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A new method for inoculation of fruit with *Guignardia citricarpa*, the causal agent of citrus black spot

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Abstract Citrus black spot (CBS) is a fungal disease, caused by *Guignardia citricarpa*, that has a high economic impact on citrus. Although *G. citricarpa* has been associated with black spot of citrus, an adequate pathogenicity test is still not available. Thus, our objective was to develop and evaluate a simple, safe, and practical pathogenicity test. We used fruits from Pera-Rio and Valencia sweet orange trees from two different orchards, located in the State of São Paulo, Brazil. Inoculation was performed by placing six disks colonized by *G. citricarpa*, onto the peel of healthy fruits, previously bagged. In the Pera-Rio sweet orange grove, initial symptoms of the false melanose type resulting from the inoculations were observed 55 days after inoculation (dai). In the Valencia grove, initial symptoms also of the false melanose type resulting from the inoculations occurred 73 dai. A total of 92.8% and 86.6% of the Pera Rio and Valencia fruits inoculated, respectively, showed symptoms of CBS. Citrus black spot symptoms were not observed in any of the control fruits.

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Introduction

Citrus black spot (CBS) is an A1 quarantine disease for countries of the European Union and for the USA (Aguilar-Vildoso et al. 2002). It is caused by *Guignardia citricarpa* (anamorph: *Phyllosticta citricarpa*) and affects practically all citrus species of economic importance, especially sweet oranges (*Citrus sinensis*). Currently, CBS is reported in Africa, Asia, Oceania, and South America and in some of these countries, it is one of the most important fungal diseases (Kotzé 1981; Peres and Timmer 2003).

The fruit are susceptible to infection for 20–24 weeks after petal fall, after which time the fruit become resistant (Kotzé 1981; Baldassari et al. 2006). The symptoms develop on mature fruit and, there are five symptom types—hard spot, false melanose, freckle spot, virulent spot (Kotzé 2000), and in Brazil, the recently described cracked spot type (de Goes et al. 2000).

Ascospores and conidia are the two sources of inoculum. Ascospores are air-borne, and produced on dead leaves on the orchard floor and usually are the main source of inoculum (Kotzé 2000), although the conidia, which are water-splash dispersed, are important in infection by the disease (Spósito 2004).

In spite of the importance of the disease and the long period that CBS has been associated with *G. citricarpa*, there is still lack of information about this

pathosystem. Among some of the difficulties that hinder research is the lack of a specific, practical, reliable, and effective inoculation method. So far, pathogenicity tests are based on the use of naturally infected citrus leaves fallen on the ground, that are collected and, after qualitative determinations for the presence of the teleomorph, the leaves are wetted and attached to the surface of the fruits during the susceptible stage (McOnie 1964). When a favourable microclimate for development of infections is maintained near the leaves, lesions develop in a few months and the pathogen can be re-isolated from the lesions. Such a method is not only impractical, but also precludes knowledge of the history of the isolate, since infections generated by this means may come from conidia or from ascospores formed in the various reproductive structures of the fungus. Lemir et al. (2000) reported a pathogenicity test conducted by inoculating ascospores produced in vitro onto lemon fruits. However, given the genetic characteristics of *G. citricarpa*, production of these structures in vitro has proven very difficult and this method has not been consistently repeatable (Timossi et al. 2003).

The objective of this work was to develop an inoculation method for *G. citricarpa* which, in addition to being effective, simple, and practical, would allow relationships regarding the history of the pathogen to be established.

Materials and methods

Sweet orange fruits from two different orchards and in different seasons were used for the pathogenicity test, one of Pera-Rio sweet orange (*C. sinensis*), located at FCAV–UNESP in Jaboticabal, SP, and the other of Valencia sweet orange (*C. sinensis*) in Araraquara, SP. At three-fourths petal fall, 200 fruits of each were selected and bagged in 18×15.5 cm clear paper bags to protect the fruit from potential natural infection. Due to fruit drop that occurs when fruit is 2–3 cm diam, it was necessary to bag many more fruits than were finally evaluated.

