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The efficacy of selected biological control agents against citrus black spot (CBS) pathogen *Phyllosticta citricarpa*

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Citrus black spot (CBS) caused by *Phyllosticta citricarpa* (McAlpine) Van der Aa (asexual state) synonym *Guignardia citricarpa* Kiely (sexual state) is one of the most devastating diseases of citrus which occurs in various citrus producing areas around the globe. Management is mainly based on monthly applications of copper fungicides and strobilurins under field conditions. In this study, bio-control agents were evaluated as alternative post-harvest treatments against citrus black spot disease. Two bio-control agents namely, *Bacillus subtilis* Cohn *and Trichoderma harzianum* Rifai were evaluated for their efficacy against *P. citricarpa*. Their efficacy was further compared with commercial fungicides Dithane 750 (Mancozeb, 750 g/kg). Results obtained showed that *T. harzianum* treatments were highly suppressive towards pathogen growth *in vitro* (85%) as compared to *B. subtillis* (4%) and Dithane 750 (12%). Treatment of artificially inoculated fruits with combined formulations of both biocontrol agents resulted in reduced CBS severity as compared to their single applications. These findings suggest that *T. harzianum* had highest suppression of pathogen growth as compared to *B. subtilis*. The results also suggest that their combined application can provide an effective disease management when compared to sole application of Dithane 750. Further studies are however, needed to determine their effectiveness under field conditions and also their efficacy can be sustained when applied with commercial fungicides.

Key words: *Bacillus subtilis*, biocontrol, dithane 750, *Trichoderma harzianum*.

INTRODUCTION

Citrus black spot caused by *Phyllosticta citricarpa* (McAlpine) van der Aa is a major disease affecting citrus production worldwide, where weather conditions are favourable (Paul et al., 2005). The disease affects all citrus species of economic importance however the disease causes more damage to sweet oranges and

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lemons due to their high susceptibility (Kotźe, 1981). The disease is of high economic importance in citrus growing countries including South Africa (Smith et al., 1997). The causal pathogen causes external blemishes on the fruit rind, making the fruit unsuitable for fresh market which in some cases, result in severe fruit drop and crop losses (Araújo et al., 2013). Worldwide, the potential economic loss is mainly due to the rejection from fresh market as a result of unsightly lesions (Paul et al., 2005; Everett and Rees-George, 2006). Potential quarantine of fruit sales to other citrus production regions can also result in economic losses of millions of dollars (Meyer et al., 2012).

Citrus black spot management relies mainly on the application of preventative fungicides during fruit susceptibility (Schutte et al., 1997). Various groups of fungicides have been used against citrus black spot with varying degree of efficacy (Rodrigues et al., 2007). However, loss of sensitivity and pathogen resistance is a common factor resulting in poor disease control by most fungicides groups (Akinnifesi et al., 2006; Rodrigues et al., 2007). Growing international concern on chemical residues on treated fruits has also resulted in need to identify and develop alternative safe measures. Biological control agents such as *Trichoderma* spp. and *Bacillus* spp. have been shown to be effective against a number of plant pathogens (Kupper et al., 2011; Abdalla et al., 2014).

Also, combined application of biological control agents has been shown to be effective against some plant diseases (Begum et al., 2010). There is limited information regarding the use of both *Trichoderma. harzianum* strain and *Bacillus subtilis* in the suppression of pathogen growth *in vitro* and disease severity *in vivo*. The main aim of this study was to evaluate the combined efficacy of biological control agents (*T. harzianum* and *B. subtilis*) in comparison with commercial fungicides (Dithane 750) against CBS pathogen *P. citricarpa*.

The specific objectives were, to determine the effect of *B. subtilis* and *T. harzianum,* as compared to Dithane 750 on *P. citricarpa* growth *in vitro* and to investigate the effectiveness of both biological control agents on citrus black spot development, when applied separately or in combination.

MATERIALS AND METHODS

Microbial culture preparations and maintenance

A pure culture of *P. citricarpa* isolate (PPRI 8774) was obtained from the National Fungal Collection, Biosystematics Division, Agricultural Research Council-Plant Protection Research Institute (ARC-PPRI), Pretoria, South Africa. The pathogen was originally isolated from an infected 'Valencia' citrus fruits from Letsetele farm in Limpopo, South Africa.

