In vitro identification and control of *Pestalotiopsis longisetula* fungus, pathogens strawberry crop

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Abstract

Over the past seven years, pestalotiopsis has been the main strawberry disease found all over the crops located in the south of Minas Gerais State, Brazil. The leaves of the plants were severely attacked by an uncommon fungus in the regional crops, mainly in 2005 and 2006. The non-immediate identification of the microorganism resulted in catastrophic pathogen control processes performed by farmers who, eagerly trying to save their crops ended up using a series of toxic agrochemicals that had been previously purchased for the control of other types of diseases. Due to these events, the present paper aims at identifying the pathogen and put chemical as well as biological products into test that might help control the disease. Results showed that no fungicide under test in the present experiment was able to inhibit the isolate at a 100% rate, even under *in vitro* conditions. Pathogen identification revealed a fungi known as *Pestalotiopsis longisetula*.

Keywords: Strawberry. Strawberry disease. *Pestalotiopsis*. Control.

Introduction

Southern Minas Gerais state is the largest strawberry-producing region in Brazil, equivalent to 33% of the total production in the country. Together, other six Brazilian states produce the remaining 77% (DIAS and SIMÕES, 2009). In 2007, the total national production was about 100 thousand tons (DIAS and SIMÕES, 2009). The strawberry crop is among the most sensitive to pests and diseases, which can cause large losses if not controlled properly. This condition has usually required producers continuous management efforts (TEIXEIRA and COSTA, 2008).

Over the past seven years, a new disease has emerged in the Brazilian strawberry fields, preventing any increase in productivity, which has, in fact, been steadily decreasing over the last decade

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(TEIXEIRA and COSTA, 2008). The first reports of the disease were of great concern, since few crops were completely eliminated, and farmers had no success in controlling the disease.

The visual symptoms observed in the field are small necrotic spots on the leaves and petioles. These spots can evolve to dark spots with black dots on the older leaves, which also become dry and brittle (GONÇALVES, 2005). The varieties Oso Grande, Sweet Charlie and Camarosa are susceptible to the pathogen (GONÇALVES, 2005).

The aim of this study was to identify the pathogen at least at the taxonomic level of the genus, and carry out laboratory tests in order to select chemicals or biological products with the potential to control the pathogenic microorganism.

Materials and methods

Diseased material collection and pathogenic microorganism isolation

Strawberry leaves showing symptoms of the disease were collected in the field (Picture 1) and observed under an optical microscope. An assembly of semi-permanent slides was set up observe pathogen structures (conidia size and width). The structures were observed through an eyepiece and X10 and X40 magnification objective, respectively, and photographed. In order to isolate the fungus, the surface of the strawberry leaves was disinfected using the following chemicals in sequence: alcohol (30 s) 70%, 2% sodium hypochlorite (4 min), and sterile distilled water. The edges of the material were removed between healthy tissue lesions and distributed on the surface of potato dextrose agar (PDA) plates. The plates were incubated at 27° C (± 2° C) to evaluate the appearance of any possible etiologic agent of the disease. Subsequently, the isolates were purified and stored in sterile water as described by Figueiredo (1967).

Picture 1. Symptoms of disease field

Koch postulate

Healthy strawberry leaves were perforated with sterile needles and then inoculated with PDA discs (0.8 mm diameter) colonized by microorganisms isolated and purified as described in the previous item. For each tested organism, seven seedlings of Oso Grande variety were used. Leaves were drilled with a set of sterile needles and disks colonized with the fungus were inoculated.

The control treatment received only PDA discs with no microbial growth. After inoculation, all the seedlings were placed in a moist chamber for 48 hours. Distilled water was sprayed onto the plants twice a day to provide suitable conditions for disease expression. The pathogenicity of the isolates was evaluated on daily basis during seven days. The organism that expressed the same disease symptoms observed in the field was subjected to re-isolation on PDA culture medium.

