

Antifungal Effect of a Local *Bacillus subtilis*, isolate TM07, on *Fusarium oxysporum* f.sp. *Lycopersici* in Morogoro, Tanzania

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Abstract

Fusarium oxysporum f.sp. *lycopersici* causes tomato wilt, a severe disease that leads to extensive yield and quality loss of tomatoes in Tanzania. Management of *Fusarium* infection is particularly challenging given the health and environmental concerns over continued use of chemical pesticides on horticultural food crops. However, there is an increasing focus towards the use of biocontrol agents to combat phytopathogens worldwide. In this study, bacterial isolates from tomato rhizosphere were screened for their antagonistic activity in vitro on *F. oxysporum*. Based on its superior effectiveness, one isolate, designated TM07, was selected and characterized as *Bacillus subtilis* by morphological, biochemical and molecular procedures. On potato dextrose agar (PDA) the isolate showed an appreciable degree of radial growth inhibition (RGI) of 40.5% of the *Fusarium*, compared to control (55.9%). Further investigations are recommended to elucidate on the mechanism behind the inhibitory effect of isolate TM07. In vivo studies are also recommended to assess the possibility of applying the *B. subtilis*, isolate TM07, as a local biocontrol agent of *Fusarium* wilt of tomato and other susceptible crops.

Keywords: Tomato wilt, *Fusarium*, biocontrol, *Bacillus*

Introduction

Plant pathogenic fungi are among important biotic components causing serious losses in agriculture and the consequent adverse economic impact (Do *et al.*, 2017). *Fusarium* wilt is among the most important fungal diseases of crops, especially vegetables in horticultural fields and greenhouses. The disease is characterized by yellowing of leaves, progressive wilting and reduced or even total loss of crop yields (Bawa, 2016). Although *F. oxysporum* is widely distributed in organic soils and plant rhizosphere, its subspecies, also referred to as *formae speciales* f. sp. special forms, show a high level of host specificity (Ajillogba and Babalola, 2013).

In Tanzania, *fusarium* wilt has been reported in many crops including: cucumber

water melon, banana and cotton (Faustine *et al.*, 2016; Luzi-Kihupi *et al.*, 2015; Majune *et al.*, 2018; Mbega and Mabagala, 2017). *Fusarium oxysporum* f. sp. *lycopersici*, which specifically attacks tomato plants is capable of surviving for long periods in the plant rhizosphere where it causes root and stem necrosis and wilting of the plants. Once established in the soil, the fungus is difficult to control because its chlamydospores can remain dormant and render the soil infective for many years (Flavel *et al.*, 2003; Pengchao *et al.*, 2013).

Fusarium wilt is commonly controlled by integrating the use of chemical fungicides with selected resistant cultivars. However, increased use of chemical fungicides has adverse impact on the environment and human health due to chemical residues. Also, cultivar selection is

a lengthy and complicated procedure for the small-scale farmer to perform (Saechow *et al.*, 2017). Biological control is an environmentally acceptable non-hazardous strategy to reduce crop damage caused by plant pathogens. The biocontrol mechanisms may involve antibiosis, competition, parasitism, microbial cell wall degradation by enzymes and induced resistance (Bawa, 2016).

Widely used fungal antagonistic biological agents include some *Bacillus* spp, *Pseudomonas* spp and *Streptomyces* spp. The *Bacillus* spp. are particularly competent because of several advantages they possess over the other bacterial antifungals. These include production of endospores which confer the *Bacillus* spp survival in adverse environmental conditions, such as extreme temperatures, pH variations and osmotic pressure (Saechow *et al.*, 2017). Furthermore, the bacilli produce diverse antifungal compounds, including: iturin, surfactin, bacilycin, and volatile metabolites (Do *et al.*, 2017).

Bacillus subtilis is found in various ecological niches including soil, water and air. It is a non-pathogenic, aerobic/microaerophilic, gram positive, rod - shaped bacterium, which produces characteristic dull, wrinkled colonies on solid medium (Matar *et al.*, 2009). It is among the most studied gram-positive bacteria. It exhibits a wide range of physiological capabilities that allow the microorganism to compete favourably with co-existing microorganisms in plant rhizosphere (Al-humam, 2016). This study was conducted to demonstrate the antagonistic potential of local *B. subtilis*, isolates on *F. oxysporum* f.sp. *lycopersici* from Morogoro, Tanzania.

