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Antifungal Effects of Different Commercial Products Against *Fusarium oxysporum* f. sp. *cubense* TR4 Causing Fusarium Wilt of Banana

Mary Amor G. Figueroa¹, Artemio S. Hagonos, Jr.²

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ABSTRACT

Banana production is seriously threatened by Fusarium wilt, also known as Panama disease worldwide. Regulatory control and the use of biological control agent, *Trichoderma harzianum* showed to be effective in minimizing the development and spread of the disease in the area. Some studies also showed the potential of some commercially-available fungicides in managing the disease. In this study, different commercial products applied singly and in combinations were evaluated against FocTR4 causing Fusarium wilt of banana and the most effective treatment combinations for the management of FocTR4 was determined under in vitro test condition. Treatments used in the experiment were as follows: T1- Control; T2- *B. subtilis* alone; T3-Fosetyl-Aluminum alone; T4-Fluopyram alone; T5- *B. subtilis* + Fosetyl-Aluminum; T6-*B. subtilis* + Fluopyram; T7-Fosetyl-Aluminum + Fluopyram and T8-*B. subtilis* + Fosetyl-Aluminum + Fluopyram. The experiment was laid out in Completely Randomized Design (CRD) with three replications. Data were statistically analyzed using the Analysis of Variance (ANOVA) and the treatment means were compared using Tukey's Honest Significant Difference (HSD). Results of the study showed that treatments of *B. subtilis* in combinations with Fosetyl-Al or Fluopyram and the combinations of these three products had totally inhibited and killed FocTR4. Single treatment of Fosetyl-Al and the combinations of the products had totally inhibited the growth and spore production of FocTR4 however, validation test revealed that Fosetyl-Al alone and its combination to Fluopyram induces chlamyospore production instead of killing the pathogen.

INTRODUCTION

Global banana production is seriously threatened by Fusarium wilt, also known as Panama disease. The disease, caused by the soil-borne fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc), wiped out the Gros Michel-based banana industry in Central America and the Caribbean in the mid-twentieth century. The effects of Foc Race 1 were overcome by a shift to resistant 'Cavendish' cultivars, which are currently the source of 99% of banana exports (Vicente et al., 2014).

Unfortunately, they also reported that a new race of Foc called Tropical race 4 (TR4) has overcome Foc resistance in 'Cavendish' clones. Perhaps even more seriously, other banana cultivars such as plantains, cooking bananas and a diverse range of dessert banana varieties (not susceptible to races 1 and 2) are also susceptible to TR4. These local varieties are mostly grown by smallholder farmers for local consumption and income generation. More than 80% of global banana and plantain production is thought to be based on TR4-susceptible germplasm. This race of Foc has caused epidemics in 'Cavendish' in the tropics different from those less severe infections previously reported in the sub-tropics.

In the Philippines, the disease was suspected to be present in the country since the 1970s, but TR4 was only confirmed in 2008. Its incidence in the surveyed farms increased from 700 cases in 2005 to 15,000 in 2007 (Molina et al., 2008). Large companies currently manage the disease following the protocol established for bacterial wilt (*Ralstonia solanacearum*) management, which

is based on quarantine, sanitation, soil disinfection and fallows (Molina, 2009).

Considering the prevalence, ubiquitous nature and the ability of fungi to cause epidemics in relatively short period of time, disease management strategies are needed to secure the productivity in today's agriculture. Chemical control measures are particularly common in management of fungal plant diseases, and most of these measures rely on the use of fungicides (Ivic, 2010). The use of biological control methods also offers an excellent alternative strategy for effective control of various diseases as well as augmentation of nutrient availability in the rhizosphere. In fact, previous studies on commercially-available microbial agents applied singly and in combinations with synthetic products showed the potential of these agents in controlling various diseases. In addition, for successful control of the disease, these products must be delivered in an appropriate state of activity and to the right place at the right time in order to achieve economical control.