Molecular identification of the fungus

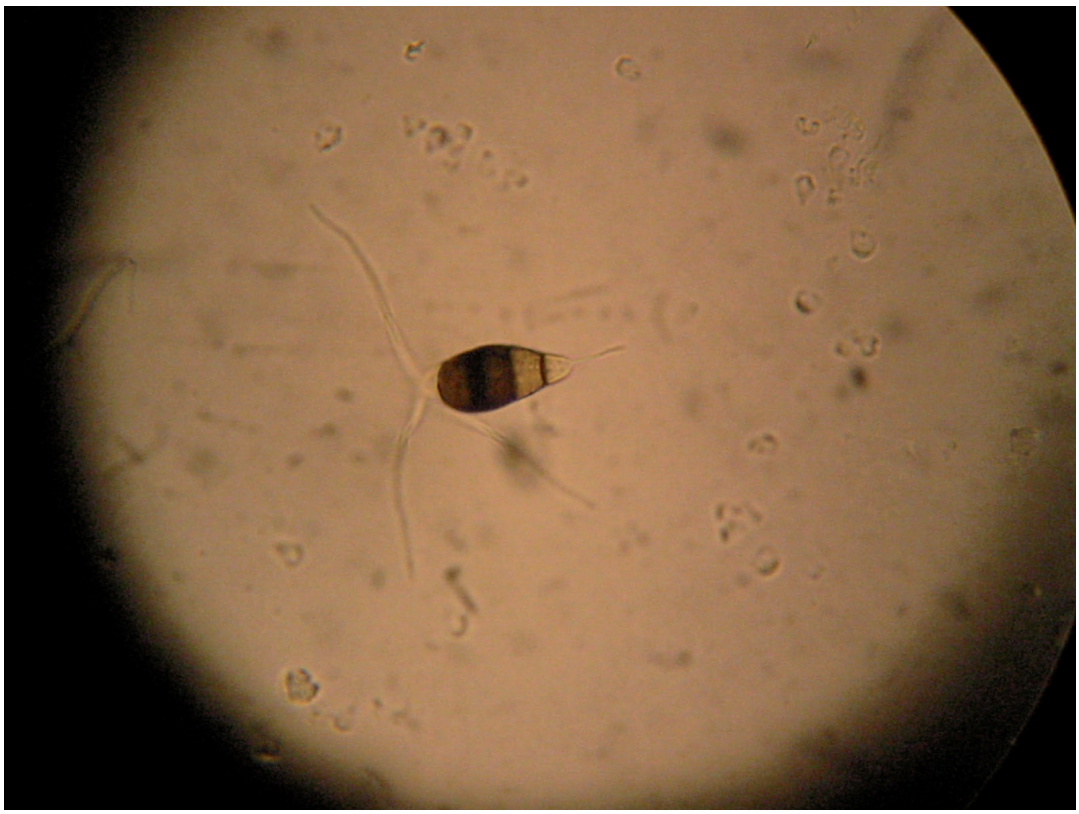
The DNA genomic was extracted from the mycelium of the pathogenic fungus, ground in a pestle and mortar under liquid nitrogen, using CTAB method. The internal transcribed space (ITS) between the codified ribosomal RNA genes 18S and 28S was amplified by PCR using ITSI 5' - TCCGTWGGTGAACCWGC- 3' and ITS4 5'-TCCTCCGCTTATTGATATGC -3' primers. The product obtained was sequenced using Big Dye Terminator Kit (Applied Biosystems) and ABI 377 sequencer. The sequence obtained was compared to those deposited in the World Data Bank (Gen Bank), using BLAST program (ALTSCHUL et al., 1997).

In vitro test with fungicides

The pathogenic fungus was cultured for seven days in Petri dishes containing PDA medium at 28° C in B.O.D chamber incubator. Concentrations on 20 μl and 200 μl of Fluanizan frowncide chemical fungicide and the trichodermil (Itafort Bioprodutos) and stubble-aid (Improcop do Brasil) biological fungicides were added to 150 ml conical flasks containing previously sterilized PDA culture medium, cooled to about 45º C. The medium was poured into Petri dishes and left for 24 hours for product sedimentation. 0.5 cm discs were removed from the edges of the pathogenic microbial colonies and placed in the center of the plate. The radial growth was measured every other day. No fungicides were added to the control treatment plates and five replicates were made for each treatment. The potential product inhibition was evaluated during a 5-day period.

Results and discussion

Young injured leaves under optical microscope showed no pathogen-associated structure, although older leaves showed some black dots on their surface. When broken, these dots released several conidia with fusiform morphological characteristics, straight and sometimes slightly curved, with four septa, and the presence of dark brown cells in the edges as well as three long appendices on the pedicel and in the basal cell (Picture 2). The size of conidium was 24.2 μm of length and width 8.3 μm. These remarks do not mean that no structures were formed on the young leaves, but rather that viewing them was not possible in this work. The healthy strawberry plants showed the formation of white mycelia clumps and a black gelatinous mass on the inoculated leaves.



Picture 2. Conidial of *Pestalotiopsis longisetula*

Strawberry leaf infection occurred regardless of plants being injured. In such event, in addition to injuries derived from environmental conditions, the fungus seems to be able to penetrate the leaf through natural openings such as stomata and lenticels. Fifteen fungal colonies grew on the PDA medium as the pathogenic fungus was isolated, but only one reproduced the symptoms of the disease in the inoculated plants. After inoculation of healthy strawberry plants, the formation of white mycelial clumps was observed on the leaves and also one black gelatinous mass. Similar symptoms were observed in mangosteen attacked by *Pestalotiopsis cruenta* (BASTOS et al., 2001).

