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Direct penetration of *Rhizopus stolonifer* into stone fruits causing rhizopus rot

J. S. Baggio, F. P. Gonçalves, S. A. Lourenço, F. A. O. Tanaka, S. F. Pascholati and L. Amorim*

Departamento de Fitopatologia e Nematologia, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, SP 13418-900, Brazil

Rhizopus rot, caused by *Rhizopus stolonifer*, is a major postharvest disease of stone fruits. The disease is related to the occurrence of mechanical and physical damage; however, observations at a Brazilian wholesale market suggest that direct penetration can occur. Therefore, the penetration mechanisms of *R. stolonifer* in stone fruits were evaluated. To identify the production of enzymes that help with direct penetration by the pathogen, esterase activity, both in mycelial discs and in spore suspensions of the fungus in water and in modified Van Etten nutrient solution, was measured. Assays were also conducted to evaluate the growth of *R. stolonifer* on glucose or cutin as a sole carbon source. The pathogen grew on both media, and higher esterase activity was observed in the cutin medium. Wounded and unwounded peaches and nectarines were inoculated with *R. stolonifer* spore suspensions in water or in modified Van Etten nutrient solution. Wounded fruit inoculated with either of the *R. stolonifer* spore suspensions developed rhizopus rot, whereas unwounded fruit developed the rot only in the presence of spores in the modified Van Etten nutrient solution. Scanning electron and light microscopic examination showed the fungus can directly penetrate the nectarine cuticle. Diisopropyl fluorophosphate, a serine hydrolase inhibitor, prevented rot development in peaches. The results provide valuable evidence for the ability of *R. stolonifer* to directly penetrate unwounded stone fruits, probably due to the production of esterase enzymes.

Keywords: esterases, infection, postharvest diseases, *Prunus*, *Rhizopus stolonifer*

Introduction

Considerable postharvest losses are reported in fruits due to physical, physiological and pathological damage, which can occur during harvesting, grading, packing and transportation to markets. Physical damage to fruit is important for pathogen penetration, especially for fungi that cause postharvest rots (Agrios, 2005). Postharvest damage during marketing can cause up to 50% loss of stone fruits (Martins *et al.*, 2006). Studies carried out in a wholesale market in Brazil showed a correlation between mechanical damage and postharvest diseases in stone fruits (Martins *et al.*, 2006; Amorim *et al.*, 2008). In Brazil, diseases are the main cause of price reduction among all postharvest damage in peaches. For example, a 1% increase in disease incidence results in a 0·91 and 1·24% reduction in sale price in wholesale and retail markets, respectively (Lima *et al.*, 2009).

Rhizopus rot, caused by *Rhizopus stolonifer*, is one of the most important postharvest diseases of stone fruits (Ogawa, 1995). The disease occurs mainly on ripe fruit, which are more prone to wounds and have higher sugar content. After 2–3 days, infected fruits become soft and watery, and they release juices with a fermented or acidic odour. Under favourable temperatures and humidity, approximately one day after the initial symptoms appear, a rapid and abundant mycelial growth can be observed on the surface of the infected fruit and the pathogen produces long mycelial stolons with black sporangia and spores (Snowdon, 1990).

Rhizopus spp. are cosmopolitan, filamentous, lower fungi found in soil, decayed fruits and vegetables, and commonly observed in packing houses (Snowdon, 1990; Massola Junior & Krugner, 2011). They can cause postharvest rots in many vegetables and fruits (Agrios, 2005), including potato, aubergine, watermelon, melon, cucumber (Harter & Weimer, 1922), sweet potato (Walker, 1972), grape (Snowdon, 1990; Tavares & Silva, 2006), strawberry (Maas, 1998), raspberry, blackberry (Davis, 1991) and stone fruits (Ogawa, 1995). According to the literature (Harter & Weimer, 1922; Davis, 1991; Ogawa & English, 1991; Maas, 1998; Tavares & Silva, 2006; Bautista-Baños *et al.*, 2014), *Rhizopus* is strictly a wound parasite, and it can only penetrate host tissues through fresh wounds, microwounds and bruises made by harvesting, handling and/or insects. This is most likely due to its inability to produce specific enzymes that help in the penetration process (Nguyen-The & Chamel, 1991). Despite these claims, the presence of rhizopus rot was observed on apparently unwounded stone fruits dur-

*E-mail: lilian.amorim@usp.br

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ing surveys carried out in a wholesale market in São Paulo, Brazil (authors' unpublished data), suggesting that *R. stolonifer* has additional penetration strategies. It is known that *R. stolonifer* spores require external nutrient sources to germinate (Medwid & Grant, 1984; Nguyen-The *et al.*, 1989); therefore, it is possible that the inability of *Rhizopus* to directly penetrate its hosts is due to its incapability to germinate on an intact fruit surface where external nutrients are not available. If the fungus cannot germinate it is unable to produce specific enzymes, such as esterases, which can help to penetrate the plant cuticle and cell wall.

The production of esterases, especially cutinases (EC 3.1.1.74), is essential for some pathogens to directly penetrate their hosts; for example *Colletotrichum graminicola* in corn and sorghum (Pascholati *et al.*, 1993), *Uromyces viciae-fabae* in beans (Deising *et al.*, 1992) and *Pestalotia malicula* in plum (Sugui *et al.*, 1998). In these studies, the enzymes were found in spore exudates and/or mucilage, and the pathogens were capable of cutin degradation. To confirm the presence and activity of these enzymes, diisopropyl fluorophosphate (DIPF), an inhibitor of serine hydrolase enzymes, including esterases and proteases (Cohen *et al.*, 1967), was used. DIPF was able to block cutinase activity, and prevent disease development on corn leaves inoculated with *C. graminicola* (Pascholati *et al.*, 1993). However, reports of the existence of enzymes that can help *R. stolonifer* penetrate directly into fruits are rare; most of them refer to enzymes of the parasitic process of establishment and host colonization by the fungus (Spalding, 1963; Wells, 1968). It has already been shown that the pathogen can synthesize enzymes that destroy plant tissues (Sommer *et al.*, 1963); however, none were identified as degraders of fruit cuticle components that may facilitate penetration by the fungus. The single report that studied the enzymatic degradation of nectarine epidermis by *R. stolonifer* showed that the pathogen penetration apparently did not involve cutinolytic enzymes (Nguyen-The & Chamel, 1991).

Despite the information that fruit infection by *R. stolonifer* occurs through wounds or directly by mycelial stolons, producing the symptoms known as nested infection, little is known about the mechanisms of fruit penetration by spores of this pathogen. Therefore, the objective of this study was to evaluate the penetration mechanisms of *R. stolonifer* in stone fruits.