The isolate was preserved on 2% Potato dextrose agar (PDA) and stored at 4°C until further use. The pathogen was identified as *P. citricarpa* using both morphological and molecular characterization. Molecular characterization was based on Polymerase Chain Reaction (PCR) technique described by Perez et al. (2007). Two biological agents namely *T. harzianum* (PPRI 8230) and *B. subtilis* (B246) were obtained from ARC-Plant Protection Research Institute and Quality Management Services (QMS) Company at Tzaneen area in Limpopo Province, South Africa, respectively. Both *B. subtilis* and *T. harzianum* were maintained in Nutrient Agar and 2% PDA, respectively and kept at 4°C until further use.

Effect of biocontrol agents on P. citricarpa growth in vitro

The growth inhibition of *P. citricarpa* by *T. harzianum* and *B. subtilis* was carried out on Potato Dextrose Agar (PDA) and Nutrient Agar (NA), respectively, using the dual culture technique. This was determined by placing 5 mm mycelial plug obtained from a 7 days old *P. citricarpa* culture, at 1 cm from the periphery of 90 mm Petri plates with PDA and incubated for 3 days at ± 28°C (Evans et al., 2003). After 3 days, a 5 mm mycelial plug obtained from 7 days old *T. harzianum* culture was placed 1 cm away from the edge, of the same Petri dish on the side of *P. citricarpa*. Control treatments were not inoculated with a biological control agent.

All plates were incubated for 7 days at ± 26°C. In *B. subtilis* treatments, dual culture method described by Etebarian et al. (2005) was followed. Half of the petri plates containing nutrient agar were streaked with 100 ml suspension of *B. subtilis* and incubated for 48 h. Thereafter, 5 mm mycelial plug of 7 days old *P. citricarpa* culture was inoculated as described above.

Fungicide solution was prepared by mixing 7.5 g of Dithane 750 with 100 ml sterile distilled water and kept at 4°C until further use (concentration). Used fungicide dilutions were according to manufacturer's instructions. For pathogen suppression, 10 ml of prepared suspension of Dithane 750 was added to 1 litre PDA after autoclaving at 40°C and dispensed into 90 mm petri plates. Each treatment was replicated four times and experiment was repeated twice.

For *in vivo* evaluation, a total of 200 healthy Valencia fruits were harvested from Letsetele citrus farm, surface sterilised with 0.1% Sodium hypochloride for 3 min, rinsed with sterile distilled water twice and air dried at room temperature for 24 h. Inoculum preparation and fruit inoculation was done according to the method described by Baldassari et al. (2009). Mature Valecia orange leaves were used for pathogen inoculum production. A 10 mm leave disk was cut with cork borer and placed on ripe orange fruit. Inoculated fruits were incubated at ± 25°C for 21 days after which lesion size was measured using a Venier caliper.

Bio-control treatments were prepared by mixing 50:50 suspensions of *B. subtilis* (1×10^7cells/mL) and *T. harzianum* culture filtrate (5×10^5 spores/Ml) and applied separately or in combination 24 h after fruits were inoculated with pathogen. For control, inoculated fruits were sprayed with sterile distilled water. All treatments were laid out in a completely randomized design (CRD) with five replicates. Disease severity was evaluated on a scale of 0 to 4 where 0= no lesion, 1 = 10% fruit area affected, 2 = 10 to 25% fruit area affected, 3 = 25 to 30% fruit area affected, 4 ≥ 50% area affected. Collected data was analysed using Statistic 10.0 software. Duncan's Multiple Range test was used to compare treatment means at P≤ 0.05 probability level.

RESULTS AND DISCUSSION

Results for *in vitro* experiment are presented in Figure 1.