Materials and Methods

Isolation and partial characterization of *Bacillus* spp. from local soils

Morphological and biochemical characterization

A total of 42 soil samples were obtained from plant rhizosphere of apparently healthy tomato plants in Mlali - Mvomero district, Morogoro, Tanzania between August and September, 2018. The sampling sites were purposefully chosen because they are known

to experience tomato wilt disease for many years. The soil samples were then taken to the laboratory at the African Seed Health Center (ASHC) - Sokoine University of Agriculture (SUA) for further studies. In the laboratory aqueous suspensions of the soils (50gm soil in 200ml distilled water) were prepared and heated at 60°C for 1h, in a water bath, to kill vegetative cells. Bacterial isolation was done by inoculating loop fulls of the cooled soil suspensions onto nutrient agar (NA). The plates were incubated at 30°C for 24h and mucoid, raised colonies of grayish colour and with, round margins were sub cultured (purified) onto secondary NA and blood agar (BA) - plates. Further characterization of the bacterial isolates was done by micro-morphological examination upon gram staining, haemolysis and by biochemical tests (Al-humam, 2016). A known *B. subtilis* (Real Bacillus SC®) obtained from Real IPM, UK, served as control.

Isolation of *Fusarium oxysporum* f. sp. *lycopersici*

Fusarium infected tomato plants were collected from tomato farms in Mvomero district between August and September, 2018. A selected plant stem was chopped into pieces, 2 cm length, split open, and disinfected with 5% sodium hypochlorite. A piece was then rinsed with sterile distilled water, dried on tissue paper and placed onto a sterile potato dextrose agar (PDA) plate at room temperature-RT, (ca 27°C), for three days to allow mycelial growth.

Upon confirmation of mycelium development by microscopy, a secondary PDA plate was prepared for demonstration of antagonistic (growth inhibition) effect by the *Bacillus* spp. on the *F. oxysporum*. A known *F. oxysporum* provided by ASHC-SUA served as the control.

Demonstration of antagonistic effect of *Bacillus* spp. on *F. oxysporum*

Demonstration of inhibition of *F. oxysporum* by *Bacillus* spp. was done by the dual culture procedure on PDA according to Matar *et al.* (2009). For this, a loop full of a 24h nutrient broth culture of the *Bacillus* isolates was streaked onto an 8.4cm diameter PDA plate

at about 2.5 cm away from the edge of the plate, and incubated for two days at 37°C. A speckle of mature (10 day old) mycelium of *F. oxysporum* was then placed at the middle of the PDA plate using a pair of sterile tweezers and the plate further incubated at RT for 10 days. A PDA plate inoculated with the *F. oxysporum*, without the *Bacillus* spp., was included as the control. Measurements of the growth inhibition zone of *F. oxysporum* by the *Bacillus* spp. were taken at day seven of incubation using a millimeter ruler and the percentage inhibition radial growth (PIGR) of the fungus was determined by the formula:

$$\text{PIGR (\%)} = \left[\frac{R1 - R2}{R1} \right] \times 100$$

Where R1 was the radial diameter of *F. oxysporum* growth on the control plate and R2 was radial growth of *F. oxysporum* growth on the plate with antagonist *Bacillus* spp.

One *Bacillus* isolate, designated TM07, which showed the most potent antagonistic effect, relative to the others, was selected for further characterization at molecular level as described elsewhere (Taale *et al.*, 2015).

Molecular characterization of *B. subtilis* (TM07) isolate

Molecular characterization was done by polymerase chain reaction (PCR) of isolate TM07, putatively identified as *B. subtilis*, by morphological and biochemical properties. The commercial *B. subtilis* - Real Bacillus SC® and distilled water served as positive and negative controls respectively.

Genomic DNA from isolate TM07 was extracted using Quick-DNA Fungal/Bacterial Mini Kit (Zymoresearch, USA) following manufacturer's instructions.

Identification of *Bacillus* isolate TM07 at genus level

The PCR mixture consisted of 18.15 µl of molecular grade H₂O, 2.5µl of 10X PCR buffer, 0.75 µl of MgCl₂ (50mM), 0.5µl of dNTP (10mM), 0.1µl of Platinum Taq DNA polymerase (Invitrogen Platinum Taq, USA), 1.0µl of primer B-K1/F (5'-TCACCAAGGCRACGATGCG-3'), 1.0µl of primer B-K1/R (5'-CGTATTCACCGCGGCATG-3')-target band 1114bp - at a final concentration of 20 µM

and 1.0 µl of extracted DNA from the isolate. The primer pairs B-K1/F and BK1/R were designed for genus identification of *Bacillus* spp. (Wu *et al.*, 2006; Taale *et al.*, 2015). *Bacillus subtilis* - Real Bacillus SC® was used as the positive control.