Thus, this study was conducted to evaluate the efficacy of different commercial products such as *B. subtilis*, Fosetyl-Aluminum and Fluopyram applied singly and in combinations and to determine the most effective treatment and treatment combinations for the management of Foc TR4 causing Fusarium wilt of banana under in vitro test condition.

LITERATURE REVIEW

Fusarium Wilt of 'Cavendish' Banana

¹ Plant Pathology Faculty, Surigao State College of Technology, Mainit, Surigao del Norte, Philippines

² Plant Pathology Faculty, University of Southeastern Philippines, Tagum-Mabini Campus, Tagum City, Philippines.

* Corresponding author's e-mail: mafigueroa03@gmail.com

Fusarium wilt, also known as Panama disease have threatened banana production worldwide. In the Philippines, the disease was suspected to be present in the country since the 1970s, but TR4 was only confirmed in 2008. Its incidence in the surveyed farms increased from 700 cases in 2005 to 15,000 in 2007 (Molina et al., 2008). Large companies currently manage the disease following the protocol established for bacterial wilt (*Ralstonia solanacearum*) management, which is based on quarantine, sanitation, soil disinfestation and fallows (Molina, 2009). To date, Cavendish remains resistant to the Fusarium strain that prevails in Central America. However, a virulent strain that can attack the Cavendish was found causing epidemics in Asia, known as Tropical Race 4 (TR4). This pathogen destroyed commercial plantations of Cavendish in Taiwan, Northern Territory of Australia, Indonesia, Malaysia, and China making their banana exports less competitive. In the Philippines, the disease was suspected to be present in the country since the 1970s, but TR4 was only confirmed in 2008. Its incidence in the surveyed farms increased from 700 cases in 2005 to 15,000 in 2007 (Molina et al., 2008).

The Pathogen

Fusarium oxysporum f.sp. *cubense* (Foc) is a soil-borne hyphomycete and is one of more than 100 formae speciales of *F. oxysporum* that causes vascular wilts of flowering plants (Domsch et al. 1980; Nelson et al. 1983). According to Wardlaw (1961) and Stover (1962), the fungus infects the roots of banana plants, colonizing the vascular system of the rhizome and pseudostem, and inducing characteristic wilting symptoms mostly after 5-6 months of planting and the symptoms are expressed both externally and internally. The fungus produces micro- and macroconidia and chlamydo spores. Besides, it over seasons in infected plants as mycelium and in the soil mostly as chlamydo spores. The latter survive in the soil for at least 20 years (Agrios, 2005).

The pathogen is broadly categorized into four races, namely: races 1, 2, 3 and 4. Races 1, 2 and 4 are pathogenic only to banana (*Musa* spp.) and race 3 is pathogenic only to *Heliconia* spp. All races are present in Australia. The pathogen is further categorized into vegetative compatibility groups (VCGs) or strains of which there are 21 recognized worldwide. The most important race of this pathogen is race 4, which affects most of the cultivated banana varieties, including 'Cavendish' banana (Daly and Walduck, 2006).

Symptomatology

According to Agrios (2005), the symptoms of Panama disease consist of yellowing of the oldest leaves or lengthwise splitting of the lower leaf sheath. Leaves may wilt and buckle at their petiole base and, later, younger leaves collapse and die. Internally, brown streaks develop on and within older leaf sheaths and these are followed by large portions of the xylem turning brick red to brown. In the meantime, the fungus, which enters the banana plant from the soil through the feeder roots, advances into the xylem vessels of the rhizome and from there into the

pseudostem, which it colonizes, resulting in discoloration and blockage.

Whereas, Somrith et al. (2011) reported that the symptoms appeared from 5 months in the younger stage to the fruit stage. The external symptom is yellowing of the leaf blade and the internal symptom exhibits reddish brown discoloration of the internal pseudostem. Infected plants collapse before maturity. Even though they can grow, their yield and the quality of fruit are decreased.