Materials and methods

Pathogen identification

One isolate of *R. stolonifer* was collected from diseased peaches obtained from a wholesale market in Brazil and identified by molecular techniques. DNA was extracted, and the ITS region of the gene was amplified by PCR using the forward primer ITS1 (5′-TTCCGTAGGTGAACCTGCGG-3′ and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′ (White *et al.*, 1990). PCR products amplified by these primers gave a fragment of 900 bp. The purified product was sequenced and compared with the DNA sequences of *R. stolonifer* deposited at GenBank (accession numbers EU622265, AF117935 and AF117936).

The fungus was grown on potato dextrose agar (PDA) (Oxoid Ltd) and kept at 25°C for 3 days under constant fluorescent light to promote mycelial growth and sporulation.

Spore germination

To test the germination of *R. stolonifer in vitro*, spore suspensions were produced by adding sterile distilled water or nutrient solution to a 3-day-old culture. The nutrient solution contained 20 g glucose, 2 g asparagine, 0·5 g KH_2PO_4 and 26 g $MgSO_4$ in 1 L water (Van Etten *et al.*, 1969). Spore concentration was determined using a haemocytometer and adjusted to 10^5 spores mL^{-1}. Four aliquots (40 μL each one) of the spore suspension were incubated in sterile Petri dishes. The Petri dishes were kept inside a plastic container with water, to provide high humidity. The containers were kept at 25°C for 4, 6, 8, 12, 24 and 48 h. Three replications were performed.

Germination of sporangiospores was noted when the germ tube length was equal to or greater than the diameter of the spore. The percentage germination was determined by counting the first 100 spores observed under a microscope at ×400 magnification. The experiment was carried out twice.

Qualitative evaluation of esterase activity

The spore suspensions, obtained as described above, were adjusted to two inoculum concentrations, 10^5 and 10^6 spores mL^{-1}. The suspensions were used immediately after preparation or kept at 25°C on a 100-rpm shaker for 4 or 8 h to promote spore germination. *Colletotrichum graminicola*, a corn pathogen known to produce cutinase, was grown on oatmeal agar and kept at 21°C for 10–14 days under fluorescent light (Pascholati *et al.*, 1993). A spore suspension, used as a positive control, was obtained by adding sterile distilled water and adjusting the concentration to 10^5 spores mL^{-1}.

To determine esterase activity, an assay was performed, based on the hydrolysis of indoxyl acetate, which results in the formation of insoluble crystals of indigo blue. The indoxyl acetate substrate (35 mg) was dissolved in 1 mL acetone and added to 49 mL 0·05 M Tris-HCl buffer (pH 8·0) to give a final concentration of 0·7 mg mL^{-1} indoxyl acetate (Pascholati *et al.*, 1993). Subsequently, spore suspensions were prepared in water or nutrient solution, at different concentrations (Table 1) and incubated at 25°C for 0, 4 or 8 h, as described above. Additionally mycelial discs of the fungus (0·5 cm in diameter) added to sterile distilled water were also tested for esterase activity. Controls were also prepared with Tris-HCl buffer alone; buffer and indoxyl acetate only; and *C. graminicola* spore suspension with buffer only or with buffer and indoxyl acetate. After adding the indoxyl acetate solution, the test tubes were incubated at 25°C for a further 30 min and 30 μL aliquots of each treatment were then placed on a polystyrene Petri dish and the formation of insoluble crystals of indigo blue observed under a light microscope. The experiment was conducted three times.

Quantitative evaluation of esterase activity

Rhizopus stolonifer was grown on a liquid medium made of 10 g glucose, 2 g asparagine, 1 g KH_2PO_4, 0·5 g $MgSO_4.7H_2O$, 0·2 mg Fe^{3+}, 0·2 mg Zn^{2+}, 0·1 mg Mn^{2+}, 5 μg biotin and 100 μg

Table 1 Qualitative evaluation of esterase activity exhibited by *Rhizopus stolonifer* and *Colletotrichum graminicola*

Code	Treatment					Esterase activity[b]
	Tris-HCl (mL)	Indoxyl acetate (mL)	*C. graminicola* (mL)[a]	*R. stolonifer* mycelium	*R. stolonifer* spore suspension	
T_1	4	–	–	–	–	–
T_2	2	2	–	–	–	–
T_3	3	–	1	–	–	–
T_4	1	2	1	–	–	+
T_5	4	–	–	5 disks	–	–
T_6	2	2	–	5 disks	–	+
T_7	3	–	–	–	1 mL in water, 10^5 spores mL^{-1}	–
T_8	1	2	–	–	1 mL in water, 10^5 spores mL^{-1}	+
T_9	1	2	–	–	1 mL in water, 10^6 spores mL^{-1}	+
T_{10}	1	2	–	–	1 mL in nutrient solution, 10^5 spores mL^{-1}	+
T_{11}	1	2	–	–	1 mL in nutrient solution, 10^6 spores mL^{-1}	+

[a]10^5 spores mL^{-1}.

[b](+) presence or (−) absence of indigo blue crystals from hydrolysis of indoxyl acetate, indicating esterase activity.

thiamine in 1 L water (Lilly & Barnett, 1951), and the same medium with glucose replaced by apple cutin, an insoluble polyester of plant cuticle (Kolattukudy, 1980). *Colletotrichum graminicola* was also grown in both media. Fungal mycelium discs (0·5 cm in diameter) were separately transferred to Erlenmeyer flasks containing 25 mL of the medium. Three Erlenmeyer flasks were used for each treatment. The pathogens were not added to the control treatments. The Erlenmeyer flasks were kept at 25°C on a shaker (100 rpm) for 4 days for *R. stolonifer* and 10 days for *C. graminicola*. The contents of the Erlenmeyer flasks were filtered (Whatman no. 1), and the resultant filtrate centrifuged at 17 787 ***g*** for 20 min at 4°C. The supernatant was filtered through a mixed cellulose esters membrane filter, with 0·22 μm pore size (MF-Millipore; Merck) and the filtrate was collected inside microtubes and assayed in a spectrophotometer. The presence of esterase activity was confirmed by detection of the hydrolysis of ρ-nitrophenyl butyrate to ρ-nitrophenol, with the resultant product measured using a spectrophotometer at 400 nm (Pascholati *et al.*, 1993). Fungal growth was quantified by fresh weight of the biomass after removal of the liquid medium.

The reaction mixture for esterase consisted of 800 μL Tris-HCl buffer (0·05 M, pH 8·0), 100 μL enzyme preparation of each fungus and 100 μL stock solution of ρ-nitrophenyl butyrate. Controls for the enzyme assays were performed with identical reaction mixtures and filtered culture medium, which had not been exposed to the fungus, as a negative control. The stock solution of 13 mM ρ-nitrophenyl butyrate was diluted in the same reaction buffer to give a final concentration of 1·3 mM. The enzyme reaction was carried out for 3 min, and each treatment was repeated six times. The protein concentration of the samples was determined by a Bradford assay (Bradford, 1976) with bovine serum albumin as the standard. Each treatment was read six times. Thus, esterase activity was expressed as the absorbance (A) variation per minute per milligram of protein (Δ_A min^{-1} mg^{-1} of protein).

