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Fungicide spray programs to manage downy mildew (dryberry) of blackberry caused by *Peronospora sparsa*

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ABSTRACT

Downy mildew (dryberry) of blackberry, caused by *Peronospora sparsa*, is a major disease of blackberry in Michoacán, México. The objective of this research was to evaluate different fungicide spray programs to manage systemic infections of downy mildew and dryberry disease in two different regions of Michoacan, Mexico in the 2007 and 2008 growing seasons. The presence of asymptomatic infections of *P. sparsa* in experimental plots was confirmed by nested PCR using published primers amplifying a fragment of 477 bp of the ITS region of ribosomal DNA. In 2007, the experiment was conducted in an 8-year-old commercial planting in Tangancicuaro. Fungicide applications began early in the growing season approximately 10 days after bud brake (July 10). In 2008, the experiment was conducted at the same location, but treatments began later (August 4) approximately 21 days after bud break at which time plants had fully expanded leaves. An additional plot was established in Atapan in 2008. Seven fungicide programs using commercial formulations and recommended rates of mefenoxam, potassium phosphite, azoxystrobin, captan, mancozeb, copper sulfate, *Bacillus subtilis*, and a sanitizing agent were tested at both locations. Results from both years and locations indicated that all programs beginning with potassium phosphite (3–4 applications) on a 10–14 day schedule after bud brake, significantly reduced the severity and incidence of downy mildew on leaves, and dryberry on fruits compared to the untreated control, regardless of the fungicide (either biological or chemical) that was applied later in the season. Programs that included 1 or 2 applications of mefenoxam, the first application in drench, early in the season also provided good control. Fungicide programs that were initiated with protectant fungicides or used protectant materials only did not provide a satisfactory level of control. Early detection of systemic infections of *P. sparsa* through the use of PCR and the use of fungicide spray programs based on early season applications of systemic fungicides such as potassium phosphite and mefenoxam provided good to excellent control of foliar downy mildew and dryberry of blackberry in Michoacán, México.

1. Introduction

Blackberry production in Mexico is concentrated primarily in the central part of the country in the state of Michoacán. Most of the blackberry production is based on the cultivar Tupi (thorny blackberry). Although some other cultivars have been evaluated in the past, "Tupi" is the most commonly planted cultivar because it is very well adapted to the subtropical climate of the region. In addition, "Tupi" has desirable characteristic for both fresh and processing markets. The production system applied this subtropical region requires chemical defoliation of plants by spraying a mixture of emulsifiable oil (3%), ammonium sulfate (20%), urea (3%) and copper sulfate (2%) at about 5–6 months after primocane emergence. Following chemical defoliation, growth regulators such as cytokinins and gibberellins are sprayed in order to promote bud brake. Mature fruit are harvested approximately 90–100 days after defoliation (Strike et al., 2008). Currently, very little information related to the management of foliar downy mildew and dryberry disease of fruit is available under this production system.

Downy mildew and dryberry disease is one of the most important diseases of blackberry in Mexico. Foliar symptoms of downy mildew appear as purple angular leaf spots usually located between veins along the mid-rib and major veins. A reddish

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discoloration is also observed on one side of the blossom pedicel. Infected young fruit lose their shine, become shriveled and eventually dry down (Rebollar-Alviter et al., 2009). One of the most typical symptoms of dryberry disease is the presence of split green or mature fruit. Although symptoms on leaves are relatively easy to detect, the lack of foliar symptoms does not insure the absence of dryberry disease. Field observations have shown that plants without foliar symptoms (asymptomatic) can have an incidence of dryberry as high as 70% (Tate, 1981; McKeown, 1988; Bresse et al., 1994; Lindqvist et al., 1998; Nordskog et al., 2003).

Peronospora sparsa Berk. was first described in England in 1862 (Francis, 1981). This pathogen has been reported to cause severe losses in boysenberry in New Zealand (Tate, 1981), and tummelberry in the United Kingdom (McKeown, 1988), arctic bramble in Finland (Lindqvist et al., 1998) and blackberry in California (Gubler, pers. communication). Downy mildew in Mexico caused severe losses in commercial plantings of blackberries cv Tupi and Brazos in the state of Michoacán (Rebollar-Alviter et al., 2009). Since foliar symptoms are not always present on *Rubus* spp. and visual inspection is not a reliable method to estimate the extent of the asymptomatic infections, PCR and real time PCR have been successfully applied to detect the pathogen (Lindqvist et al., 1998; Koponen et al., 2000; Hukkanen et al., 2006, 2008).

Studies conducted in New Zealand on Boysenberry demonstrated that the disease could be successfully controlled with the use metalaxyl and mancozeb (Tate, 1981, 1983). On *Rubus fruticosus* and *Rosa* spp. the disease was controlled with a mixture of Cimoxanil + mancozeb + oxadixyl, fluazinam and Fosetyl-aluminum (O'Neil et al., 2002). Under low disease pressure, 2 applications of Metalaxyl-M, 3 of phosphorous acid and 3 of azoxystrobin plus 1 of dichlofluanid applied 21 days before flowering significantly reduced fruit losses due to downy mildew on boysenberry. In contrast, under high disease pressure, only phosphorous acid (3 sprays) gave acceptable disease control (Walter et al., 2004). In Finland, in a greenhouse study potassium phosphite (phosphorous acid) resulted in only moderate control of *P. sparsa* on artic bramble when applied 4 days before inoculation during flowering compared with other agrochemicals with different modes of action such as plant activators (Hukkanen et al., 2008).

