.1 g) were recorded, and each section was triturated in a sterile mortar with 2 ml sterile water. After sedimentation, serial dilutions of supernatant were plated on NA medium containing 300 g ml^{-1} Rif and incubated at 30°C. The population density of KY-21 was measured using the dilution plating method on selective medium and was calculated as the number of CFU per gram FW. Each treatment was replicated three times.

Defense-related enzymes activities assay in banana

The pathogen of *Foc* 4 was inoculated in PDA liquid medium and cultured at 28°C, 250 rpm for 4 days. The cultures were filtered through double- layered gauze and diluted in PDA liquid medium to a concentration of 10^5 (CFU) ml^{-1}.The inoculation of KY-21 and *Foc* 4 were made in the population dynamics of KY-21 in banana. The middle sections of the stems of the bananas were sampled every two days up to 17 days after inoculation. The 2.0 g of fresh samples were placed in a pre-cooled mortar and grinded in 10 ml of 50 mM phosphate buffer (pH 6.4). The extract was centrifuged, and the supernatant was used as enzyme extract to assay peroxidase (POD) and polyphenol oxidase (PPO) activities. Bananas treated with sterile distilled water were taken as control. Treatments were arranged in a randomized complete block design with three replications, each treatment was replicated three times.

POD activities were assayed according to Liu et al. (2010). The 1 ml reaction mixture contained 30 l guaiacol, 900 l 50 mM sodium phosphate buffer (pH 6.4), 20 l crude extract, and 100 l 600 mM H_2O_2. Changes in absorbance at 470 nm were recorded every 30 s for up to 3 min. POD activity was calculated as the change in absorbance units min^{-1} g^{-1} FW.

PPO activity was determined based on the method described by Liu et al. (2010). The 1 ml reaction mixture contained 300 l of 20 mM catechol, 700 l of 50 mM sodium phosphate buffer (pH 6.4), and 20 l crude extract. The increase in absorbance was measured at 420 nm for 1 min. The PPO activity was expressed as units min^{-1} g -1 FW. Enzymatic assays of POD and PPO were conducted in three replicates. One unit was defined as a 0.01 absorbance change per minute.

Greenhouse assay

Biocontrol assays were conducted under greenhouse conditions. The 30 days old tissue cultured banana plantlets (cv. Baxi) were transplanted to plastic cups (15 x 15 x 10 cm) containing a mixed soil of clay and vermiculite. The plantlets were managed as the methods described. The experiment was laid out in a randomized block design with three replications. In each treatment, there were 20 plants per replication. The inoculum of KY-21 and *Foc* 4 were made as the methods described in the population dynamics in banana and defense-related enzymes activities assay in banana.

After 35 days, a 10 ml suspension of strain KY-21 (5 x 10^5 CFU ml^{-1}) was inoculated into the soil around each plantlet. Control plantlets were inoculated with sterile distilled water. Two weeks after inoculation, treated and control plantlets were inoculated with a 10 ml suspension of strain *Foc* 4 (5 x10^5 CFU ml^{-1}) with the same method described above. Disease symptoms were assessed as soon as the disease was visible. The wilt incidence and vascular discoloration were scored after 2 months with the method described by Saravanan et al. (2003). Severe wilt incidence (SWI) was evaluated as the sum of wilt incidence on a grade 4 and 5. Similarly, severe vascular discolouration incidence (SVDI) was evaluated as the sum of vascular discoloration incidence of grade 4 and 5. The wilt index (WI) and vascular discoloration index (VDI) were calculated.

Statistical analysis

All of the data were analyzed using analysis of variance (ANOVA) in SPSS statistical software. Mean separation was performed with LSD test. Differences at P < 0.05 were considered significant.

RESULTS

Isolation and antifungal activity assays of KY-21

From the rhizosphere soil of banana's root, 41 bacteria were screened. Among these isolates, 4 strains were found to be effective in inhibiting pathogen growth by the dual- culture method. The diameter of the inhibition zone were more than 0.2 cm (Table 1) . The strain that had the most effective inhibition (1.0 cm of diameter of inhibition zone) was named KY-21 and further studied.