Enzyme activity and fungal mass data were subjected to analysis of variance and Tukey's test ($P < 0·05$) using STATISTICA software (Statsoft).

Inoculation of peaches and nectarines with *R. stolonifer*

Each experiment consisted of four treatments with 30 replications. Mature fruits (soluble solids from 8·35 to 8·85 Brix, and firmness from 0·77 to 1·83 kgf) were surface-disinfected with 0·5% sodium hypochlorite solution for 3 min. The fruits were placed individually on plastic nests without touching each other. Treatments evaluated were: (i) wounded fruit inoculated with a spore suspension in water (W-W); (ii) unwounded fruit inoculated with a spore suspension in water (U-W); (iii) wounded fruit inoculated with a spore suspension in nutrient solution (W-N); (iv) unwounded fruit inoculated with a spore suspension in nutrient solution (U-N); and a control treatment with water or nutrient solution that did not contain pathogen spores.

Fruits in treatments (i) and (ii) were wounded (1 mm in diameter by 3 mm in depth) with a hypodermic needle. A 30-μL aliquot of *R. stolonifer* spore suspension (10^5 spores mL^{-1}) was placed over the equatorial region of the wounded and unwounded fruits. The fruits were then incubated at 25°C in the dark, in a humid chamber for 24 h. Rhizopus rot incidence was assessed for 5 days after inoculation. Two cultivars of peaches (Dourado and Chiripá, two experiments each) and two cultivars of nectarines (Sunripe and Josefina, one experiment each) were used.

The final incidence of disease among the different treatments was compared by nonparametric comparison tests of multiple proportions (Zar, 1999). If the null hypothesis of equal proportions was rejected, a Tukey-type multiple comparison testing among the proportions was carried out based on the angular transformation (Zar, 1999).

Effect of DIPF on peaches inoculated with *R. stolonifer*

The influence of DIPF on the spore germination of *R. stolonifer* and the infection of peach by the pathogen was evaluated on polystyrene Petri dishes and inoculated peaches, respectively.

Spore suspensions were prepared in nutrient solution as described above. Aliquots of 20 μL *R. stolonifer* spore suspension (10^5 spores mL^{-1}) were placed on a Petri dish surface and 20 μL of a stock solution of 200 μM DIPF (Sigma-Aldrich) in Tris-HCl buffer (0·05 M, pH 8·0) added. The control treatment consisted of the spore suspension alone. The Petri dishes were kept inside a germination box with a wet filter paper to produce a moist chamber. The germination boxes were kept at 25°C for 24 h in the dark. Four aliquots per dish and three dishes per

treatment were used. Germination assessment was based on the observation of 100 spores under the light microscope.

Mature peaches were surface-disinfected with 0·5% sodium hypochlorite solution for 3 min, and placed individually on plastic nests without touching each other. Treatments evaluated were: (i) 40 μL *R. stolonifer* spore suspension; (ii) 20 μL spore suspension and 20 μL of a stock solution of DIPF at the time of inoculation; (iii) 20 μL spore suspension and 20 μL DIPF 1 h after inoculation; (iv) 20 μL spore suspension and 20 μL DIPF 2 h after inoculation; (v) 20 μL spore suspension and 20 μL DIPF 4 h after inoculation; (vi) 20 μL spore suspension and 20 μL DIPF 6 h after inoculation; (vii) 40 μL nutrient solution without *R. stolonifer* spores; and (viii) 20 μL nutrient solution and 20 μL DIPF without *R. stolonifer* spores. Rhizopus rot incidence was assessed 3 days after inoculation, and fruits that had softened in the region of pathogen deposition were considered diseased. Five peaches per treatment were used and the experiments were repeated once. The disease incidence was compared among the different treatments by nonparametric comparison tests of multiple proportions (Zar, 1999).

Nectarine penetration by *R. stolonifer* observed under the scanning electron microscope (SEM)

Nectarines were surface-disinfected with 0·5% sodium hypochlorite solution for 3 min and received the same treatments previously described for inoculation of peaches and nectarines with *R. stolonifer*. The fruits were kept on nests inside plastic containers, without touching each other, and then incubated in a humid chamber at 25°C in the dark for 10 h. Six fruits per treatment were used. Three fruits were washed with distilled water and brushed with a delicate paintbrush to remove the pathogen structures from the nectarine surface.

Fragments (1 × 1 cm) were excised from the inoculated regions of fruit from all treatments for analysis by scanning electron microscopy. The fragments were exposed to 2% (w/v) osmium tetroxide (OsO_4) vapour for 12 h (Kim, 2008), and transferred into a container with silica gel present to dry. The fragments were sputter-coated (SCD 050 Bal Tec) and examined with an LEO 435 VP scanning electron microscope (Leo Elektronenmikroskopie GmbH).

Nectarine penetration by *R. stolonifer* observed under the light microscope

Unwounded nectarines were surface-disinfected and inoculated with 30 μL of *R. stolonifer* spore suspension (10^5 spores mL^{-1}) in nutrient solution or with 30 μL nutrient solution without the pathogen (control). The fruits were kept inside plastic containers and then incubated in the dark in a humid chamber at 25°C for 12 h.

Fragments (1 × 1 cm) were excised from the inoculated regions and fixed in Karnovsky solution (Karnovsky, 1965). The samples were subsequently dehydrated through a graded alcohol series and embedded in methacrylate (Historesin; Leica Instruments). Using a rotating microtome, 5-μm-thick sections were cut and stained. For histological analysis, samples were stained with toluidine blue (O'Brien & McCully, 1981) and permanent slides were mounted in the synthetic resin Entellan. Slides were viewed under a Zeiss Axioskop 2 microscope and digital images were captured with an attached camera connected to a computer.

Table 2 *Rhizopus stolonifer* spore germination (%) in water or nutrient solution, after different periods of incubation

	Treatment			
	Water		Nutrient solution	
Incubation (h)	Mean[a]	Range[b]	Mean[a]	Range[b]
4	1·6	1–3	7·3	6–8
6	5·3	2–8	83·6	82–88
8	7·6	6–8	90·5	85–95
12	7·8	4–13	96·2	95–98
24	7·9	5–11	100·0	
48	9·6	4–15	100·0	

[a]Values obtained from two experiments of 100 spores each.
[b]Minimum and maximum values of spore germination from the two experiments.

Results

Spore germination

The spore germination of *R. stolonifer* was higher in the presence of nutrient solution than in water (Table 2; Fig. 1). Spore germination rates in water ranged from 1·6% at 4 h, to 9·6% at 48 h, whereas in nutrient solution the germination rates were higher than 80% after 6 h, and reached 100% at 24 h.