Disease Cycle

Based on the review of Pegg, Moore and Bentley (1996), *Fusarium oxysporum* f. sp. *cubense* is a soil-borne pathogen that infects plants through lateral or branching rootlets, penetrates the vascular tissue and proliferates in the xylem. It restricts movement of water and induces wilting and premature death. The fungus can survive for many years in soil as a saprophyte or as chlamydo spores. Chlamydo spores are stimulated to germinate by root exudates, particularly those from wounded roots, which subsequently become infected.

Microconidia are produced in the xylem within 2-3 days on its adaxial side and move upwards in the xylem but become trapped at the end walls in the vessels. However, hyphae penetrate these end walls and sporulate. Infection stimulates the formation of tyloses and the secretion of gels in the xylem vessels within 24-48 hours, which together with trapped microconidia at the perforation plates, restrict water movement. Eventually as the plant dies, the fungus grows out of the vascular system into the parenchyma after 48-96 hours where it sporulates profusely and conidia and chlamydo spores are returned to the soil (Ploetz (1992).

Disease Epidemiology

The pathogen is spread primarily in infected rhizomes (suckers), which are used traditionally for the vegetative propagation of banana. Less frequently, the pathogen is spread as spores in soil, running water, and on farm equipment and machinery (Agrios, 2005). PCARRD-DOST (2006) supported those means of spread and added that the natural and initial site of infection is through the root tips of the plant.

Management

There are no economical methods exposed to eliminate Foc TR4 in the soil. Current management practices involved implementation of strict quarantine measures to prevent the transfer of diseased planting materials into new areas. Infected banana plants, including those within a 6-m radius, must be immediately eradicated to minimize the spread of the disease PCARRD-DOST (2006).

The most effective control can be or achieved by planting banana varieties resistant to the existing races of the pathogen. Planting pathogen-free rhizomes in pathogen-free soil is also effective. Likewise, the use of tissue culture-produced propagative material free of the pathogen is helpful (Agrios, 2005).

There are reports, however, that maintenance of an active micro-flora in the soil was advocated as a general control measure to be used in conjunction with somaclones

showing some resistance. Also, various organisms found to suppress Foc in laboratory and glasshouse experiments have been advocated as biocontrol agents, but little success has been reported using strains of *Trichoderma*, *Pseudomonas*, *Streptomyces*, non-pathogenic *Fusarium* of both rhizospheric and endophytic microbes in the field (Zhang et al., 2013). Today, large companies currently manage the disease following the protocol established for bacterial wilt (*Ralstonia solanacearum*) management which is based on quarantine, sanitation, soil disinfection and fallows (Molina, 2009).

A Chinese research team also reported that crude extract of Chinese chive (*Allium tubererosum*) inhibited the in vitro growth of Foc 'tropical race 4'. In field trials in an area heavily infested by Foc 'tropical race 4', Chinese chive in rotation with banana, reduced incidence of *Fusarium* wilt by 88 %-97 % and a disease severity index by 91-96 % (Huang et al., 2012). The results of further work indicated that volatiles released from root exudates or leached roots and leaves may be responsible (Zhang et al., 2013).

Effects of Fosetyl-Al

Fosetyl-Al is a systemic fungicide which is the breakdown product of Foli-R-Fos that has an active ingredient of Phosphonate, the anion of Phosphonic acid, which is used as fungicides against the pathogens belonging to the order Peronosporales (Davis et al., 1994). This fungicide is known for its direct effect on the fungus that stops plant infection by inhibiting spore germination and the penetration of the pathogen into the plant or blocks mycelial development and sporulation in the case of a curative treatment and its indirect effect of slowing down significantly the invasion of the pathogen by reinforcing the defensive reactions of the plants. This enables the host's hypersensitive response to seal-off the invading organism (Bayer Crop Science, 2013).

Fosetyl-al is rapidly absorbed by the plant roots or leaves and is translocated in both an upward and downward direction, particularly to the growing points. In this way, the foliage and root growth developing between treatments is protected, including the root hairs of the plants receiving foliar spray application. It also exhibits certain level of curative activity. Best results, particularly against the diseases that develop rapidly, are generally obtained when treatments commence prior to the onset of infection (Bayer Crop Science, 2012).