Morphological characterization tests showed that the pathogenic fungus belonged to *Pestalotiopsis* sp genus. This was confirmed by the molecular identification test, which resulted in 98% similarity with that genus. The isolate was deposited in the collection of Valley University Sapucahy microbiological. *Pestalotiopsis* genus is complex, and its classification to a specific level is severely hampered by the enormous variation in morphology accepted for different species of the genus (KRUSCHEWSKY, 2010). Traditionally, the taxonomic characterization of this fungus has been based on conidial morphology (JEEWON et al., 2004), spore production and association with teleomorph, described only for some species (METZ et al., 2000). Since the establishment of the genus, several taxonomic studies have been carried out to suggest a better classification for *Pestalotiopsis* species (KRUSCHEWSKY, 2010).

This paper reports on the attack of *Pestalotiopsis* sp. in strawberry crops for the first time in Minas Gerais state, but it is not the first record in Brazil. Other reports have been made regarding similar attacks in Jarinu, São Paulo state, Brazil (CAMILI et al., 2002). The morphological and molecular characterization of the pathogen led to identification of *Pestalotiopsis longisetula* species. The spread of this disease has not been studied so far, but it is supposed to have arisen from the extensive mar-

keting of seedlings amongst farmers in Minas Gerais and São Paulo states. The disease has also been diagnosed in Egypt (EMBABY, 2007) and Hawaii (KEITH et al., 2006), among other place. In Egypt, the fungus was isolated from strawberry cv. Tamar and Yael fruits showing rot and frequent presence of *Pestalotiopsis longisetula*, compared to other isolated pathogenic fungi (EMBABY, 2007). Other species of this fungus have been described as pathogens other crops, such as cashew, which was attacked by *Pestalotiopsis funerea* conifers (BAJO et al., 2008), guava by *Pestalotiopsis psidii* (CARDOSO et al., 2002), *Carapa guianensis* (*Meliaceae*) by *Pestalotiopsis microchaeta* and also some ornamental plants (HOPKINS; MCQUILKEN, 2000).

In *in vitro* test, control treatment had grown over the whole plate after five days of evaluation. Trichodermil fungicides and stubble-aid were not effective in controlling the fungus under the concentrations in use, and frowncide fungicide inhibited 40% of the pathogen growth under 200 μl concentration, showing some effectiveness in controlling *Pestalotiopsis* sp fungus. This fungicide is used to control other strawberry diseases, such as anthracnose and *Dendrophoma obscurans* fungus (FORTE, 2005). However, when these fungicides are applied under field conditions their effectiveness does not avoid the problems caused by the attack of *Pestalotiopsis* sp fungus. (personal communication). Few studies on the control of *Pestalotiopsis* sp fungus. have been reported worldwide. Lisboa-Padulla et al. (2009) reduced the incidence of *Pestalotiopsis* sp fungus in seeds of Brazil wood by using captan, benomyl + thiram carboxim fungicides.

Conclusion

The fungus that attacked strawberry crop was identified as *Pestalotiopsis* sp. but their morphological characteristics and symptoms are the same as described for *P. longisetula*. The correct identification of the microorganism prevents pathogens from being empirically controlled.

Frowncide fungicide can help controlling *Pestalotiopsis* sp. fungus *in vivo* testing should be performed to confirm *in vitro* results.

Identificação e controle *in vitro* do fungo patogênico *Pestalotiopsis longisetula* patógeno da cultura do morango

Resumo

A pestalotiose tem sido, nos últimos sete anos, a principal doença da cultura do morango em todas as regiões produtoras do sul de Minas Gerais, Brasil. As folhas das plantas foram severamente atacadas por um fungo nada comum nas lavouras mineiras, principalmente nos anos de 2005 e 2006. A não identificação imediata do microrganismo resultou em processos de controles desastrosos do patógeno quando realizados pelos agricultores que, na ânsia de salvar o patrimônio, aplicaram uma série de defensivos químicos disponíveis em seus estoques, mas que foram adquiridos para o controle de outras doenças. Devido a todos esses acontecimentos o presente trabalho objetivou identificar o patógeno e testar produtos químicos e biológicos que pudessem contribuir para o controle dessa doença. Os resultados mostraram que, dos fungicidas testados neste experimento, nenhum foi capaz de inibir o isolado em 100%, mesmo em condições *in vitro*. A identificação do patógeno mostrou tratar-se do fungo *Pestalotiopsis longisetula*.

Palavras-chave: Morango. Doenças do morango. *Pestalotiopsis*. Controle

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