Differences in the morphology of the spores and germ tubes of *R. stolonifer* in nutrient solution and water were also observed (Fig. 1). When in nutrient solution, the structures of the fungus became swollen, thickened and granular in appearance. The germ tubes tended to clump together to form mycelial pellets after extended periods of incubation.

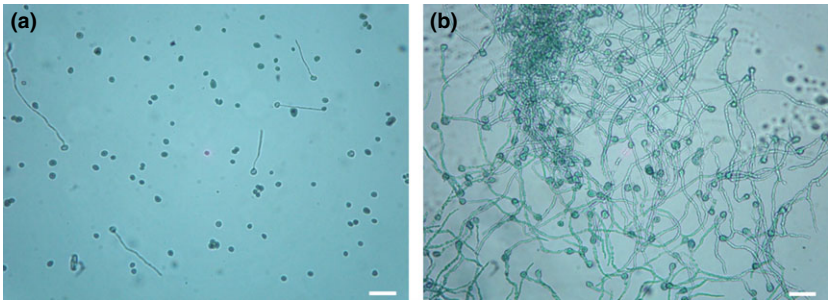

Figure 1 Germination of *Rhizopus stolonifer* spores in water (a) and nutrient solution (b) after 8 h of incubation. In (b) thick hyphae can be seen. Bars represent 25 μm.

Qualitative and quantitative evaluation of esterase activity

For qualitative esterase activity, the indigo blue colour was observed 30 min after addition of indoxyl acetate to the *C. graminicola* and *R. stolonifer* suspensions (Table 1). In the case of the *R. stolonifer* spore suspension in nutrient solution (10^6 spores mL^{-1}, treatment T_{11}) with shaking, the darkest blue colour was observed after 8 h (data not shown). The presence of indigo blue crystals was detected inside and surrounding the spores (not shown).

Rhizopus stolonifer and *C. graminicola* were able to grow on culture media that had glucose or cutin as a sole carbon source. The highest fresh mass weight values were obtained when the fungi were grown on glucose medium, and *R. stolonifer* grew more prolifically than *C. graminicola* (Table 3).

The values of esterase activity were 0·198 and 0·140 Δ_{OD} min^{-1} mg^{-1} protein for *C. graminicola* and 0·011 and 0·044 Δ_A min^{-1} mg^{-1} protein for *R. stolonifer*, in glucose and cutin culture media, respectively.

Table 3 Fresh mass of *Rhizopus stolonifer* and *Colletotrichum graminicola* colonies grown on culture media containing glucose or cutin as a sole carbon source

	Fresh mass (g)[a]	
Carbon source	*R. stolonifer*[b]	*C. graminicola*
Glucose	5·97 Aa	4·99 Ab
Cutin	4·50 Ba	3·91 Bb

[a]Values obtained by mean of three repetitions. Values followed by the same upper case letters in the columns and the same lower case letters in the rows do not differ among them according to Tukey's test ($P < 0·05$).

[b]Evaluations carried out after 4 and 10 days for *R. stolonifer* and *C. graminicola*, respectively.

Rhizopus rot on peaches and nectarines

In all experiments, infection of peaches and nectarines by *R. stolonifer* did not occur in unwounded fruits when the spores were suspended in water (U-W treatment), whereas infection occurred in unwounded fruits when spores were suspended in nutrient solution (U-N treatment; Figs 2 & 3). Both treatments with wounded fruits showed more than 80% of rhizopus rot incidence in all experiments (Figs 2 & 3).

Rhizopus rot occurred in 96·7 and 93·3% for Dourado and Chiripá peaches, respectively, in the U-N treatment

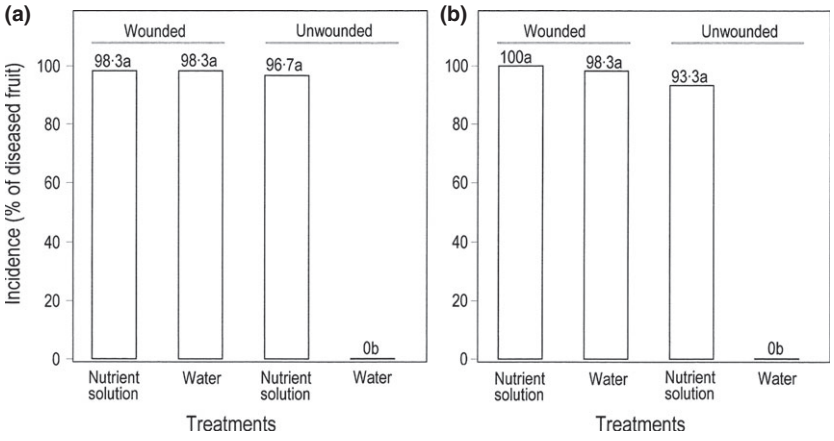

Figure 2 Incidence of rhizopus rot in Dourado (a) and Chiripá (b) peach cultivars 5 days after inoculation with *Rhizopus stolonifer*. The data represent the mean of two experiments of 30 fruits each. Bars followed by the same letter do not differ significantly at the 5% level by a nonparametric comparison test of multiple proportions (Zar, 1999).

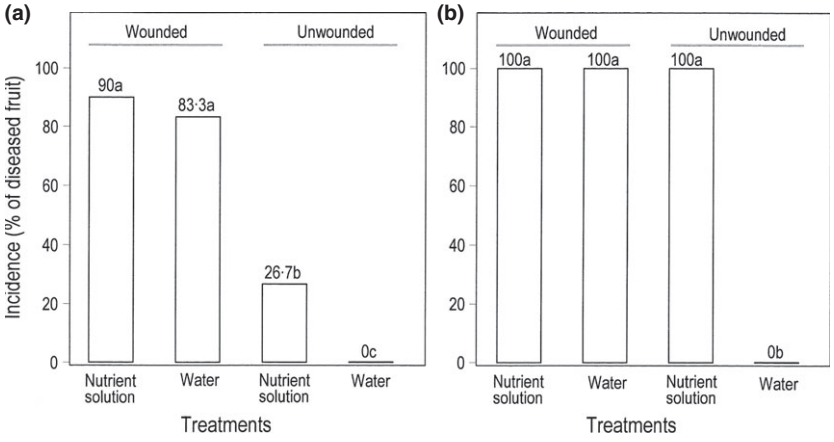

Figure 3 Incidence of rhizopus rot in Sunripe (a) and Josefina (b) nectarine varieties 5 days after inoculation with *Rhizopus stolonifer*. Bars followed by the same letter do not differ significantly at the 5% level by a nonparametric comparison test of multiple proportions (Zar, 1999).

Table 4 Disease incidence of peaches with rhizopus rot symptoms treated with diisopropyl fluorophosphate (DIPF) at different periods following inoculation with *Rhizopus stolonifer*

Time of DIPF addition to the fruit after inoculation (h)	Incidence (%)[a]
Control[b]	100 a
0	50 b
1	50 b
2	10 b
4	30 b
6	30 b

Values followed by the same letters in the column do not differ significantly at the 5% level by a nonparametric comparison test of multiple proportions (Zar, 1999).
[a]Values obtained by mean of two experiments of five fruits each.
[b]Fruit not treated with DIPF.