Many researches have shown the potential of this fungicide in controlling bacterial and fungal organisms belonging to the classes other than Oomycetes. Taylor and Washington (1984) reported, fosetyl-al has significantly reduced cankers on peach caused by *Phytophthora cactorum* when applied as curative treatments. On the other side, application of these phosphonates at higher concentration was found to provide some control against Panama disease of banana caused by *Fusarium oxysporum* f.sp. *cubense* (Davis & Bruce, 1996). However, the degree of control varied with the banana growing season and from one locality to another. Preliminary investigation in

the study of Davis and his co-workers (1994) on Foc in culture confirmed that potassium phosphonate inhibited mycelial growth, but showed that the degree of inhibition was extremely sensitive to phosphate levels in the growth medium and that at concentrations of phosphate likely to be present in plant tissues, phosphonate effects were evident only at concentrations in the region of 25 mM.

On the other hand, Landschoot and Cook (2005) claimed that phosphonates were not effective as substitute for phosphate fertilizers but USDA scientists observed that phosphonates have a delayed phosphorus response in the plants. Subsequent research revealed that there is a conversion of phosphites into phosphates in the soil with the aid primarily of soil-borne bacteria. This result helps in the enhancement of quality and yield of the plant.

Effects of *Bacillus subtilis*

The biological control of plant diseases is an excellent alternative strategy for effective control of various diseases as well as augmentation of nutrient availability in the rhizosphere. Several studies have indicated the good potentials of *B. subtilis* as an effective biological control agent of different plant pathogens (Saha et al., 2012; Serafini, 2000; Pukall et al., 2005; Killani et al., 2011, Seema and Devaki, 2012; Abd Allah, 2005; Pleban et al., 1997).

Bacillus subtilis is an ubiquitous naturally- occurring saprophytic bacterium that is commonly recovered from soil, water, air, and decomposing plant material. It is a biofungicide which was started to be used as a seed-dressing biofungicide, with registrations in more than seven crops (Backmann et al. 1994). Preliminary studies conducted by Saha and his comrades (2012) revealed in vitro evidences which indicated multiple modes of action of *B. subtilis* including antibiosis, effective colonization and plant growth promotion which revealed their potential for field application and commercial use as biocontrol agents.

B. subtilis bacteria produce a class of lipopeptide antibiotics including iturins that help the bacteria out-compete other microorganisms by either killing them or reducing their growth rate (CPL Scientific Publishing, 2001). In addition, Warkentin (2012) and Edgecomb, together with Manker (2006), conveyed that this bacterium produces fungicidal metabolites such as lipopeptides that would breakdown pathogen cell membranes causing the pathogen to collapse and die. They also added that this bacterium can be used as Plant Growth Promoting Rhizobacterium (PGPR) because of its ability to produce 2,3 butanediol which is a volatile compound known to trigger plant growth promotion.

Serafini (2001) reported that *B. subtilis* inhibits plant pathogen spore germination, disrupts germ tube growth, and interferes with the attachment of the pathogen to the plant. He further discussed that this bacterium is also reported to induce systemic acquired resistance (SAR) against bacterial pathogens and boosted the plant's responses against the antagonists.