The inoculum consisted of disks of completely expanded Pera-Rio orange leaves from greenhouse-grown trees. After autoclaving at 120°C for 30 min, four disks were placed in transversely equidistant pairs on 2% water agar in Petri dishes with the abaxial side down. A disk containing culture medium and a colony of the isolate of *G. citricarpa* known to be pathogenic obtained from a Pera-Rio fruit was then placed between the leaf disks (Fig. 1a). The dishes were maintained in BOD chambers at 25°C with a 12/12 h photoperiod for 30 days. Subsequently, pycnidia, conidia, and possible pseudothecia and ascospores were observed on the leaf disks (Fig. 1b) that were then used for inoculum in the tests.

The inoculation was conducted by placing six disks in contact with the peel of the previously bagged, healthy fruits, with a diameter of 20 to 30 mm that were then covered using a rayon fabric.. The fruits were then covered with 18×15.5 cm clear paper bags to prevent infection by other pathogens normally associated with citrus fruits at their early stages of development. To ensure favourable infection conditions, the bags covering the fruits were wetted daily for 7 days. The fruits remained bagged until ripe. In November, 28 fruits of Pera-Rio were inoculated and 30 fruits of Valencia were inoculated in February. One fruit of the same age was not inoculated or bagged and was marked and maintained beside each inoculated fruit as a control.

During the experiment, weekly inspections were conducted to determine the time of appearance of the first symptoms and to observe the effectiveness of the method. Two supplementary evaluations were performed at 120 days and at 150 days after inoculation (dai), when the fruits were ripe and before those with symptoms dropped.

Re-isolations were made at harvest to confirm the presence of the pathogen on fruits with characteristic

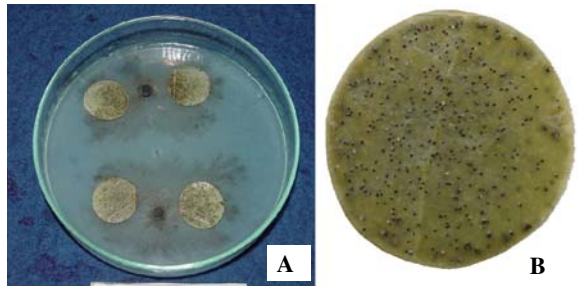

Fig. 1 Production of inoculum; **a** Pera-Rio orange leaf disks colonised by *Guignardia citricarpa*. These disks, previously autoclaved, were placed on the surface of water agar together with 5 mm diam disks of *G. citricarpa*; **b** colonised leaf disks containing pycnidia, conidia, and immature pseudothecia of *G. citricarpa*

symptoms. Lesion fragments of about 5 mm^2 were removed and immersed in 70% ethanol for 1 min and then in a 1:3 *v/v* mixture of sodium hypochlorite: sterile water for 3 min, and rinsed in sterile water for 30 s. After drying of the material on sterile filter paper, the fragments were placed in Petri dishes containing oatmeal (OA) medium to characterization of *Guignardia* species colonies as described by Baayen et al. (2002) and Baldassari et al. (2008).

Results

Characteristic symptoms of CBS and the re-isolation of typical colonies of *G. citricarpa* confirmed the occurrence of infections and, consequently, the effectiveness of the method. On Pera-Rio sweet orange, initial symptoms resulting from the inoculations were observed 55 dai. Those symptoms were of the false melanose type (Fig. 2a), and were present on six of the 28 inoculated fruits. Later, at 120 dai, after general evaluation of all inoculated fruits, 17 fruits showed characteristic CBS symptoms (Fig. 2b). At the final evaluation at 150 dai, an additional three fruits were found with typical symptoms of the disease, and the symptoms had become more evident, and now showed the freckle spot type of lesion (Fig. 2c). Therefore, of the 28 fruits inoculated, 26 fruits showed typical CBS symptoms at a level of infection of 92.8%. No characteristic CBS symptoms were observed on any fruit of the controls. A temporal sequence of the expression of CBS symptoms in inoculated Pera-Rio fruits is presented in Fig. 2.

In Valencia, the initial symptoms resulting from the inoculations occurred at 73 dai and were also of the false melanose type. On this variety, periodic evaluations were done, and a final evaluation was performed at 225 dai, when 26 of the 30 fruits showed typical symptoms at a level of infection of 86.6%. In Valencia, hard-spot symptoms were observed on two fruits in the last evaluation. The occurrence of characteristic CBS symptoms was not observed in any of the control fruits. In both sweet orange varieties, of the 58 fruits inoculated, typical symptoms were observed on 52 fruits, at an infection level of 89.6%.