(Fig. 2). When the peaches were wounded, the disease incidence was higher than 95% (Fig. 2). rhizopus rot incidence was 27 and 100% for Sunripe and Josefina nectarines, respectively, in the U-N treatment (Fig. 3).

Effect of DIPF on peaches inoculated with *R. stolonifer*

Rhizopus stolonifer spores treated with DIPF showed 100% germination and did not exhibit abnormalities in their development (data not shown). Some inoculated peaches treated with DIPF showed rhizopus rot symptoms; however, the disease incidence did not exceed 50%, whereas 100% of the non-treated fruits were infected (Table 4). In some fruits, DIPF was not able to prevent mycelial growth by *R. stolonifer* on peach surfaces; however, rot was not observed and the mycelia were formed only as the result of spore germination in the nutrient solution (Fig. 4).

Nectarine penetration by *R. stolonifer* observed under the SEM and light microscopes

There was neither spore germination nor penetration on unwounded nectarines inoculated with the spore suspension of *R. stolonifer* in water (Fig. 5a). In contrast, fungal growth around the wound was observed on wounded fruit inoculated with the spore suspension in water (Fig. 5b). Regardless of the presence of wounding, rhizopus rot developed on nectarine surfaces when nutrient solution was used (Fig. 5c,d). A swollen hypha at the end of the germ tube was observed on the intact surface of wounded nectarines inoculated with spore suspension in water. The same was observed on wounded and unwounded fruits inoculated with spore suspension in nutrient solution (Fig. 5e). *Rhizopus stolonifer* spores in water or nutrient solution placed on wounded nectarines germinated and penetrated the fruit through the unwounded surface, showing that direct penetration can occur.

Direct penetration of the intact nectarine surface by *R. stolonifer* was observed by light microscopy after inoculation of the fruit with a spore suspension in nutrient solution (Fig. 6a). The edge of the hypha became swollen, and a penetration peg breeched the intact cuticle of the nectarine surface (Fig. 6a). The same process was also observed under the SEM, with a swollen penetration structure (appressoria-like) at the end of the germ tube (Fig. 6b,c). When *R. stolonifer* was removed from the fruit surface, appressoria-like structures remained on the nectarine surface (Fig. 6d). It appeared that these appressoria-like structures were involved in penetration, as degradation of the fruit surface was observed (Fig. 6b,c,d).

Discussion

This study showed that *R. stolonifer* is able to directly penetrate unwounded stone fruits when spores germinate in an external nutrient supply. Esterases, especially cutinases, are produced by the fungal mycelia and germ tubes after spore germination. Penetration of healthy fruits by *R. stolonifer* is inhibited when an esterase inhibitor is used.

This study confirmed that *R. stolonifer* was unable to germinate without nutrients in the spore suspension, in accordance with the results of Nguyen-The *et al.* (1989).

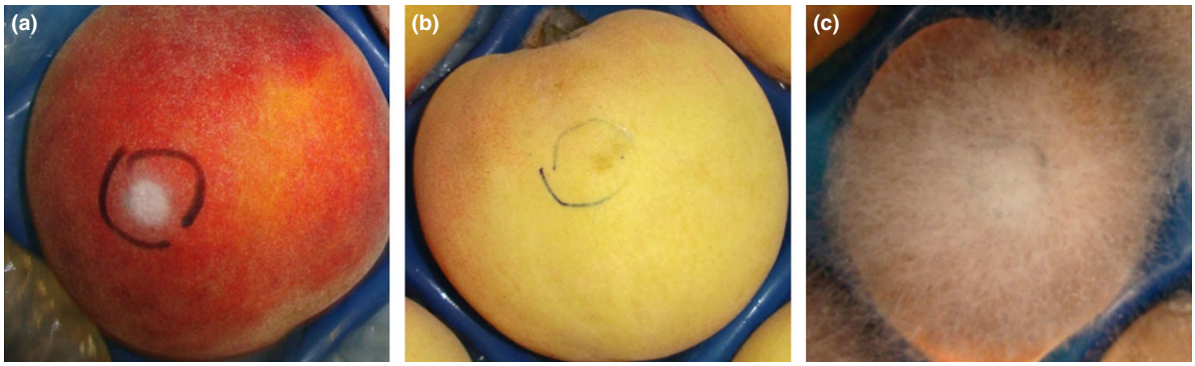

Figure 4 Peaches, 3 days after inoculation with a drop of *Rhizopus stolonifer* spore suspension in nutrient solution, treated (a, b) or not treated (c) with diisopropyl fluorophosphate (DIPF) 2 h after inoculation. Mycelial growth was observed around the site of deposition of the spore suspension in (a).