As observed by the inhibition zone method, cell-free filtrate of the antagonistic strain showed antifungal activity

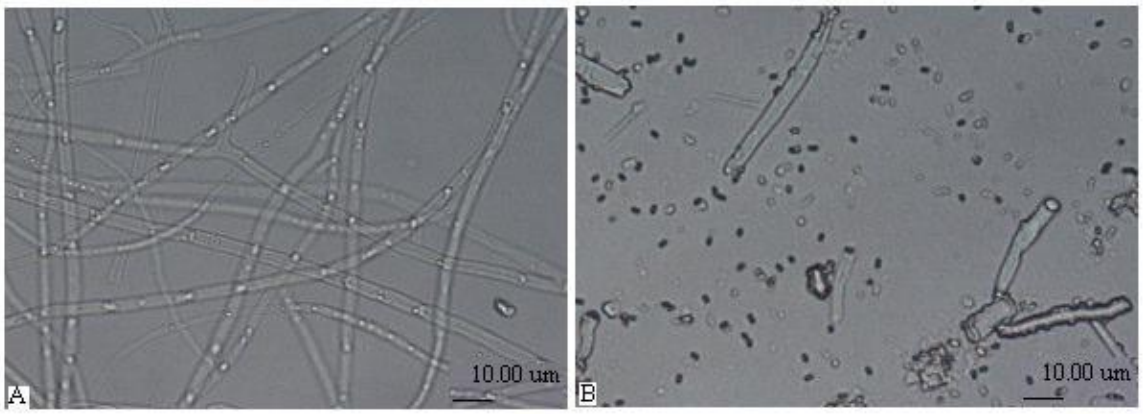

Figure 1. The effect of the fermentation filtrate on the mycelium's growth of *F. oxysporum (*A) Normal mycelium, (B) Deformity mycelium.

Table 2. Physiological and biochemical characters of KY-21.

Testing items	Results	Testing items	Results
Gram reaction	+	Hydrolysis of:	
Oxidase	_	Starch	+
atalase	+	Tyrosine	+
V-P reaction	+	Carbon utilization[b]:	
Nitrate reduction	+	Glucose	+
Indole reaction	_	Arabinose	+
Gelatin liquefaction	+	Xylose	+
Growth in 5% NaCl[a]	+		

+, positive, _, negative. a: could grow in NA liquid medium containing 5% of NaCl, b: could utilize the following compounds as sole carbon sources.

against *Foc* 4 (diameter of the inhibition zone were more than 0.3 cm). The mycelium growth of *Foc* 4 at the edge of the inhibition zone was vesicular and constricted (Figure 1).

Morphological and physiological characteristics of strain KY-21

Colonies of KY-21, which were grown on NA agar medium at 30°C for 16 h, were gray-yellow pigmented, circular, rough and rugose. The colony was low convex, with irregular diffused edges in appearance. The cells were rod-shaped, 1.8 to 2.4 m long, and 0.5 to 0.8 m wide. They were Gram-positive and occurred mostly in singles with some pairs.

Strain KY-21 showed positive for oxidase and catalase, could utilize glucose, xylose, arabinose and mannitol, reduce of nitrate to nitrite, hydrolyze tyrosine and starch and grow in 7% NaCl (Table 2). Based on these morphological, physiological and biochemical properties, the strain was identified as *B. subtilis*, according to the methods described in Bergey's Manual.

Phylogenetic analysis of strain KY-21

To precisely determine the phylogenetic position of strain KY-21, 1464 bp of the 16S rDNA sequence was aligned with those of the most closely related isolates by the Clustalx program. Phylogenetic distances were calculated according to the neighbor-joining method. A consensus tree obtained from 1,000 bootstrap replicates was drawn (Figure 2). Bootstrap analysis showed the strain was grouped with *B. subtilis* (GenBank accession AB501113) with a sequence similarity of 100%. The phylogenetic analysis of the 16S rDNA sequence revealed that the KY-21strain belongs to *B. subtilis*.

