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Assessment of the antimicrobial activity of *Lentinula edodes* against *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

Bacterial spot of tomato (*Solanum lycopersicum*) caused by *Xanthomonas campestris* pv. *vesicatoria* (Xcv) is a devastating disease of tomato world-wide. In the southeastern United States, high summer temperatures and humidity are ideal conditions for this disease, resulting in defoliation, fruit spotting and a significant reduction in fruit yield. Current organic production practices restrict the use of synthetic chemicals for disease control; hence, there is a need for the development of new and effective biopesticides to mitigate plant diseases. Among several biological agents with potential for disease control, *Lentinula edodes* (shiitake mushroom) has been shown to have antibacterial properties. A controlled-environment study was therefore conducted to validate *L. edodes* mycelia culture filtrate (Le_{mcf}) foliar application to control bacterial spot of tomato using the cultivar Agriset 761. *Lentinula edodes* mycelia culture filtrate foliar spray significantly suppressed bacterial spot incidence in tomato foliage *in vitro* but was not effective *in vivo*. The phytotoxicity symptoms in Le_{mcf}-treated tomato foliage were attributed to the presence of 422.78 μg of oxalic acid per milliliter of Le_{mcf} (quantified by high-pressure liquid chromatography). Plant height and flowering were normal in Le_{mcf}-treated plants. Additionally, Le_{mcf} seed treatment did not adversely impact tomato germination but significantly enhanced the germination of marginal tomato seeds subjected to biotic stress (Xcv). Our results suggest that after eliminating oxalic acid from Le_{mcf}, the product may be a potential biopesticide for managing bacterial spot of tomato. Future greenhouse or field experiments should be conducted after eliminating oxalic acid from *L. edodes* culture filtrates.

1. Introduction

The demand for and sale of organic food commodities have increased approximately twenty-fold in recent years, due partially to a significantly decreased or no burden of synthetic chemical residues in organic crops compared to conventionally grown crops (Baker et al., 2002). Of organically produced vegetables, one of the most widely produced is tomato (*Solanum lycopersicum* L.) (Dimitri and Oberholtzer, 2009). Bacterial spot of tomato results in significant economic losses and is considered a worldwide challenge in tomato production (Potnis et al., 2015; Ritchie, 2007). Bacterial leaf spot (BLS) is caused by four different bacteria, *Xanthomonas euvesicatoria* (*X. campestris pv. vesicatoria*), *X. vesicatoria*, *X. perforans* and *X. gardneri*, collectively known as xanthomonads (Potnis et al., 2015; Ritchie, 2007). Xanthomonads infect tomato world-wide over wide range of temperatures (Potnis et al., 2015). Disease symptoms appear on leaves and fruits and decrease the total and marketable fruit yield up to 50% (Abbasi and Weselowski, 2015). In organic tomato production, BLS is managed by crop rotations, certified disease-free seeds and seedlings, the proper disposal of infected plant debris, multiple applications of copper bactericides and biological control (Baysal et al., 2007; Strange and Scott, 2005; Potnis et al., 2015). Out of 174 biopesticide ingredients registered with the United States Environmental Protection Agency (USEPA, 2016), only one (bacteriophages) is registered as a standard control against BLS. However, phage efficacy against BLS is limited by temperature, UV irradiation, desiccation and copper bactericide exposure (Iriarte et al., 2007). Bacterial spp.; *Bacillus*, *Pseudomonas*

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and fungal spp.; and *Trichoderma* and *Crinipellis perniciosa* have been tested against BLS under field conditions and reported to be effective (Fontenelle et al., 2011; Ji et al., 2006; Udayashankar et al., 2011; Cavalcanti et al., 2006, 2007). However, most biocontrol agents are effective under a limited range of environmental and soil conditions (García et al., 2003; Huang et al., 2000; Iriarte et al., 2007). Copper-resistant xanthomonads, developed due to the excessive use of chemical control measures, have been reported worldwide since 1960 (Abbasi et al., 2014; Potnis et al., 2015). The effectiveness of copper-based bactericides against BLS is further reduced when weather conditions are conducive for the development and spread of this disease, due to the novel xanthomonad virulence factors, which enable them to infect tomato over a wide range of temperatures (Potnis et al., 2015). Alternative strategies to overcome resistance in *Xanthomonas* spp., i.e., copper bactericides combined with biofungicides were found to result in moderate success against BLS (Abbasi and Weselowski, 2015). Therefore, BLS management in organic tomato production is a major challenge, and conventional disease control measures in organic tomato production need to be combined or replaced with more effective natural compounds to circumvent bacterial resistance to bactericides.

Mushrooms have been known to have antibacterial properties and are considered one of the most reliable sources for future novel compounds (Barseghyan et al., 2015). *Lentinula edodes* (Shiitake mushroom) an edible, medicinal fungus of the *Basidiomycetes* family, is used as functional food and is well known for its antibacterial properties (Giavasis, 2014). Various antibacterial compounds, including lentinan, lenthionine, eritadenine, protocatechuic acid, p-hydroxybenzoic acid and oxalic acid, have been isolated from *L. edodes* fruiting bodies (Bender et al., 2003; Enman et al., 2008; Reis et al., 2012; Hatvani, 2001; Bisen et al., 2010). Ergosterol, flavonoids, oxalic acid, and lenthionine in *L. edodes* have been identified as active metabolites against bacterial pathogens; *Micrococcus luteus*, *Bacillus cereus* and *B. megaterium*, *Streptococcus pyogenes* and *Staphylococcus aureus*, respectively (Bender et al., 2003; Hatvani, 2001; Kitzberger et al., 2007). Eritadenine (Enman et al., 2008), lentinan, oxalic acid and sugars have been identified in *L. edodes* mycelia culture filtrate (Le_{mcf}). Pacumbaba et al. (1999) reported the *in vitro* inhibition of the phytopathogenic bacteria *Xanthomonas*, *Erwinia amylovora*, *Ralstonia solanacearum* and *Pseudomonas syringae* by *L. edodes* mycelia leachates.