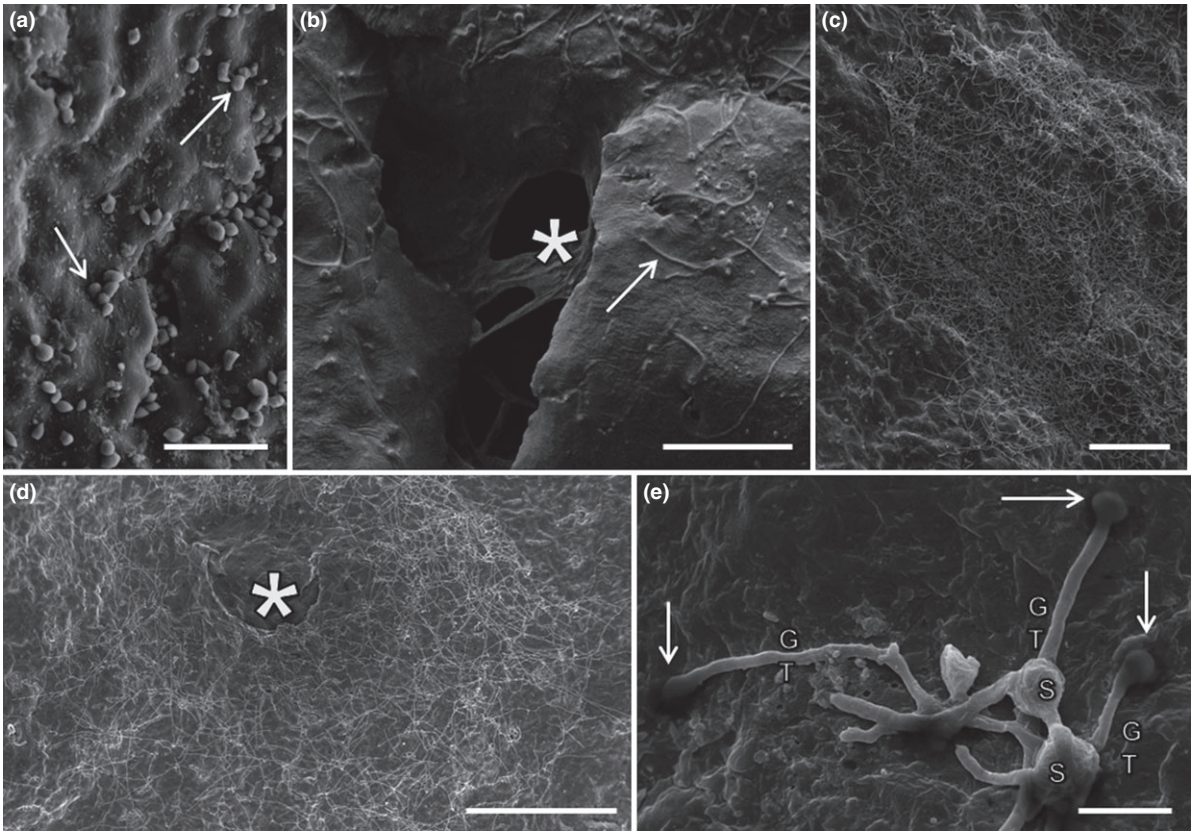

Figure 5 Scanning electron microscopy of *Rhizopus stolonifer* on nectarines. (a) Unwounded nectarine with nongerminated *R. stolonifer* spores (arrows) in water, bar = 50 μm; (b) germinated *R. stolonifer* spores (arrow) in water around a nectarine wound (*), bar = 200 μm; (c) unwounded nectarine surface with germinated *R. stolonifer* spores in nutrient solution, bar = 300 μm; (d) germinated *R. stolonifer* spores in nutrient solution around a nectarine wound (*), bar = 1 mm; (e) appressoria-like structures (arrows) at the end of germ tubes (GT) of germinated spores (S) in nutrient solution and penetration of the intact surface by the pathogen, bar = 20 μm.

Thus, spore germination requires exogenous sources of carbon and nitrogen as the spores contain insufficient endogenous carbon to germinate in water (Medwid & Grant, 1984). The fungus requires from 3 to 5 h in nutrient solution to germinate (Harter & Weimer, 1922) and produce infective hyphae (Srivastava & Walker, 1959). When in nutrient solution, germinated spores become swollen and form thickened mycelia, as previously observed for *Rhizopus arrhizus* and *R. stolonifer* (Buckley *et al.*, 1968).

Rhizopus stolonifer spores treated with indoxyl acetate showed a blue colour, as a consequence of the formation of insoluble indigo blue crystals due to esterase activity. These crystals were observed in the *C. graminicola* spore matrix (Pascholati *et al.*, 1993) and *U. viciae-fabae* (Deising *et al.*, 1992). These fungi produce esterases, especially of the cutinase type, which facilitate spore adhesion to the host cuticle and direct penetration. In the present study it was noted that germinated *R. stolonifer* spores produced greater amounts of esterases than nongerminated spores. This observation suggests that *R. stolonifer* spores have constitutive esterase enzymes. Therefore, any incapacity of *R. stolonifer* to penetrate directly is most probably due to the absence of spore germination and esterase production.

Rhizopus spores release several kinds of amino acids, enzymes and other proteins during their germination (Van Etten *et al.*, 1969). The fungus invades the host using pectinases, such as polygalacturonases and pectin methyl esterases, which macerate the host tissue during infection and colonization (Srivastava *et al.*, 1959; Spalding, 1963; Wells, 1968). In strawberries, the fungus infects wounded fruits and after its establishment secretes a pectolytic enzyme responsible for degrading the middle lamella, causing subsequent cellular breakdown and fruit softening (Maas, 1998). Germinating *Rhizopus* spores can release these enzymes, and pectolytic enzymes are produced even when the germination process is inhibited and new fungal colonies are not formed (Sommer *et al.*, 1963). According to those authors, only these enzymes were produced by *R. stolonifer* and were responsible for colonization. However, the present study demonstrates that the fungal spores can also produce esterases, which may be important for the infection process.

The indoxyl acetate solution added to the mycelial discs of *R. stolonifer* showed a darker blue colour than

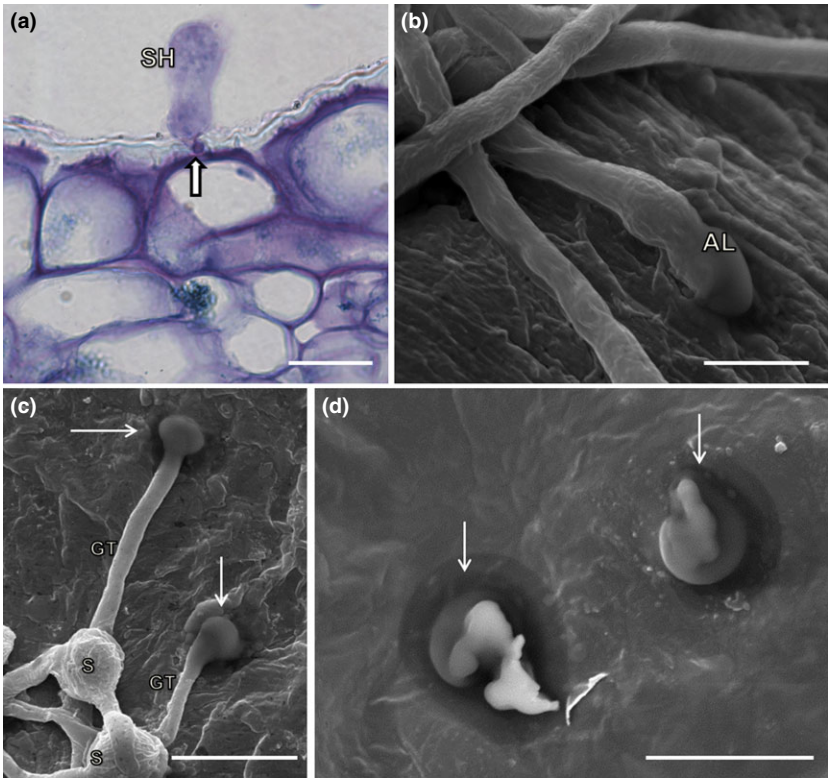

Figure 6 Light and scanning electron microscopy of *Rhizopus stolonifer* on nectarines. (a) Swollen hypha (SH) of *R. stolonifer* and a penetration peg (arrow) invading the intact nectarine surface, 12 h after pathogen inoculation, bar = 20 μm; (b) appressoria-like (AL) structures at the end of *R. stolonifer* germ tube and direct penetration into unwounded nectarine, bar = 10 μm; (c) *R. stolonifer* spores (S), germ tubes (GT) and appressoria-like structures (arrows) in nutrient solution on unwounded nectarine, bar = 20 μm; (d) appressoria-like structures (arrows) of *R. stolonifer* after removal of the pathogen mycelium with water and paint brush, bar = 20 μm.

the spore suspension, suggesting that the vegetative structures produce higher amounts of esterases, as has been observed in tomatoes inoculated with *R. stolonifer* (Velázquez-Del Valle *et al.*, 2005). Both inoculation methods produced 100% infection, but the mycelium was more invasive than the spores due to the higher production of pectolytic enzymes responsible for cellular breakdown (Velázquez-Del Valle *et al.*, 2005).