The production of antibiotics by the *Bacillus* spp. and

their uses in the biological control of plant pathogens have been reported in many reviews. *B. cereus* produces lytic enzymes and antibiotics; and *B. subtilis* possesses a lytic factor in its cell wall (Pukall et al. 2005). In an earlier report, Young et al. (1974) stated that *B. subtilis* produces at least five different antibiotics, namely: subtilin, bacitracin, bacillin, subtenolin, and bacilonycin that accounts for the inhibition. Moreover, Pukall et al. (2005) identified four toxin-producing strains of *Bacillus* spp., namely *B. pumilis*, *B. fusiformis*, *B. subtilis*, and *B. mojavensis* apart from normal toxin producer, *B. cereus*. Several researches have reported the production of toxins by *Bacillus* spp. The *Bacillus subtilis* used in the study of Killani together with his co-workers (2011) successfully inhibited the growth of all the root/soil-borne fungal pathogens isolated from cowpea in-vitro. They described that the production of antibiotics by *Bacillus* spp. contributes a big factor. Similarly, in the study conducted by Seema and Devaki (2012), *B. subtilis* showed 50% inhibition of *Rhizoctonia solani* in in vitro test. They concluded that this may be due to the presence of the antibiotic like inturin A and surfactin produced by *B. subtilis* that has been supported by Akihiro et al. (1993). They described that *B. subtilis* produced antifungal peptide antibiotic inturin A and surfactin and further discussed that this inturin A has a strong antifungal activity when compared to surfactin. Moreover, many experiments had established and revealed the potential of this bacterium against pathogens causing diseases. For instance, Abd-Allah (2005) reported that *B. subtilis* protected peanut seeds from the negative effect of *Sclerotium rolfsii* and significantly increased growth parameters of pods compared to the negative control. Likewise, application of a chitinase-producing strain of *Bacillus cereus* directly to soil significantly protected cotton seedlings from root rot disease caused by *Rhizoctonia solani* (Pleban et al., 1997).

Effects of Fluopyram

Fluopyram is a succinate dehydrogenase inhibitor (SDHI) fungicide. Succinate dehydrogenase (SDH) is an enzyme complex couples the oxidation of succinate to fumarate in the mitochondrial matrix (or in the cytoplasmic membrane of bacteria) with the reduction of ubiquinone (UQ), a hydrophobic membrane bound electron carrier, to ubiquinol (UQH₂) in the membrane during aerobic respiration (Horsefield et al., 2006). In addition to its function as a dehydrogenase in the respiratory system, complex II plays an important role in the tricarboxylic acid cycle. The mitochondrial SDH complex is composed of a membrane-peripheral domain and a membrane anchor domain (Avenot and Michailides, 2010). SDHI fungicides play an important role in plant protection against many phyto-pathogenic fungi by specifically binding to the ubiquinone-binding site (Q-site) of the mitochondrial complex II, thereby inhibiting fungal respiration. These fungicides have been used internationally since the late 1960s and are highly effective against basidiomycete pathogens such as rusts

or *Rhizoctonia* sp and even nematodes. Due to their unique mode and site of action, they show no cross resistance with other chemical classes such as strobilurins, benzimidazoles or anilinopyrimidines and therefore are excellent candidates for managing fungicide resistance development and optimizing diseases control (Avenot et al., 2008; Leroux et al., 2003; Stammler et al., 2007; Zhang et al., 2007).

MATERIALS AND METHODS

Experimental Design and Treatments

The study was laid-out in Completely Randomized Design (CRD) with eight treatments and replicated three times with five plates per replicate or a total of 120 plates. Treatments used in the study were as follows: T1- Control; T2- *B. subtilis* alone; T3-Fosetyl-Aluminum alone; T4- Fluopyram alone; T5- *B. subtilis* + Fosetyl-Aluminum; T6-*B. subtilis* + Fluopyram; T7-Fosetyl-Aluminum + Fluopyram and T8-*B. subtilis* + Fosetyl-Aluminum + Fluopyram. Five hundred millilitre of water solutions were prepared for these products with the following recommended rates based on their product labels: 5 ml of *B. subtilis*, 2.14 g of Fosetyl-Aluminum and 0.50 ml of Fluopyram.

Isolation and Mass Production of the Pathogen

Isolation protocols for *F. oxysporum* f.sp. *cubense* described by Vicente et. al (2014) was adopted in this experiment. The pure culture of the pathogen was then mass produced in PDA and were incubated for seven days.

Test of Efficacy of Different

Commercial Products against FocTR4

To test the efficacy of different commercial products under laboratory condition, different treatments with different concentrations applied singly and in combinations was tested against *Fusarium oxysporum* f. sp. *cubense* (Foc) TR4 using poisoned food technique by Burgess et.al (2008). All products were calibrated based on their rates per one liter water solution and were added to the previously-sterilized medium and shaken prior for pour-plating. Pure culture disks of Fo were obtained using sterilized cork borer and were planted at the center of the treated medium. Thereafter, all plates were then incubated under room temperature. Observations and measurement of the zones of growth was done after seven days of incubation.