Lentinula edodes culture filtrates enhanced the efficacy of the biocontrol yeasts *Cryptococcus laurentii* and *Pichia membranifaciens* against *Penicillium expansum* (blue mold pathogen in post-harvest apples) in apple fruits and induced resistance in the host (Tolaini et al., 2010; Wang et al., 2013). *Lentinula edodes* induced resistance against *Clavibacter michiganensis* subsp. *Michiganensis* in tomato (Silva et al., 2013). However, little work has been done to evaluate *L. edodes* as a source of biopesticide. In this study, we evaluated the efficacy of Le_{mcf} against BLS *in vitro* and *in vivo*, assessed the effect of Le_{mcf} on tomato plant growth and flowering, and determined the effect of Le_{mcf} on seedling emergence in biotic and physiologically stressed tomato seeds.

2. Materials and methods

2.1. Chemicals, microorganisms, and tomato cultivar

All of the chemicals were of analytical grade and were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). *Lentinula edodes* strain no. 38196 from American Type Culture Collection (ATCC, Rockville, MD, USA) was used for this study. *Xanthomonas campestris* pv. *vesicatoria* was isolated locally from tomato and maintained in pure culture on nutrient agar at −20 °C. All of the plant assays were performed on tomato cultivar Agriset 761.

2.2. Lentinula edodes mycelial culture filtrate (Le_{mcf}) collection

Lentinula edodes mycelia were grown for two weeks on agar medium as described by Pacumbaba et al. (1999). Axenic portions of agar blocks (approximately 5 mm^3) with two-week-old mycelia were aseptically transferred to 100 ml of liquid malt and yeast (MY) medium (20 g of malt extract, 2.0 g of yeast extract, and 20 g of sucrose, pH 5.33) in 250 ml Erlenmeyer flasks and fermented (25, 250 rpm) for 30 d in three biological replicates. The blank was MY medium without *L. edodes* mycelia. After fermentation, the contents of the flasks were filtered through sterile Whatman no. 42 filter papers, and the mycelia were discarded. *Lentinula edodes* mycelia culture filtrate (Le_{mcf}) was filter-sterilized by passing through 0.22 μm sterile syringe filters and stored at −80 °C.

2.3. Lentinula edodes mycelia culture filtrate (Le_{mcf}) activity against X. campestris pv. vesicatoria (Xcv) in vitro

The *in vitro* antibacterial activity of Le_{mcf} was determined by modifying a protocol developed by Wiegand et al. (2008). Briefly, Xcv cells were isolated in pure culture by streaking on nutrient agar plates. Loopfuls of bacterial cells from freshly grown Xcv colonies were suspended in 10 ml of Muller Hinton broth in capped 15 ml glass tubes in three replicates. The inoculated glass tubes were incubated (32 °C, 12 h), and the Xcv population was estimated by reading the optical density at 625 nm (OD_{625}) on a spectrophotometer (Biotek, Winooski, VT, USA) and confirmed by plating serial dilutions. After adjusting to an approximate concentration of 10^8 cfu ml^{-1} in MH broth, 100 μl of Xcv cell suspension was treated with the same volume of Le_{mcf} in a 96-well microtiter plate. The bactericidal activity of Le_{mcf} *in vitro* was compared to that of the positive control, 100 ppm of streptomycin sulfate and untreated control, i.e., Xcv cell suspension. For sterility control, 200 μl of saline (0.9% sodium chloride, w/v) was added to three wells, and the 96-well plate was incubated at 32 °C for 16 h. Then, 100 μl from all treatments and control wells were plated on agar petri plates (10 g of bacteriological agar, 0.1 g of thymine, 4.5 g of sodium chloride, and 9 g of tryptone per 900 ml of distilled-deionized water). The Petri plates were incubated at 32 °C for 24 h, and the colony-forming units (cfu) were counted. The Xcv inhibition percentage was calculated by the following formula:

$$Inhibition\ (\%) = (1 - O/C) \times 100 \quad (1)$$

C is the number of cfu in the untreated control (overnight bacterial culture), and O is the number of cfu in the Le_{mcf}-treated sample. The experiment was repeated three times with three biological replicates (BR) in each treatment group and control.

2.4. Efficacy of L. edodes mycelia culture filtrate (Le_{mcf}) against bacterial leaf spot (BLS) in vitro

This bioassay was conducted to evaluate the efficacy of Le_{mcf} against BLS in artificially inoculated *in vitro* tomatoes compared to streptomycin sulfate (100 ppm, used as the positive control for the antimicrobial agent). *In vitro* tomato plants were grown by germinating surface-sterilized tomato seeds (30% Clorox, drop of Tween-20, submerged for 20 min, rinsed thrice with distilled water, and blotted dry between sterile Whatman filter paper layers) on 50 ml of Murashige and Skoog (MS) medium (10 g of agar, 2.2 g of MS basal medium, 1000 ml of water, 121 °C, 15 psi, 15 min) in