Rhizopus stolonifer, grown on a medium with cutin as the sole carbon source, grew and produced esterase enzymes. Previous investigations of *Pestalotia malicola* also revealed intense esterase activity when the fungus was grown on cutin medium (Sugui *et al.*, 1998). In the present study, the esterase activity of *R. stolonifer* was fourfold higher on cutin medium than glucose medium. This observation suggests that *R. stolonifer* must produce cutinase-type enzymes in order to use this carbon source. This is supported by an investigation of *Fusarium solani* f. sp. *pisi*, a pea pathogen, which could grow on cutin medium and exhibited esterase activity, 99·7% of which was due to cutinase (Stahl & Schäfer, 1992); mutants lacking the cutinase gene did not produce esterases and their growth was inhibited on the cutin media. Cutinase is important for fungal pathogenicity, and essential for fungal penetration through the host cuticle layer (Ettinger *et al.*, 1987). For example, cutinase-deficient mutants of *Colletotrichum gloeosporioides* lost their pathogenicity and ability to cause anthracnose on papaya (Dickman & Patil, 1986). The insertion of cutinase genes in *Mycosphaerella* sp., a pathogen that penetrates papaya exclusively through wounds, enabled it to penetrate unwounded fruits directly (Dickman *et al.*, 1989).

Diisopropyl fluorophosphate is an inhibitor of serine hydrolase enzymes, including esterases and proteases (Cohen *et al.*, 1967), and it is able to block cutinase activity (Pascholati *et al.*, 1993). Diisopropyl fluorophosphate can prevent disease development on corn leaves inoculated with *C. graminicola* (Pascholati *et al.*, 1993). In the present study, DIPF decreased infection of unwounded peaches by *R. stolonifer*, most probably due to the inhibition of cutinase, but it did not inhibit mycelium formation on the peach surface. Although some treated peaches became infected, this was probably a result of the product's volatility or the pathogen's fast development on the fruit.

Rhizopus stolonifer infection has already been reported for several hosts. The infection on grapes can occur due to the release of cellular substances caused by injuries to berry pedicels or incomplete connections between berry and pedicel (Lisker *et al.*, 1996; Tavares & Silva, 2006). Furthermore, it was found that the removal of the raspberry receptacle during harvest resulted in a cavity, which released substances that promoted a favourable humidity and nutrient supply for *Rhizopus* development (Davis, 1991). However, none of the previous reports showed direct penetration of *Rhizopus* into the hosts. The addition of peach juice to *R. stolonifer* spore suspension was found to promote pathogen growth and nectarine infection (Nguyen-The *et al.*, 1989). The

authors reported that cuticular microcracks became larger and the pathogen could develop and produce pectinolytic enzymes inside these microcracks, which hydrolysed the fruit epidermis cell wall. In the present study, no microcracks were observed using the scanning electron microscope, showing that, in their absence, *R. stolonifer* penetrated unwounded nectarine surfaces. Therefore, it seems that wounds are more important for juice release than for pathogen penetration. The released substances from wounds enabled *R. stolonifer* germination and the subsequent production of enzymes capable of breaking the fruit surface. As observed on grape surfaces inoculated with *R. stolonifer* (Lisker *et al.*, 1996), the present investigation also showed that spores germinated on nectarines near the wounds and then developed around and inside the wounds. Similarly, pathogen penetration was not observed through disruptions or natural openings such as stomata (Lisker *et al.*, 1996). An SEM study of *P. malicola* by Sugui *et al.* (1998) showed that germ tubes of this pathogen penetrated the host cuticle of quince fruit and plums directly and the pathogen developed over and under the cuticle surface, as observed for *R. stolonifer* in nectarines in the present investigation.

This study showed that *R. stolonifer* was able to infect and cause fruit rot in unwounded peaches and nectarines due to spore germination in nutrient solution placed on the fruit surface. In contrast, pathogen spores suspended in water were not able to penetrate unwounded stone fruit tissues. The disease incidence in unwounded fruits inoculated with *R. stolonifer* in nutrient solution was as high as in wounded inoculated fruits, showing that even in the absence of wounds the pathogen is able to penetrate its host if there is an external source of nutrients provided. When the spores were suspended in water the fruit surface most probably had insufficient nutrients to support spore germination and, consequently, pathogen penetration. These results may have practical implications for stone fruit transportation and storage because one damaged fruit can release nutrients essential for pathogen spore germination and subsequent penetration of nearby healthy and unwounded fruits.

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References

Agrios GN, 2005. *Plant Pathology*. 5th edn. San Diego, CA, USA: Elsevier Academic Press.

Amorim L, Martins MC, Lourenço SA, Gutierrez ASD, Abreu FA, Gonçalves FP, 2008. Stone fruit injuries and damage at the wholesale market of São Paulo, Brazil. *Postharvest Biology and Technology* **47**, 353–7.

Bautista-Baños S, Bosquez-Molina E, Barrera-Necha LL, 2014. *Rhizopus stolonifer* (soft rot). In: Bautista-Baños S, ed. *Postharvest Decay: Control Strategies*. London, UK: Academic Press, 1–44.

Bradford MA, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–54.

Buckley PM, Sommer NF, Matsumoto TT, 1968. Ultrastructural details in germinating sporangiospores of *Rhizopus stolonifer* and *Rhizopus arrhizus*. *Journal of Bacteriology* **95**, 2365–73.

Cohen JA, Oosterbaan RA, Berends F, 1967. Organophosphorus compounds. *Methods in Enzymology* **11**, 686–702.

Davis RP, 1991. Postharvest soft rot (leak disease). In: Ellis MA, Converse RH, Williams RN, Williamson B, eds. *Compendium of Raspberry and Blackberry Diseases and Insects*. St Paul, MN, USA: American Phytopathology Society, 23–4.

Deising H, Nicholson RL, Haug M, Howard RJ, Mendgen K, 1992. Adhesion pad formation and the involvement of cutinase and esterases in the attachment of uredospores to the host cuticle. *The Plant Cell* **4**, 1101–11.

Dickman MB, Patil SS, 1986. Cutinase deficient mutants of *Colletotrichum gloeosporioides* are nonpathogenic to papaya fruit. *Physiological and Molecular Plant Pathology* **28**, 235–42.