Population dynamics of KY-21 in bananas

Rrifampicin resistant mutants of KY-21were detected in the root, corm, and pseudostem after inoculation. These results showed that the KY-21 strain could colonize and transfer in banana tissues and display good ability of colonization. In different parts of the banana, population levels of KY-21 were initially increased, and then tended

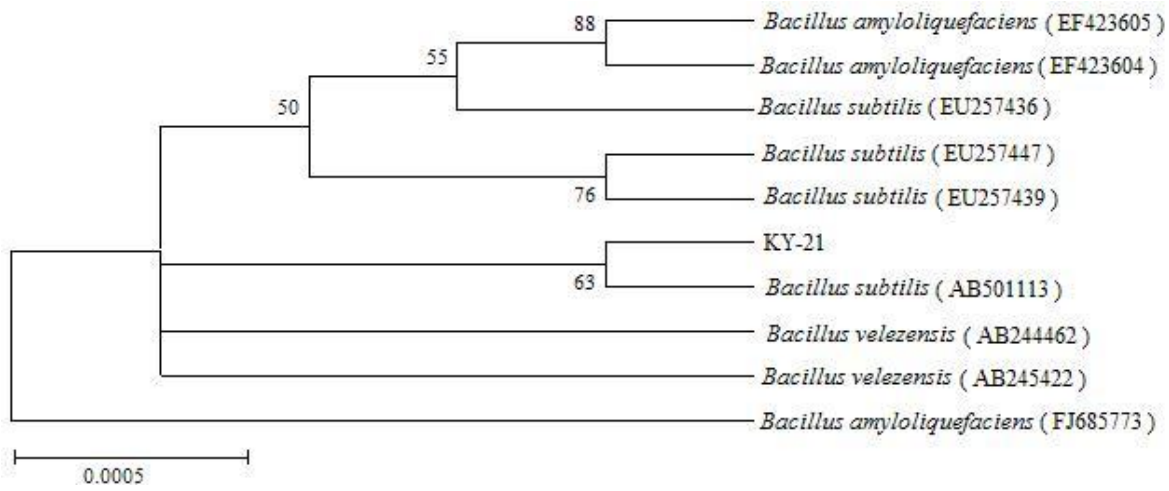

Figure 2. Phylogenetic consensus tree showing relationships of strain KY-21 and related species based on their16S rDNA sequences. The numbers at each branch points show the bootstrap values.

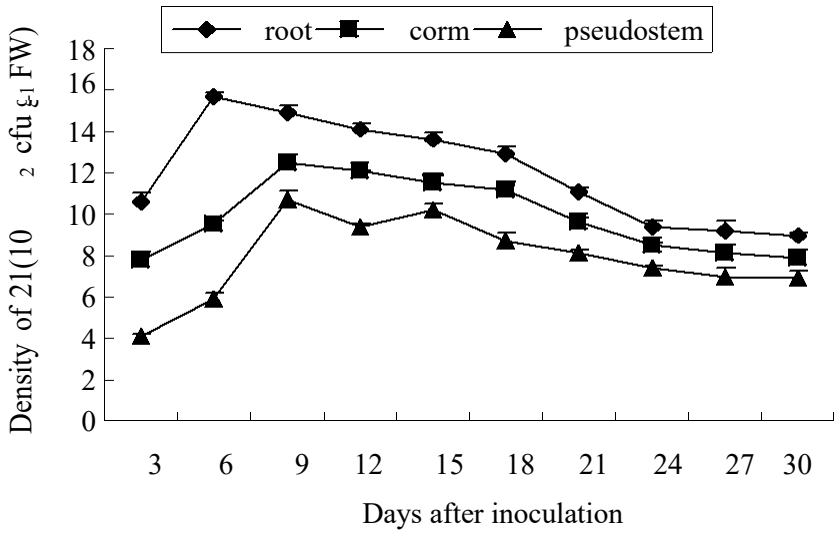

Figure 3. Population dynamics of KY-21 in banana error bars represent standard error of the mean.

to decrease. The highest population levels of KY-21 in the root, corm, and pseudostem were appeared at 6, 9 and 9 d after inoculation respectively. Population levels in the root were higher than that in the root and corm, with the lowest population levels found in the pseudostem. After 17 days, the KY- 21 strain was still detectable in these different parts of the banana. These results are presented in Figure 3.