Discussion

Currently, the usual inoculation method is restricted to that developed by McOnie (1964), by placing naturally infected citrus leaves directly on fruit. Although McOnie (1964) mentioned that the presence of conidia was rare on old leaves, containing over 50% pseudothecia, the potential for their presence must not be underestimated since the fungus characteristically produces a large number of pycnidia on fallen leaves (Kiely 1948). This method has the disadvantage of the lack of precise knowledge about the source and origin of the inoculum. In addition, the method does not allow the evaluation of the pathogenicity of isolates that exist in mycological collections and limits the possibility of qualitative and quantitative standardisation of the inoculum.

The inoculation method, in which a suspension of ascospores is used, as described by Lemir et al. (2000), is a highly promising and suitable method, since it overcomes the difficulty of inoculum standardisation, and allows previous knowledge about the isolate to be employed. However, the problem in adopting such a method is that it is difficult to obtain pseudothecia and ascospores regularly and selectively,

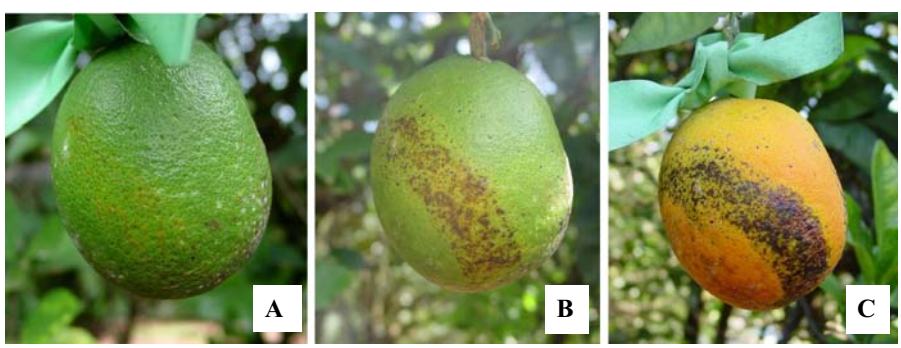

Fig. 2 A temporal sequence of the expression of CBS symptoms on inoculated Pêra-Rio fruits; **a** initial symptoms of false melanose-type 56 dai; **b** symptoms of false melanose-type 120 dai; **c** symptoms of false melanose and freckle spot-type 150 dai

under laboratory conditions. Although these authors were successful in obtaining these structures under artificial conditions and were equally successful in the inoculation of lemon fruits, several attempts conducted by various investigators to obtain the teleomorph have been unsuccessful or were sometimes limited to a single isolate (Timossi et al. 2003). However, in our study probably only a few sexual structures were obtained, but were not quantitatively determined.

According to the literature (Kotzé 1981; Baayen et al. 2002), *G. citricarpa*, in contrast to *G. mangiferae*, does not form the teleomorph under artificial conditions, or in cases where it does, produces a small number of ascospores in association with large numbers of conidia, making it difficult to standardise the inoculum and to conduct pathogenicity tests.

In the present work, due the nature of *G. citricarpa*, the source of inoculum consisted of conidia, which is useful since the history of the isolate is known. Consequently, in addition to the fact that this is a practical and effective technique, it allows individual isolates to be evaluated regardless of host or origin, and thus allows a relationship to be established with regard to origin and pathogenicity.

With regard to the symptoms produced, a predominance of false melanose-type symptoms was observed, both on Pera-Rio and Valencia fruits. However, the presence of hard-spot symptoms in two Valencia sweet orange fruits was observed at 256 dai. Therefore although in previous studies conidia typically produced false melanose-type symptoms, it is apparent that they may also produce hard spot-type symptoms.

At present, the majority of the literature on the *G. citricarpa*-*Citrus* spp. pathosystem deals with research on control of the pathogen, especially with the use of fungicides. Although the disease was described over a century ago, little progress has been made with regard to basic aspects that are essential to provide direction for new research and broaden our knowledge of the pathogen. One of the elements that has significantly hampered the advancement of research on this host–pathogen interaction has been the lack of a more suitable and practical inoculation methodology; hence this new inoculation methodology that has been developed is important.

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