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Research Paper

Biocontrol of *Fusarium mangiferae* responsible for mango malformation using bacterial isolates

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ABSTRACT

Fusarium species are known to be responsible for mango malformation, a serious pre-harvest disease causing major yield and financial losses to the industry worldwide. This study confirmed the identity and pathogenicity of the species associated with the disease in the northern provinces of South Africa. Thirty-five fungal isolates were obtained from infected orchards, of which 32 were identified as the malformation pathogen *Fusarium mangiferae*. Seventy-seven bacterial isolates, obtained from mango orchards as well as unrelated environments, were screened for their antifungal properties and the modes of action of the best isolates against *Fusarium* were established. The study demonstrated that only five bacterial isolates (*Alcagenes faecalis*) were able to significantly inhibit the growth of the pathogen. The modes of action of the bacteria were a combination of competition for space and secondary metabolites produced by the bacteria, which included volatiles, phenolic compounds and siderophores. An *in vivo* trial was performed to determine if exposure to the pathogen and antagonist could induce resistance by increasing secondary metabolites and specifically mangiferin. Chemometric analysis of the data obtained from the *in vivo* trial revealed that the total soluble secondary metabolite concentrations after application of the selected biocontrol agents or pathogen inoculation, were lower when compared to the control group.

1. Introduction

Mango Malformation Disease (MMD) is one of the most threatening pre-harvest diseases of mango, causing major yield and financial losses to the industry worldwide. The spread of the disease is a major concern for South African producers (Schoeman et al., 2015). Although several *Fusarium* species have been associated with MMD, including *F. mangiferae, F. sterilihyphosum, F. proliferatum, F. tupiense* and *F. mexicanum,* only *Fusarium mangiferae* and *F. sterilihyphosum* have been isolated from malformed tissue in South Africa (Britz et al., 2002; Freeman et al., 1999, 2014; Liew et al., 2016; Marasas et al., 2006). Koch's postulates were upheld for *F. sterilihyphosum* isolated from diseased plants in Brazil (Lima et al., 2009), however, no results are currently available for South African mango trees artificially inoculated with genetically identified isolates. Thus, it is imperative to confirm, *via* Koch's postulates, the pathogenicity of isolates isolated from malformed tissue from South Africa. To date, management of the disease mainly involves sanitation and the continuous application of synthetic fungicides (Soto-Plancarte et al., 2015). Such fungicides are regarded as harmful to the environment and their persistent use may induce fungal resistance. Therefore, the discovery of alternative pathogen control methods has become a priority (Dröby et al., 2009; Sharma et al., 2009).

In the past two decades, biocontrol agents have emerged as potential, effective strategies for disease control of several crops (Bale et al., 2008). The market for microbial inoculants around the globe is increasing at an annual rate of approximately 10% (Berg, 2009) and may provide long-term eco-friendly protection to crops (Lo, 1998). Commercially available products are applied pre- and postharvest for a large range of fruits including citrus, grapes and peaches (Calvo-Garrido et al., 2014). In South Africa, Avogreen™ and Mangogreen™ have been developed, but their use has been limited, due to inconsistent efficacy under commercial conditions (Demoz and Korsten, 2006). Although many biocontrol agents are used as growth promoters, to the best of our knowledge, none have been successfully tested against mango flower malformation. A bio-fertilizer produced by REAL IPM, known as REAL Bacillus TFP® contains *Bacillus subtilis.* The product can be applied to a wide-range of indoor and outdoor crops to promote the growth of plants. A preliminary trial on mango trees performed by the supplier resulted in an increased mangiferin production by the trees following the application of the product.

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Several studies have reported the confinement of malformation to the inflorescence and vegetative growing regions of the plant is the direct result of increased mangiferin levels, in response to infection by the pathogen (Chakrabarti and Ghosal, 1989; Kumar et al., 2011). For these reasons, the effect of the application of the South African product, REAL Bacillus TFP® on mangiferin levels and the resulting reduction in malformation, has been included in the current study.

The first step towards the development of effective biological control of a pathogen is the identification of antagonistic organisms. Regnier et al. (2010) previously reported that bacterial isolates isolated from unconventional sources (mine effluents in this case) displayed promising antifungal activities against certain postharvest pathogens of fruit. In our study, isolates obtained from the orchard as well as isolates previously isolated from mine tailing effluents, were screened for the control of MMD.

To fully understand the efficacy of biocontrol agents, a thorough study of the internal dynamics of the interaction between the antagonist, pathogen and host must be conducted. Successful biocontrol agents generally act *via* more than one mode of action that work in combination to inhibit the growth and spread of the pathogen (Govender et al., 2005). Competition for space and nutrients, cell wall-lytic enzymes, siderophores, phenolic compounds and antibiotics are all possible modes of action of microorganisms (Sparado and Droby, 2016). Plants synthesize many different secondary metabolites as a defence response to biotic and abiotic stressors and induction of host resistance might increase compounds that play a role in the control of the pathogen (Nielson et al., 2013).

The present study focused firstly on the confirmation of the identity of the *Fusarium* species responsible for mango malformation disease in northern South Africa, secondly, screen microorganisms isolated from different environments for their ability to control this pathogen, and finally to determine the putative modes of action of these microorganisms, including their ability to induce the production of defence metabolites by the host.