Amplification consisted of 30 PCR cycles in a thermocycler (SimpliAmp thermal cycler, Applied Biosystems, Singapore). The cycling program was: initial denaturation at 94°C for 5 min. followed by 30 cycles of denaturation at 94°C for 1 min., annealing at 56°C for 45s and elongation at 72°C for 30s. The PCR was ended with a final extension at 72°C for 10 min. and the amplified product cooled at 4°C.

Identification of *Bacillus* isolate TM07 at species level

Specific primer pair Bsub5F and Bsub3R (target band 600bp) was used in PCR to identify isolate TM07 at species level (Taale *et al.*, 2015). The PCR mixture consisted of 18.15 µl of molecular grade H₂O, 2.5 µl of 10X PCR buffer, 0.75 µl of MgCl₂ (50mM), 0.5 µl of dNTP (10mM), 0.1 µl of Platinum Taq DNA polymerase (Invitrogen Platinum Taq, USA), 1.0 µl of each primer (20µM)(Bsub5F: 5'-AAGTCGAGCGGACAGATGG3', Bsub3R: 5'-CCAGTTTCCAATGACCCTCCCC3', and 1.0 µl of extracted DNA of each isolate. Amplification consisted of 30 PCR cycles in a Thermocycler (SimpliAmp thermal cycler, Applied Biosystems, Singapore). The cycling program was; initial denaturation at 94°C for 5 min. followed by 30 cycles of denaturation at 94°C for 1 min., annealing at 60°C for 45s and elongation at 72°C for 30s. The PCR was ended at 72°C for 10 min. and the amplified product cooled at 4°C.

Agarose gel electrophoresis

The DNA fragments were separated by loading 10 µl of each PCR product mixed with 2 µl of loading dye to 1.5% agarose gel containing 10,000X DMSO Gel red Nucleic Acid stain (Biotium, USA). All Purpose Lo DNA Marker 50bp-2000bp (Bionexus, USA) was used as standard for the determination of fragment length. The agarose gel was run in Tris Borate EDTA (TBE 1.0X) buffer for 120 min. under

120V, 50mA and photographed under ultraviolet light illumination.

Results

Isolation and partial characterization of *Bacillus subtilis* isolates

Upon purification of the *Bacillus* spp. isolates on NA, 17 samples gave rise to grayish mucoid colonies which were, roundish and wrinkled; suggestive of *Bacillus* spp. On BA, scanty hemolysis was present around the colonies (Fig. 1). Smears from the colonies showed rod shaped gram positive bacteria in short chains (Fig. 2). Furthermore, the cultures tested catalase and oxidase positive. These isolates were then tested for antagonism against *F. oxysporum*



Figure 1: Colonies of *Bacillus* sp. isolate TM07 on blood agar

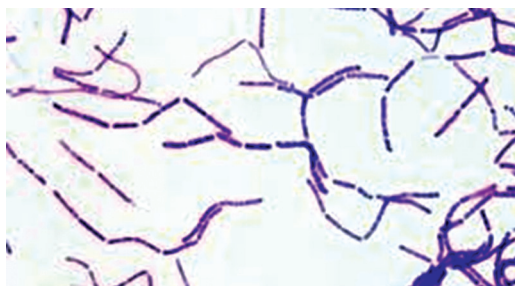


Figure 2: Micromorphological image of gram positive *Bacillus* sp. isolate TM07

Characterization of *Fusarium oxysporum* f.sp. *lycopersici*

On PDA, *F. oxysporum* mycelium appeared cotton like velvet with a pinkish colour at the center after 10 days of incubation at RT (Fig. 3).



Figure 3: Mature *Fusarium oxysporum* f.sp. *lycopersici* mycelium on potato dextrose agar plate

Demonstration of antagonistic effect of *Bacillus* spp. isolates on *F. oxysporum*

The 17 *Bacillus* spp. isolates tested for antagonism on *F. oxysporum* showed variable degrees of radial growth inhibition (RGI), with isolate TM07 presenting the largest RGI of 40.5% relative to the others (Fig. 4). The positive control, *B. subtilis* - Real Bacillus SC®, here encoded as Co₃, (Table 1) gave an RGI of 55.9%. For the negative control, *F. oxysporum* growth covered the entire surface R1 (8.4cm) of the PDA. There was a statistically significant difference in RGI of *F. oxysporum* by *Bacillus* spp. isolates ($p < 0.05$ - one sample t-test)