In Mexico, most of blackberry production is exported to the USA, and very few fungicides are registered for managing the disease. Currently registered materials include phosphorous acid-derived fungicides and some strobilurin fungicides. In order to extend the efficacy of the currently available fungicides, effective fungicide programs need to be developed considering the biochemical and physical mode of action of the materials being used and their risk for fungicide resistance development. In addition, the biology and sources of primary inoculum of the disease needs to be considered. The objective of this research was to evaluate different fungicide programs to manage foliar downy mildew and dryberry of blackberry under the forced production system in the state of Michoacán, Mexico.

2. Materials and methods

2.1. Molecular detection of downy mildew

Total DNA was extracted from symptomatic fruits, symptomatic and asymptomatic leaves collected from test plots at Atapan (Los Reyes Municipality) and Tangancicuaro, using the methodology cited by Bainbridge et al. (1990). Ten plants per plot and 10 symptomatic and asymptomatic leaves and fruits were selected in each test plot during the previous season of establishing the experiments. To avoid plant phenols effects on the PCR reaction 0.5 g of polyvinyl pirrolidone (PVP) was added to 500 μL lyses solution while grinding plant tissue for DNA extraction.

PCR protocols published by Lindqvist et al. (1998) and Aegerter et al. (2002) were used to amplify the Internal Transcriber Spacer (ITS) region of rDNA operon. PCR products with some modifications were run on each case. In the first case to detect *P. sparsa* a master mix was done using Lindqvist protocol: 0.1 mM of dNTP, 0.25 μM of primer sets PR3 (5′-GGCTGGCTGCTACTGGGCA-3) and PR4 (5′-GCCGACTGGCCACGCGGA-3′), 1U of *Taq* DNA polimerasa (Promega, USA), 1.5 mM de $MgCl_2$, 1× Buffer, and 1 μl of total DNA. PCR amplifications were performed with an initial denaturing process held at 94 °C for 1 min; then 35 denaturing cycles at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 45 s with a final extension at 72 °C for 3 min.

For the protocol of Aegerter et al. (2002), the reaction mixture consisted of 0.2 mM of dNTP's, 0.1 μM of a forward primer PS3 (5′-ATTTTGTGCTGGCTGGC-3′) and a reverse primer PS1 (5′-TGCCA-CACGACCGAAGC-3′), 2 units of *Taq* DNA polimerasa (Promega, USA), 2.5 mM of $MgCl_2$, 1× Buffer, 0.5% of milk, and 1 μl total DNA. Thermocycle program consisted of 94 °C for 5 min for initial denaturing followed by 40 cycles at 94 °C for 2 min, 55 °C for 2 min, 72 °C for 2.5 min and the finally 10 min a 72 °C as a final extension.

In order to improve the sensitivity on symptomatic and asymptomatic leaves, a nested PCR was run using Aegerter et al. (2002) protocols, and PS3/PS1 primers were used for the first amplification, expecting a fragment of approximately 660 bp. From this amplification 1 μl was used as template and PR3/PR4 primers (Lindqvist et al., 1998) were considered for two nested PCR, to amplify a fragment of 477 bp. Also, a dilution of 1:30 of the PCR product obtained in the first amplification was used for nested PCR.

All PCR amplifications were carried out in a Peltier Thermal Cycler PTC-200 (BIORAD, Mexico); the PCR products were verified by loading 5 μl on a 1.2% agarose electrophoresis gel. The gel was stained with ethidium bromide (3 mg/l) and the bands were visualized under a Gel Doc 2000 UV transilluminator (BIORAD, USA). The remaining PCR amplified products were purified with the QIAquick PCR purification kit (Qiagen, USA), following the manufacturer instructions. These PCR-products were sequenced in both directions in an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) to ensure there were no misreadings.

Sequences corresponding to both strands were assembled and edited using BioEdit software version 7.0.5 (Hall, 1999) and a consensus sequence of each sample was created. Since the basic local alignment search tool (BLAST) from the NCBI find regions of local similarity between sequences with significant alignments, consensus sequences were submitted to BLASTN 2.2.19. Sequences obtained were deposited in GenBank-NCBI.

2.2. Field experiments

During the 2007 growing season, the experiment was established in an 8-yr-old commercial planting of the cultivar Tupi with 2 m between rows and 40 cm between plants in Tangancicuaro, Michoacán. Chemical defoliation of plants consisted of an application of 20% ammonium sulfate, 3% emulsifiable oil and 2% copper sulfate and 3% urea, sprayed to run-off the third week of June. Five days later, plants were "activated" with a solution of 50 ppm of Thidiazuron (cytokinin effect) and 7 days after this treatment a second application of thidiazuron at 25 ppm plus gibberellic acid at a rate of 25 ppm was sprayed to the plants. Fungicide applications were initiated approximately 10 days after bud brake (after plants were activated) when shoots were 5–15 cm-long (First week of July). In 2008, the experiment was conducted in the same location. This year treatment applications began approximately