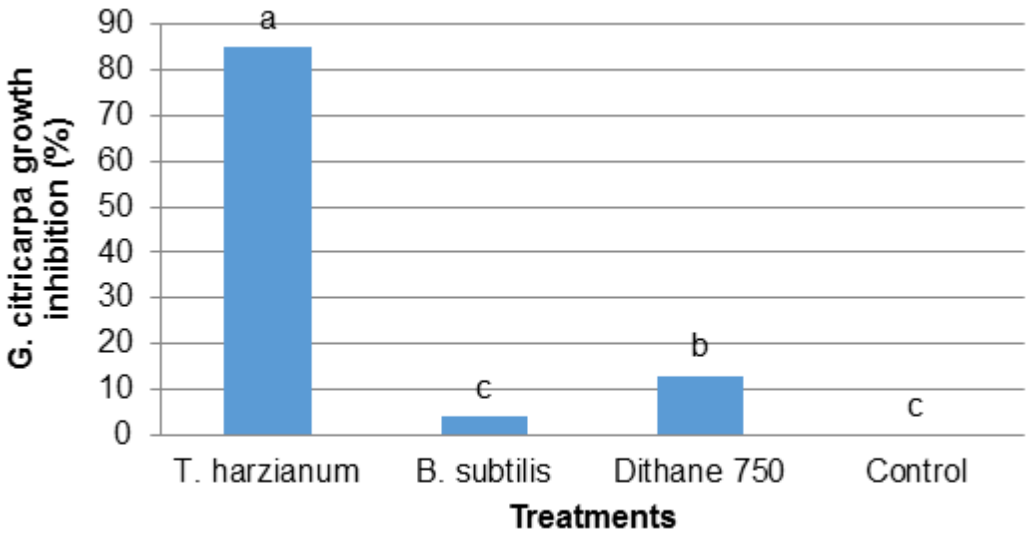

Figure 1. Inhibitory effect of bio-control agents and fungicide on *P. citricarpa* after 30 days of inoculation.

Table 1. Effect of *T. harzianum*, *B. subtilis* and Dithane 750 on CBS development on artificially inoculated citrus fruit.

	Pathogen lesion size (mm)[a]			
Treatments	**1**	**7**	**14**	**21**
T. harzianum	0.0[ab]	0.0[a]	0.0[a]	0.2[a]
B. subtilis	0.0[a]	0.3[a]	0.8[ab]	1.3[b]
Dithane 750	0.0[a]	0.2[a]	1.1[b]	1.2[b]
Inoculated control	0.0[a]	1.2[b]	2.8[c]	2.6[c]
Un-inoculated control	0.0[a]	0.0[a]	0.0[a]	0.0[a]

[a]Lesion size on inoculated fruit was measured from days 1 to 21. [b]Numbers followed by the same letters are not significantly different according to Duncan's Multiple Range Test.

The table is not correct. The treatments comparison is wrong because there are a lot of variations (culture medium, organisms, fungicide, seeding of the organism, etc.). The treatments can be compared with its control, but not with each other Dual culture test results which showed highest pathogen growth inhibition occurring in *T. harzianum* treatments at 85%. P. *citricarpa* growth inhibition was lowest in *B. subtilis* treatment at 4%. The same results regarding the suppressive effect of *B. subtilis* was also observed in inoculated fruits (Table 1), where lesion size was significantly higher than *T. harzianum* treatments at 1.3 and 0.2 mm, respectively. However, the level of inhibition was not significantly different in comparison with Dithane 750. Dithane 750 is currently one of the fungicides used in the management of Citrus black spot (CBS), however loss of pathogen sensitivity is a concern (Kupper et al., 2011).

Various reports have shown positive efficiency of *T. harzianum* in the management of other plant diseases (Thilagavathi et al., 2007; Begum et al., 2010). The same results was also observed in our study where *T. harzianum* was able to significantly suppress the growth *P. citricarpa in vitro* and also reduce disease severity in artificially infected citrus fruits (Table 2). Treatment of infected fruits with *B.subtilis* also resulted in a significantly high number of fruits, showing disease symptoms and

severity (1.8).

However, when *B. subtilis* was applied in combination with *T. harzianum*, a significant reduction in disease severity was observed. In previous reports, application of strain mixture of *B. subtilis*, *Pseudomonas* and *Trichoderma* spp. resulted in an increased suppression of *B. cinerea* than when each biocontrol agent was applied alone (Thilagavathi et al., 2007). It has been suggested by various authors that when applying biocontrol agents as combination in disease management, they should be compatible to achieve consistent control (Raaijmakers et al., 1996).

Table 2. Effects of *T. harzianum* and *B. subtilis* applied separately or in combination on CBS occurrence and severity.

Treatments	CBS infected fruits (%)	CBS severity[a]
T. harzianum	10.5±0.2	1.0[bb]
B. subtilis	16.3±1.3	1.8[c]
combined	9.6±1.2	0.2[a]
control	50.5±1.3	5.3[d]

[a]Disease severity was rated on a scale of 0 to 4. [b]Numbers within a same column followed by the same letter are not significantly different according duncan's multiple range tests.