Changes of defense-related enzymes activities

The POD activity in plants increased up until 7 days after inoculation with KY-21, and then gradually decreased through 17 days. The highest activity of POD was 2.1-fold more than that of the control. A similar trend of POD activity was observed following treatment with *Foc* 4, but the highest level of POD was 1.7-fold higher compared to the control (Figure 4A). There was a similar trend in PPO activity after inoculation with KY- 21 and *Foc* 4 in bananas. With both KY-21 and *Foc* 4, the PPO activity increased and reached the highest level at 7 days, and then the activity gradually decreased through 17 days. The highest levels of PPO activity in the treatment of KY-21 and *Foc* 4 were 1.9-fold and 1.5-fold higher than the control, respectively (Figure 4B).

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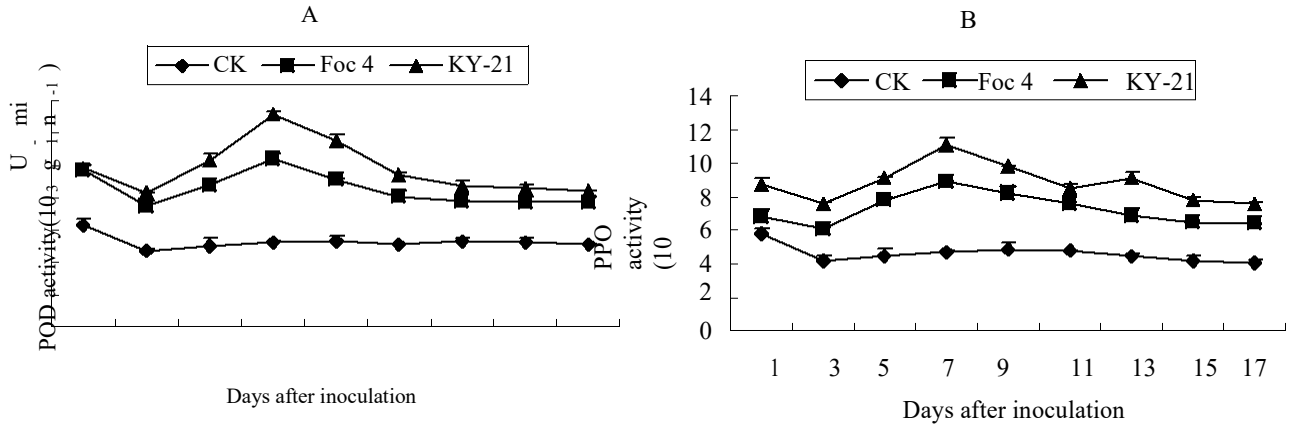

Figure 4. Induction of defense enzymes activities in banana by application of KY-21 (A) peroxidase(POD), (B) polyphenol oxidase(PPO) error bars represent standard error of the mean.

Table 3. Biocontrol activity of KY-21 against Fusarium wilt of banana in greenhouse.

Treatment	SWI (%)	SVDI (%)	WI (%)	VDI (%)
control	68.4 a	48.3 a	76.7	71.7
KY-21	35.0 b	18.3 b	65.0	59.7

SWI, severe wilt incidence; SVDI, severe vascular discolouration incidence, WI, wilt index; VDI, vascular discoloration index. Means within same column followed by different letters mean significant differences (LSD P < 0.05).