Dickman MB, Podila GK, Kolattukudy PE, 1989. Insertion of cutinase gene into a wound pathogen enables it to infect intact host. *Nature* **342**, 446–8.

Ettinger WF, Thukral SK, Kolattukudy PE, 1987. Structure of cutinase gene, cDNA, and the derived amino acid sequence from phytopathogenic fungi. *Biochemistry* **26**, 7883–92.

Harter LL, Weimer JL, 1922. Decay of various vegetables and fruits by different species of *Rhizopus*. *Phytopathology* **12**, 205–12.

Karnovsky MJ, 1965. A formaldehyde–glutaraldehyde fixative of high osmolality for use in electron microscopy. *Journal of Cell Biology* **27**, 137–8.

Kim KW, 2008. Vapor fixation of intractable fungal cells for simple and versatile scanning electron microscopy. *Journal of Phytopathology* **156**, 125–8.

Kolattukudy PE, 1980. Biopolyester membranes of plants: cutin and suberin. *Science* **208**, 990–1000.

Lilly VG, Barnett HL, 1951. *Physiology of the Fungi*. New York, NY, USA: McGraw-Hill.

Lima LM, Caixeta-Filho JV, Kassouf AL, Amorim L, 2009. Valoração de atributos de qualidade em pêssegos comercializados no Estado de São Paulo. *Revista de Economia e Sociologia Rural* **47**, 465–84.

Lisker N, Keren-Shacham Z, Sarig P, Zhtkhi Y, Ben-Arie R, 1996. The biology and pathology of the fungus *Rhizopus stolonifer*, cause of black mould disease of tables grapes in Israel. *Plant Pathology* **45**, 1099–109.

Maas JL, 1998. *Compendium of Strawberry Diseases*. St Paul, MN, USA: American Phytopathology Society.

Martins MC, Lourenço SA, Gutierrez ASD, Jacomino AP, Amorim L, 2006. Quantificação de danos pós-colheita em pêssegos no mercado atacadista de São Paulo. *Fitopatologia Brasileira* **31**, 5–10.

Massola Junior NS, Krugner TL, 2011. Fungos fitopatogênicos. In: Amorim L, Rezende JAM, Bergamin Filho A, eds. *Manual de Fitopatologia: Princípios e Conceitos*. São Paulo, Brazil: Ceres, 149–206.

Medwid RD, Grant DW, 1984. Germination of *Rhizopus oligosporus* sporangiospores. *Applied and Environmental Microbiology* **48**, 1067–71.

Nguyen-The C, Chamel A, 1991. Enzymatic degradation of isolated plant cuticles and nectarine fruit epidermis by culture filtrates of phytopathogenic fungi. *Agronomie* **11**, 101–8.

Nguyen-The C, Hugueney R, Arnoux M, 1989. Contribution à l'étude des voies de pénétration de parasites fongiques des nectarines *Monilinia laxa* (Ascomycète-Discomycète) et *Rhizopus stolonifer* (Zigomycète-Mucorale). *Agronomie* **9**, 271–6.

O'Brien TP, McCully ME, 1981. *The Study of Plant Structure: Principles and Selected Methods*. Melbourne, Australia: Termarcarphi Pty. Ltd.

Ogawa JM, 1995. Rhizopus rot. In: Ogawa JM, Zehr EI, Bird GW, Ritchie DF, Uriu K, Ueymoto JK, eds. *Compendium of Stone Fruit Diseases*. St Paul, MN, USA: American Phytopathology Society, 16.

Ogawa JM, English H, 1991. Stone fruit and their diseases. In: Ogawa JM, English H, eds. *Diseases of Temperate Zone Tree Fruit and Nut Crops*. Oakland, CA, USA: University of California, Division of Agriculture and Natural Resources, 125–242.

Pascholati SF, Deising H, Leite B, Anderson D, Nicholson RL, 1993. Cutinase and non-specific esterase activities in the conidial mucilage of *Colletotrichum graminicola*. *Physiological and Molecular Plant Pathology* **42**, 37–51.

Snowdon AL, 1990. *A Colour Atlas of Postharvest Diseases and Disorders of Fruit and Vegetables: General Introduction and Fruits*. London, UK: Wolfe Scientific.

Sommer NF, Creasy MT, Maxie EC, Romani RJ, 1963. Production of pectolytic enzymes by *Rhizopus stolonifer* sporangiospores after 'lethal' gamma irradiation. *Applied Microbiology* **11**, 463–6.

Spalding DH, 1963. Production of pectinolytic and cellulolytic enzymes by *Rhizopus stolonifer*. *Phytopathology* **53**, 929–31.

Srivastava DN, Walker JC, 1959. Mechanisms of infection of sweet potato roots by *Rhizopus stolonifer*. *Phytopathology* **49**, 400–6.

Srivastava DN, Echandi E, Walker JC, 1959. Pectolytic and cellulolytic enzymes produced by *Rhizopus stolonifer*. *Phytopathology* **49**, 145–8.

Stahl DJ, Schäfer W, 1992. Cutinase is not required for fungal pathogenicity on pea. *The Plant Cell* **4**, 621–9.

Sugui JA, Pascholati SF, Kunoh H, Howard RJ, Nicholson RL, 1998. Association of *Pestalotia malicola* with the plant cuticle: visualization of the pathogen and detection of cutinase and non-specific esterase. *Physiological and Molecular Plant Pathology* **52**, 213–21.

Tavares SCCH, Silva ILSS, 2006. Doenças da uva. In: Oliveira SMA, Terao D, Dantas SA, Tavares SCCH, eds. *Patologia Pós-colheita: Frutas, Olerícolas e Ornamentais Tropicais*. Brasília, Brazil: EMBRAPA Infromação Tecnológica, 821–85.

Van Etten JL, Koski RK, El-Olemy MM, 1969. Protein synthesis during fungal spore germination. IV. Transfer ribonucleic acid from germinated and ungerminated spores. *Journal of Bacteriology* **100**, 1182–6.

Velázquez-Del Valle MG, Hernández-Lauzardo AN, Bautista-Baños S, Rodríguez-Ambríz SL, 2005. Determinación de la actividad poligalacturonasa en el proceso de maceración causado por *Rhizopus stolonifer* en jitomate. In: *II Congresso Nacional de Fitopatología/VII Congresso Internacional de Fitopatología*, 2005. Chihuahua, Mexico: Sociedad Mexicana de Fitopatología, A.C, Abstract L-33.

Walker JC, 1972. *Plant Pathology*. 3rd edn. New Delhi, India: Tata McGraw-Hill Publishing Company Ltd.

Wells JM, 1968. Growth of *Rhizopus stolonifer* in low-oxygen atmospheres and production of pectic and cellulolytic enzymes. *Phytopathology* **58**, 1598–602.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York, NY, USA: Academic Press, 315–22.

Zar JH, 1999. *Biostatistical Analysis*. Upper Saddle River, NJ, USA: Prentice-Hall.