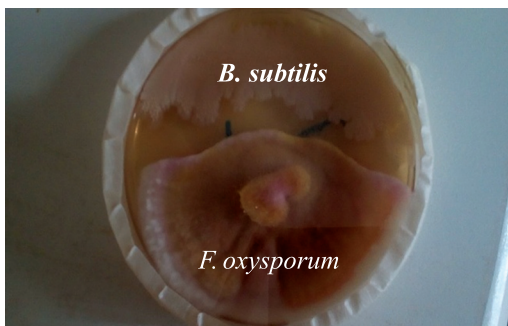


Figure 4: Antagonism of *Bacillus* isolate TM07 on *F. oxysporum* f.sp. *lycopersici* on PDA plate (diameter 8.4cm)

Molecular characterization of *B. subtilis* (TM07) isolate

At the genus level the isolate showed a characteristic *Bacillus* sp., DNA band of

Table 1: Percentage radial growth inhibition of *Fusarium oxysporum* by *Bacillus* spp. isolates

| Isolate designation | | Radial growth (cm) of <i>F. oxysporum</i> in test plate (R2) | Percentage radial growth inhibition (RGI) |
|---------------------|-------|--|---|
| Co3* | | 3.7 | 55.9 |
| 1 | TM07 | 5.0 | 40.5 |
| 2 | TM02 | 5.4 | 35.7 |
| 3 | TM04 | 5.5 | 34.5 |
| 4 | TM05 | 6.2 | 26.2 |
| 5 | TM01 | 6.4 | 23.8 |
| 6 | TM06 | 6.5 | 22.6 |
| 7 | TM03 | 6.0 | 28.6 |
| 8 | TM08 | 6.2 | 26.2 |
| 9 | TM09 | 5.0 | 28.6 |
| 10 | TM010 | 5.0 | 28.6 |
| 11 | TM011 | 5.0 | 28.6 |
| 12 | TM012 | 5.0 | 28.6 |
| 13 | TM013 | 4.2 | 26.2 |
| 14 | TM014 | 4.2 | 22.6 |
| 15 | TM015 | 4.0 | 28.5 |
| 16 | TM016 | 4.0 | 22.6 |
| 17 | TM017 | 3.0 | 16.7 |

P < 0.05, *df* = 42 Co3* Control isolate *Bacillus subtilis* - Real *Bacillus* SC®

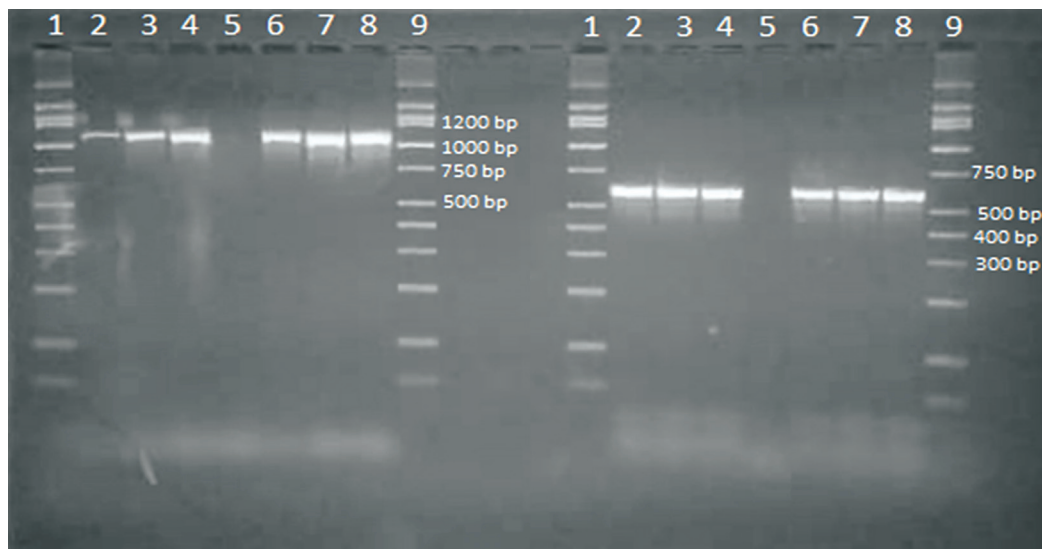


Figure 5: Two agarose gel electrophoreses of PCR products of *Bacillus* sp. (TM07) showing characteristic bands size 1114bp (left) and 600bp (Right) for genus and species level identification respectively. Lanes 1 and 9 are DNA markers; Lanes 2, 3 and 4 are the PCR product of bacteria isolate TM07; Lane 5 is distilled water (negative control); Lanes 6, 7 and 8 are DNA samples of Real *Bacillus* SC® (positive control)