2. Materials and methods

2.1. Isolation and identification of fungal isolates

For this study, an infected 'Sensation' orchard at the Agricultural Research Council-Institute for Tropical and Subtropical Crops research farm in Nelspruit (Mpumalanga, South Africa) and a 'Kent' orchard at Bavaria Estate in Hoedspruit (Limpopo, South Africa) were sampled. All fungal cultures used in this study were obtained from Ms Schoeman [Agricultural Research Council-Institute for Tropical and Subtropical Crops (ITSC), Nelspruit, Mpumalanga Province, South Africa]. The pathogens were isolated from malformed panicles, leaves and apical buds that were randomly sampled twice a month during six consecutive months (July–December) over the 2013 and 2014 flowering seasons The collected samples were cultured on Potato Dextrose Agar and incubated at 28 °C ± 2 °C for 6 days. Pure cultures were obtained using single spore and hyphal tip isolation techniques. Identification was based on the morphological characteristics of single-spore isolates cultured on carnation leaf agar as described by Leslie and Summerell (2006). Macro- and microconidial size, shape and number septa, as well as presence or absence of chlamydospores were used to identify the isolates. Fungal isolates were identified genetically using a modified method of Kvas et al. (2008) and Nik et al. (2013), by analyzing the partial gene sequences of translation elongation factor 1α. Briefly, the fungal isolates were cultured on Nash and Snyder semi-selective medium by placing a plug in the centre of the plate. The plates were then incubated at 27 °C for two weeks. Approximately 100 mg of mycelium was used to extract genomic deoxyribonucleic acid (DNA) from pure cultures of each individual fungal strain using a ZR Fungal/Bacterial DNA MiniPrep™ extraction kit (Zymo Research, Irvine, California, USA). Gel electrophoresis was performed to confirm DNA isolation. For polymerase chain reaction (PCR) amplification, a set of primers EF1 + EF2 [5′- ATGGGTAAGGAGGACAAGAC-3′ and 5′-GGAAGTACC AGTGATCATGTT-3′, respectively] previously used by Kvas et al. (2008) were purchased from Inqaba Biotec (Pretoria, South Africa). The PCR conditions, as described by Nik et al. (2013), were used for amplification as follows: 94 °C for 1 min, followed by 34 cycles of 94 °C for 30 s., 61 °C for 45 s, and 72 °C for 1 min. To prevent the amplification products from ligating (5′ end attaching to 3′ end), the phosphates on the 5′end were removed with ExoSAP (Zymo Research, USA) in order to keep the DNA molecules linear for sequencing. The DNA sequencing was done by Inqaba Biotec using a Hitachi 3500xL genetic analyzer sequencer (Tokyo, Japan). Once the DNA sequences were received, the electropherograms were visualized and corrected where necessary to confirm the identity of the individual nucleotides, using Chromas and BioEdit molecular software. The identities of the isolates were determined by searching known sequences in GenBank using the Blast search of the National Centre for Biotechnology Information (NCBI).

2.2. Screening of bacteria for inhibition of Fusarium mangiferae

Bacterial isolates (77), from agricultural soils (31), gold mine tailings dam (21) in Mpumalanga Province (South Africa) and from an organic mango orchard (25) in Hoedspruit (Limpopo Province, South Africa), were isolated using SCN-containing media and screened for inhibitory activity against seven genetically identified *F. mangiferae* isolates selected on their growth rate. Fresh pure bacterial isolates were obtained by reviving isolates from the culture collection (Department of Biotechnology and Food technology, Tshwane University of |technology, Pretoria, South Africa). Selected bacterial isolates were identified using 16SRNA analysis. The genomic DNA was extracted using the QIAmp DNA Mini kit (Southern Cross Biotechnology, Claremont, Western Cape, South Africa) according to the manufacturer's specifications. The 16S rRNA genes were amplified using PCR and the primer set 1387R (5′-GGGCGGAGTGTACAAGGC-3′) and 63F (5′-CAGGCCTA ACACATGCAAGTC-3′). The PCR products were purified using a QIAquick Purification Kit (Southern Cross Biotechnology, Claremont, Western Cape, South Africa), sequenced by Inqaba Biotec (Pretoria, South Africa) and analyzed by Finch TV (Geospiza). The identities of the isolates were determined by searching known sequences in GenBank using the Blast search of the National Centre for Biotechnology Information (NCBI).

The inhibitory action of all the screened bacterial isolates were compared to that of the commercial product REAL Bacillus TFP® (Real IPM, South Africa (Pty) Ltd.). The 77 bacterial isolates plus the commercial product were screened for inhibitory activity against the seven identified *Fusarium mangiferae* isolates. A loop of each bacterial isolate was streaked (2.5 cm streaks) in a triangular configuration 2 cm from the centre of the plate. A *Fusarium* spore suspension (10 uL) was then inoculated into the middle of the plate. The control plates consisted of just the fungal isolate. The bacterial isolate was replaced with sterile water. The selected seven *Fusarium mangiferae* isolates suspensions were used and the experiment was done in triplicate. The plates were incubated at 30 °C for seven days and the diameter (vertical and horizontal) of the fungal growth measured using a caliper (Absolute Digimatic-Mitutoyo Corporation, Japan). The average mycelial growth of all seven fungal isolates tested per antagonist, was compared to the control to establish an inhibitory measurement. Data were expressed as percentage inhibition of mycelial growth as described by Plaza et al. (2004).

2.3. Modes of action of the five most effective antagonists

2.3.1. Competition for space

The dot dual culture assay and growth curves were used to evaluate competition for space. In the dot dual culture assay a loop of each bacterial isolate was streaked (2.5 cm streaks) in a triangular