In our study, results show that treatment of CBS infected fruits with a combination of both *T. harzianum* and *B. subtilis* formulations significantly reduced number of citrus, showing CBS symptoms which also reduced disease severity (Table 2). Citrus fruit treatment with both *T. harzianum* and *B. subtilis* significantly reduced the number of diseased plants and suppressed disease symptom appearance. This combined effect corresponds with previous findings where combined application of *B. subtilis* and yeast isolates reduced gray mould in treated apple fruits (Zangoei et al., 2014). Considering the good performance of both biocontrol when used in combination, it is anticipated that their use against citrus black spot could be beneficial when applied as a post-harvest treatment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abdalla SA, Algam SAA, Ibrahim EA, El Naim AM (2014). *In vitro* screening of Bacillus isolates for biological control of early blight disease of tomato in shambat soil. World J. Agric. Res. 2(2):47-50.

Araújo WL, Maccheroni W J, Aguilar-Vildoso CI, Barroso PAV, Saridakis HO Azevedo JL (2001). Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can. J. Microbiol. 47:229-236.

Baldassari RB, Reis RF, de Goes A (2009). A new method of inoculation of fruit with *Guignardia citricarpa*, the causal agent of citrus black spot. Eur. J. Plant Pathol. 123:1-4.

Begum MF, Rahman MA, Alam MF (2010). Biological control of Alternaria fruit rot of chilli by *Trichoderma* species under field conditions. Mycobiology 38 (2):113-117.

Etebarian HR, Sholberg PL, Eastwell KC, Sayler RJ (2005). Biological control of apple blue mould with *Pseudomonas fluorescens*. Microbiol. 51:591-598.

Evans HC, Holmes KA, Thomas SE (2003). Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri* in Equador and preliminary assessment of their potential biocontrol agents of cocoa diseases. Mycol. Prog. 2:149-160.

Everett KR, Rees-George J (2006). Reclassification of an isolate of *Guignardia citricarpa* from New Zealand as *Guignardia mangiferae* by sequence analysis. Plant Pathol. 55:194-199.

Kotźe JM (1981). Epidemiology and control of citrus black spot in South Africa. Plant Dis. 65:945-950

Kupper KS, Correa EB, Moretto C, Goes A (2011). Control of *Guignardia citricarpa* by *Bacillus subtilis* and *Trichoderma* spp. Rev. Bras. de Fruitic. 33 (4):1111-1118.

Meyer L, Jacobs R, Kotzè JM, Truter M, Korsten L (2012). Detection and molecular identification protocols for *Phyllosticta citricarpa* from citrus matter. South Afr. J. Sci. 108(3-4):53-59.

Paul I, Van Jaarsveld AS, Korsten L, Hattingh V (2005). The potential global geographical distribution of citrus black spot pathogen *Guignardia citricarpa* (Kiely): Likelihood of disease establishment in the European Union. Crop Prot. 24:297-308.

Smith IM, McNamara DG, Scott PR, Holderness M (1997). Quarantine pests of Europe. CAB International, Wallingford.

Raaijmakers JM, Leeman M, Van Oorschot MMP, van der Sluis I, Schippers B, Bakkers PAHM (1996). Dose response relationships in biological control of Fusarium wilt of radish by *Pseudomonas* spp. Phytopathology 85:1075-1081.

Rodrigues MBC, Andreote FD, Spósito MB, Aguillar-Vildoso CI, Araújo WL, Pzzirani-Kleiner AA (2007). Resistance to benzimidazole by *Guignardia citricarpa*. Braz. Agric. Res. 42:323-327.

Schutte GC, Mansfield RI, Smith H, Beeton KV (2003). Application of Azoxystrobin for control of benomyl-resistant Guignardia citricarpa on Valencia oranges in South Africa. Plant Dis. 87:784-788.

Thilagavathi R, Saravanakumar S, Raupathi N, Samiyappan R (2007). A combination of biocontrol agents improves the management of dry root rot (*Macrophomina phaseolina*) in greengram. Phytopathology 46:157-167.

Zangoei E, Etebarian HR, Sahebani N (2014). Biological control of apple gray mould by mixtures of *Bacillus subtilis* and yeast isolates. Afr. J. Food Sci. 8(3):155-163.