Greenhouse experiments

At 35 days after inoculation, leaf chlorosis was observed in control plantlets treated with *Foc* 4, which was earlier than those treated with KY-21. After 60 days, with 68.4% of the control plantlets showing a severe wilt symptom development, leaf yellowing happened extensively, and 48.3% control plantlets showed severe vascular discoloration. In contrast, the plantlets treated with KY-21 exhibited 35% severe wilt symptom and 18.3% severe vascular discoloration, WI and VDI were also significantly lower than the control. Plantlets inoculated with KY-21 showed significantly reduced development of disease compared to the control (Table 3).

DISCUSSION

A *Bacillus* against *Foc* 4 was successfully isolated using the cell wall of the pathogen as the sole carbon source. The antagonistic KY-21 strain selected through the sequential screening procedure showed a significant capability to biocontrol *Foc* 4. To our knowledge, this screening model is novel. Compared with our previous work, the method is more effective in screening biocontrol agents against the disease caused by *Foc* 4.

The mycelium growth of *Foc* 4 was seriously inhibited by the fermentation filtrate of KY-21, probably due to the antifungal metabolites produced by KY- 21. These compounds dissolve the cell wall of the pathogens hyphea, which leads to leaking out of the intracellular substances and blocks normal growth.

B. subtilis have been identified as potential biological control agents. These strains could produce a wide range of antifungal compounds, such as subtilin, TasA, subtilosin, bacilysin, mycobacillin and some enzymes which can degrade fungal cell wall (Berg, 2001). It was suggested that these antibiotic production plays a major role in plant disease suppression (Knox et al., 2000; Leelasuphakul et al., 2006). In addition, some antagonistic mechanisms of these *Bacillus* species involves in the competition for nutrients and space, the induction of plant resistance, etc. (Guerra-Cantera et al., 2005; Van loon et al., 1998). In our study, the growth inhibition of *Foc* by KY-21 may be due to the production of antimicrobial compounds mentioned above.

Some bacteria deriving from the rhizosphere can enter and colonize in internal tissues of plant. This involves in passive or active colonization mechanisms. Bacterial cells can enter plant tissues passively through some tiny wounds and root tips. In the process of active penetration, the cell wall degrading enzymes play an important role (Hardoim et al., 2008). In this research, the passive and active mechanisms may be involved in the KY-21 colonization. KY-21 is able to colonize and transfer into banana tissues and display good ability of

colonization.

Many abiotic and biotic factors are known to affect the survival of introduced antagonists, such as inoculation method and competition from other microbes as well as the host plant itself (Kim et al., 1997; Roamos et al., 1991; McGuire et al., 2000). Successful colonization of potential biological antagonists in plants plays an important role in biological control against pathogens that propagate in vascular tissue, such as *Foc*. Those antagonists, which efficiently colonize and survive in plants, could be effective in managing the vascular disease. In this study, KY-21 was still detected in the root, corm and pesudostem tissue at 20 days after inoculation. These results showed that the strain KY- 21 is able to colonize and transfer into banana tissues and display good ability of colonization.

Plants possess various inducible defense mechanisms to protect themselves against pathogen attack. One of the defense responses was to the accumulation of defense related enzymes. The level of defense related enzymes is known to play a crucial role in the degree of host resistance. POD and PPO are believed to be one of the most important factors of the plant's biochemical defense against pathogens, and are actively involved in the self-regulation of plant metabolism after infection (Kavitha and Umesha, 2008; Jetiyanon, 2007; Dutta et al., 2008). POD is involved in substrate oxidation and cell wall lignification, the PPO can oxidizes phenolic compounds to quinines. Both of these defense mechanisms are associated with disease resistance.

In this study, bananas showed higher enzyme activity of POD and PPO after inoculation with KY-21 compared with the control. The activities of POD and PPO still maintained high levels at 15 days after inoculation. These results indicate that the increase in activity of defense related enzymes was another antagonistic mechanism against Fusarium wilt pathogen by KY-21.

ACKNOWLEDGEMENT

This work was financially supported by the Grant No. ITBBZX200823, 200903049-2, and 808174.

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