Validation Test

This was conducted to validate the effect of each treatment either the product causes fungistatic or fungistasis effect. This was conducted by transferring the culture disk of the pathogen from the treated medium after seven days unto the untreated culture medium. All plates were then incubated for another seven days for observation.

Data Gathered

Diameter of Colony

The diameter of the fungal colony was recorded by taking the average of two long and two short diameters taken at right angles for each colony measured in millimetre (mm) using a ruler.

Zone of Inhibition (ZI)

Zone of inhibition (ZI) of the different rates of commercial preparation of *B. subtilis* was determined by measuring the growth of *F. oxysporum* f.sp. *cube* TR4 after 10 days of incubation and % inhibition was calculated using the formula below (Alwathnani and Perveen, 2012):

$$\text{Inhibition (\%)} = (C-T/C) \times 100$$

Where, C is the average colony diameter in control plate and T is the average colony diameter in treated plates.

Number of Spores

Spores were counted using a haemocytometer. A four mm disc of FocTR4 were cut from the near center portion of the culture plate using a cork borer and placed in a test tube with 10 ml of sterilized distilled water and were shaken well to dislodge the spores. One drop of this spore suspension as placed in a haemocytometer and the spores were counted (Ramirez-Peralta et al, 2013). The spores were counted after the termination on the laboratory test. Basic procedure of using haemocytometer in the counting of spores was followed using the formula of Quimio and Quimio (2001):

$$\text{Number of Spores} = A+B+C+D+E$$

(50,000 for microspores and 2,000 for macrospores)

Validation Test

Results of the validation test will be based on the growth of the pathogen. If the transferred culture disk of the pathogen from the treated plate grew (+), this means that the product only causes fungistasis effect or just inhibited the growth of the pathogen. Whereas, if the disk does not grow (-), then the product causes fungistatic effect or totally killed the pathogen.

Statistical Analysis

The study was analysed using Analysis of Variance (ANOVA) in Completely Randomized Design and the differences among treatment means was compared using Tukey's Honest Significant Difference (THSD).

RESULTS AND DISCUSSION

Inhibitory Effects of Different Commercial Products Applied Singly and in Combinations Against FocTR4

Treatment effects of different commercial products applied singly and in combinations against FocTR4 is presented in Table 1. Inhibitory effects of the products on the morphological characteristics of FocTR4 and validation of their efficacy is shown in Table 2. Analysis of Variance revealed significant differences among treatment means in all parameters.

Results showed that treatments of Fosetyl-Al alone and the combinations of the three products, *B. subtilis*, Fosetyl-Al and Fluopyram had totally inhibited (100.00%) the growth of FocTR4 after 10 days of observation. Results also showed that single treatment of *B. subtilis* had significantly inhibited (51.03%) FocTR4 with colony diameter of 39.38mm. Single treatment of Fluopyram, on the other hand, showed lower inhibition (12.78%) with 70.10mm FocTR4 colony diameter. Fosetyl-al alone and the combinations of the three products products also inhibited the production of FocTR4 spores with zero micro- and macrospores. Comparably, lower macrospores production of FocTR4 was obtained by the plates with single treatment of Fluopyram and in the control plate with average number of spores of 1,333.00 and 5,000.00 which subsequently produces 11,983,333.33 and 25,983,333.33 microspores. Highest number of FocTR4 population count was observed in plates treated with *B. subtilis* alone with micro- and macrospores of 58,866,666.67 and 43,333.00, respectively.