approximately 1114 bp size at between the 1000 bp and 1200 bp bands of the reference DNA marker upon agarose gel electrophoresis of the PCR product. At the species level, the isolate was identified as *B. subtilis* by a DNA band of approximately 600 bp, between the 500 bp and 750 bp bands of the DNA marker. These bands were evident also with the PCR product of the control isolate (Real Bacillus SC®) (Fig. 5).

Discussion

In this study, 17 local soil *Bacillus* spp isolates from tomato farms in Morogoro, Tanzania were examined for their antagonistic potential (growth inhibition) against the fungal agent of tomato wilt disease, *F. oxysporum* f.sp. *lycopersici*. This study has shown in vitro that local *Bacillus* spp. isolates can inhibit the growth of *F. oxysporum* f.sp. *lycopersici* at varying degrees. One isolate, confirmed to be *B. subtilis*, designated *B. subtilis*-TM07, demonstrated a relatively stronger growth inhibitory effect on the fungal agent.

Fusarium wilt is a disease of economic importance in tomato and other horticultural crops worldwide (Njau *et al.*, 2012). In Tanzania, this disease causes devastating losses among small scale horticulturists who cannot afford application of chemical pesticides. Some farmers in Mvomero district, for instance, claim to suffer up to 100% wilting of tomatoes, and some soils have been permanently abandoned due to *F. oxysporum*. Although tomato wilting can be caused by a diversity of physical and biological agents, eg. the bacterium *Ralstonia* sp, farmers in the district believe *Fusarium* is the major cause of the wilting disease, popularly referred to as. “*Mnyauko wa fusari*” (Personal Communication).

Fusarium oxysporum is known to have a diversity of hosts, with its subspecies (*formae speciales*-f.sp) attacking specific annual and perennial crops. Although the family *Solanaceae* are particularly vulnerable, other crops including banana and cashew are equally affected by *Fusarium* in Tanzania (Karangwa *et al.*, 2016; Mbasu *et al.*, 2020).

The mechanism behind *Fusarium* wilting disease may involve antibiosis, competition for nutrients, parasitism or fungal cell degradation

by bacterial enzymes (Bawa *et al.*, 2016; Abriouel *et al.*, 2011; Do *et al.*, 2017). Screening of microbial extracts has revealed structural diversity of natural compounds with broad biological activities, such as antimicrobial, antiviral, immunosuppressive and antitumor effects (Sansinenea and Ortiz, 2011).

According to the 2016/2017 Annual Agricultural Survey Report, Morogoro region had the largest planted area with tomatoes in mainland Tanzania, with a production of over 150,000 tons, per annum of the crop. Tomato farming is, therefore, an important sector of Tanzania's economy by provision of food for households and labour in the value adding chain (MMA, 2008).

Given that tomato production is dominated by small scale producers, the potential of reducing poverty by enhancing production is enormous. It is, therefore, important to protect the tomato industry by developing appropriate strategies to address pest infestation, which ranks second highest challenge faced by farmers (Mutayoba and Ngaruko, 2018). Biological control has a sustainable potential in preventing *Fusarium* wilt, given its environmental safety. *Bacillus subtilis*, singly or in combination with other biological agents (eg. *Trichoderma harzianum*, *Pseudomonas fluorescens*) (Basco *et al.*, 2017) could replace chemical pesticide to a great extent in the future.

Conclusion

This study suggests that local rhizosphere *B. subtilis* could be used to inhibit the growth of *F. oxysporum*. Given this property, *B. subtilis* is a potential antifungal agent for protection of tomatoes and other crops susceptible to *F. oxysporum*. Additional in vitro studies are essential to further elucidate the mechanism behind the antagonism. For example, screening of microbial extracts eg. bacteriocins and lipopeptides will reveal the diversity of the biologically active compounds.

Furthermore, studies are recommended to establish the antifungal potential of the *Bacillus* spp. under field conditions. Eventually, a local *B. subtilis* based brand - product could be developed for biological management of *F. oxysporum* f.sp. *lycopersici*, in future, thus

reducing to a big extent the habitual application of chemical pesticides by tomato farming communities.

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