Results of the validation test of the efficacy of the products (Table 1& 2) showed that the inhibition of single treatments of these products and the combination of Fosetyl-Al and Fluopyram was mainly due to its fungistasis effect which only inhibit the growth of the fungus as indicated by the positive symbols (+). In the case of Fosetyl Aluminum, presence of chlamydospores (survival structure) were clearly observed which might implies the possibilities of the product to induce chlamydospore production. On the other hand, treatment of *B. subtilis* combined with Fosetyl-Al or Fluopyram and the combinations of the three products showed that their combinations can cause fungistatic effect which totally kills the fungus as indicated by the negative symbol

Table 1. Effects of single and combined treatments of different commercial products on the colony diameter, percent growth inhibition and spore population count per millilitre of FocTR4 after 10 days of incubation and validation of their effects toward the pathogen.

Treatments	Colony Diameter (Mm)	% Zone Of Inhibition **	Foc Tr4 Spore Population Count/ Millilitre		Validation Test
			Micro**	Macro**	
Control (SDW only)	80.41 ^d	0.00 ^d	25,983,333.33 ^c	5,000.00 ^a	(+)
<i>B. subtilis</i>	39.38 ^b	51.03 ^b	58,866,666.67 ^d	43,333.00 ^b	(+)
Fosetyl-Aluminum	0.00 ^a	100.00 ^a	0.00 ^a	0.00 ^a	(+)
Fluopyram	70.10 ^c	12.78 ^c	11,983,333.33 ^b	1,333.00 ^a	(+)
<i>B. subtilis</i> + Fosetyl-Al	0.00 ^a	100.00 ^a	0.00 ^a	0.00 ^a	(-)
<i>B. subtilis</i> +Fluopyram	0.00 ^a	100.00 ^a	0.00 ^a	0.00 ^a	(-)
Fosetyl-Al + Fluopyram	0.00 ^a	100.00 ^a	0.00 ^a	0.00 ^a	(+)
<i>B. subtilis</i> + Fosetyl-Al + Fluopyram	0.00 ^a	100.00 ^a	0.00 ^a	0.00 ^a	(-)
CV (%) =	2.92	0.67	5.21	19.45	

(-). Data were replicated thrice with three plates per replication.

Means having the same superscript means there were no significant differences at 1% level, THSD.

a = Colony diameter (mm) was measured by computing the average of the two longest and two shortest growth diameters in plates.

b = Percent of zone of inhibition was computed using the formula: $C-T/C \times 100$, where, C is the average colony diameter in control plate and T is the average colony diameter in treated plates.

Data were subjected to arc-sine transformation prior to analysis.

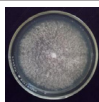
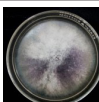

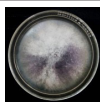

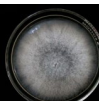
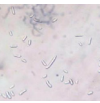
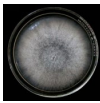
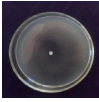
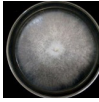

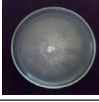
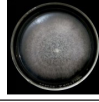
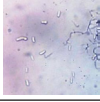
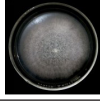
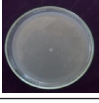
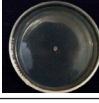
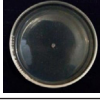
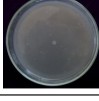
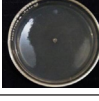

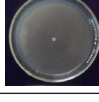

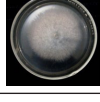



c = Foc TR4 population count per millilitre was computed using the formula: $(A+B+C+D+E) \times 50,000$ (for microspores) and 2,000 (for macrospores).

d=Validation test was conducted by transferring the agar disc from the treated plates onto a fresh medium to test the efficacy of the products on and after treatment application:

(+) means that FocTR4 grow on the fresh medium after

transfer. Using of Fosetyl-Al against FocTR4 can totally inhibit the hyphal growth. The results may be attributed by active ingredient of fungicide. Where in, fosetyl-Al and other salts of phosphorous acid has a break down product of Phosphite ion, which is the main active ingredient against Oomycete pathogens (Alviter, 2006). Investigation on the Phytophthora sp. concluded that phosphite interferes with key phosphorylating enzymes and phosphorous metabolism in general, affecting the synthesis of different phosphorous-containing compounds essential for its growth and development such as Nicotinamide adenine dinucleotide (NAD), Adenosine triphosphate (ATP) and nucleotides. Moreover, Fosetyl-Al possess excellent systemic activity against several diseases caused by Phytophthora sp. and can inhibit mycelial growth and sporulation of the fungus (Boughalleb, 2006). Khaskheli and his comrades (2017) reported that the mycelial growth of Fusarium nivale causing mango malformation disease was significantly inhibited by Fosetyl-Aluminium in their in vitro study. Furthermore,

Table 2. Inhibitory effects of *B. subtilis*, Fosetyl-Al and Fluopyram applied singly and in combinations against the morphological characteristics of Foc TR4. A) Colony growth of Foc TR4 after 10 days of observation; B) Effects on the hyphal growth and structures of Foc TR4 taken from the Foc-inoculated agar block in the treated medium; C) Foc TR4 spore population per millilitre taken from the near center of the treated medium; D) Growth of Foc TR4 in fresh and untreated medium after transfer from treated medium to validate their effects toward the pathogen.

Treatments	Colony Growth	Hyphal Growth	Spore Population	Validation Test
	A	B	C	D
Control (SDW only)				
<i>B. subtilis</i> alone				
Fosetyl-Aluminum alone			No spores	
Fluopyram alone				
<i>B. subtilis</i> + Fosetyl-Al			No spores	
<i>B. subtilis</i> + Fluopyram			No spores	
Fosetyl-Al+ Fluopyram			No spores	
<i>B. subtilis</i> + Fosetyl-Al + Fluopyram			No spores	

Alviter (2006) discussed that the effects of phosphite on *Phytophthora* sp. grown at low level phosphates shown to be greater, suggesting an antagonistic relationship between phosphite and phosphate under in vitro conditions. The effect of Fosetyl-al on the structures of FocTR4 induces chlamydospore (Figure 2B).

However, in the spore population count, plates treated with *B. subtilis* showed highest number of micro and macrospores as compared to other treatments. Also, plates treated with fosetyl-aluminum has no spore formation. These cases might be correlated to the concept of the second hypothesis of compensation on which a specific “weakness” can be compensated by a specific “strength” (Bashi and Rotem, 1974, 1975a and Bashi et al, 1973). In order to compensate the activities of *B. subtilis* on degrading the cell wall of the hyphal structure of FocTR4, the pathogen produced more spores for it to compensate the degradation of its hyphae and in order for it to survive.

The same goes with the reaction of FocTR4 to the effect of fosetyl-Al on which it induced itself to produce chlamydospores for it to compensate the effect of the product against its spores as previously discussed, fosetyl-al targeted mainly its spore formation by affecting the synthesis of different phosphorous-containing compounds essential for its growth and development.

On the other hand, Fluopyram is a succinate dehydrogenase inhibitor (SDHI) fungicide. Succinate dehydrogenase (SDH) is an enzyme complex couples the oxidation of succinate to fumarate in the mitochondrial matrix (or in the cytoplasmic membrane of bacteria) with the reduction of ubiquinone (UQ), a hydrophobic membrane bound electron carrier, to ubiquinol (UQH₂) in the membrane during aerobic respiration (Horsefield et al., 2006). In addition to its function as a dehydrogenase in the respiratory system, complex II plays an important role in the tricarboxylic acid cycle (Kreb’s Cycle). The mitochondrial SDH complex is composed of a membrane-peripheral domain and a membrane anchor domain (Avenot and Michailides, 2010). Nevertheless, SDHs are classified as medium to high risk for resistance development because of their single-site mode of action.

CONCLUSION

Based on the results, generally, it can be concluded that Fosetyl-Al alone and its combination to *B. subtilis* or Fluopyram and the combination of the three fungicides can potentially mitigate Foc TR4 population which causes the most devastating disease of banana. However, further validation of their effects under pot and field tests should be undergone. Moreover, the combination of these products to other available strategies to improve its efficacy can be explored in the future.

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