



Penetration by Botryosphaeriaceae species in avocado, guava and persimmon fruit during postharvest

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Abstract

Botryosphaeriaceae species have a wide host range and a worldwide distribution. These fungal species can colonize several plant organs, such as the trunk, leaves and fruit. Some Botryosphaeriaceae species cause important diseases on persimmon, avocado and guava fruit. However, there is a lack of information regarding the mechanisms of penetration by Botryosphaeriaceae species on these tropical and subtropical fruits. This study aimed to better understand the mechanisms involved in fungal penetration, host specificity and aggressiveness of *Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum* on avocado (*Persea americana*), guava (*Psidium guajava*) and persimmon (*Diospyros kaki*) fruit. Scanning electron microscopy (SEM) image analysis showed that in avocado fruit, the three studied Botryosphaeriaceae species penetrated through lenticels. In guava fruit, penetration through stomata was verified for *Botryosphaeria dothidea* and *Neofusicoccum parvum*. In persimmon fruit, an appressoria-like structure was observed for *B. dothidea*, which suggests direct penetration. Disease incidence in wounded fruit was 24% higher than in non-wounded fruit. *L. pseudotheobromae* and *N. parvum* showed differences in aggressiveness in guava fruit. The longest incubation period was observed for *N. parvum* inoculated on guava, with an average of 4.5 days, and the shortest incubation period was verified for *B. dothidea* inoculated on avocado, with an average of 2.8 days. The area under the disease progress curve (AUDPC) did not differ between Botryosphaeriaceae species on avocado, whereas on guava and persimmon fruit, the AUDPC was lower for *B. dothidea*. The information regarding penetration mechanisms and aggressiveness is important to improve postharvest disease control strategies.

KEYWORDS

Botryosphaeria dothidea, *Diospyros kaki*, *Lasiodiplodia pseudotheobromae*, *Neofusicoccum parvum*, *Persea americana*, postharvest diseases, *Psidium guajava*, scanning electron microscopy

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1 | INTRODUCTION

Botryosphaeriaceae species are the causal agents of numerous diseases in a diverse array of plants. These fungal species cause various disease symptoms, depending on the host and tissue affected, such as leaf spot, fruit and root rot, dieback and trunk canker. In addition to the ability to infect different plant tissues, the wide host range of these species contributes to the spread of pathogens worldwide (Slippers et al., 2017; Yang et al., 2017). Increased global trade and international air travel facilitates the transport of fresh material and favours the introduction of pathogens such as Botryosphaeriaceae species into new regions. Diseases caused by Botryosphaeriaceae species can remain latent, which complicates the identification of diseased plants during quarantine programmes (Slippers et al., 2017). In many woody plants, Botryosphaeriaceae species are usually present as endophytes, and stress factors, such as drought, moist soils or warm weather conditions, can promote symptom expression (Slippers et al., 2017).

Recent advances in molecular techniques have made the identification of several new species of plant pathogenic Botryosphaeriaceae possible (Wingfield et al., 2012). Crous et al. (2006) proposed a reorganization of Botryosphaeriaceae phylogenetic lineages based on sequences of the 28S rRNA gene. At the species level, characterization is possible by combining information about the large subunit of the nuclear ribosomal RNA gene (LSU-rpb2), the internal transcribed spacer 1 and 2, including the intervening 5.8S nrDNA gene (ITS), translation elongation factor 1- α (*tef1*) and the β -tubulin gene (*tub2*) (Yang et al., 2017). Advancements in molecular methods have contributed to the identification of new Botryosphaeriaceae species in new areas or the infection of new hosts (Wingfield et al., 2012).

Fruit rot caused by Botryosphaeriaceae species has been reported in field and postharvest such as the rot of pitaya (Valencia et al., 2005), jackfruit (Ni et al., 2008), mango (Marques et al., 2013), avocado (Eskalen & McDonald, 2011; Feijo et al., 2019; Ni et al., 2009; Valencia et al., 2019), guava (Cedeno et al., 1998; Nogueira Júnior et al., 2016) and persimmon (Nogueira Júnior et al., 2017; Palou et al., 2013). Symptoms caused by *Botryosphaeriaceae* species on fruit can be observed in the peduncle (stem end) and in the area surrounding the fruit calyx (stylar end). During postharvest storage, the lesions turn dark brown to black and can produce white to grey mycelia (Palou et al., 2013). In avocado, the first symptoms on fruit affected by Botryosphaeriaceae species are areas with slight softening and pulp discoloration followed by the development of mycelia and pycnidia in the stem end (Valencia et al., 2019). Guava fruit infected with these species usually displays a reddish-brown lesion on the stylar end. The grey mycelia become dark with disease progress, producing black globose pycnidia (Nogueira Júnior et al., 2016). In all of the above-mentioned fruit, lesions grow as the fruit ripens, leading to complete fruit colonization by these pathogens. In general, symptoms observed during postharvest can be caused by infection in early stages of fruit development in the field. The pathogen might be able to grow systemically in the plant, staying latent until fruit ripening or storage (Slippers & Wingfield, 2007). In

addition, infection of other plant tissues, such as shoots, petioles, leaves and flowers, can be a source of inoculum for fruit infection (Michailides, 1991).

Botryosphaeria dothidea, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum* are species that can be found in tropical, subtropical or temperate climate zones (Burgess et al., 2019). The incidence of these fungal species causing dieback in perennial plants may be related to the climatic conditions of each region. In China, for example, 65% of diseased samples collected in regions with temperate and subtropical monsoon climates were identified as *B. dothidea*. In regions with a subtropical monsoon climate, *Lasiodiplodia theobromae* and *N. parvum* were present in 23% and 5% of the samples, respectively (Yan et al., 2013). In Australia, species distribution was also correlated with weather conditions (Burgess et al., 2019). In tropical conditions, stylar-end rot of guava, stem-end rot of avocado and mango and fruit rot in persimmon caused by Botryosphaeriaceae species are serious postharvest diseases (Costa et al., 2010; Firmino et al., 2016; Nogueira Júnior et al., 2016, 2017). The incidence of postharvest diseases, like the ones caused by Botryosphaeriaceae species, depreciates the value of the fruit, alters their consistency, taste and colour and reduces the fruit's shelf life (Chitarra & Chitarra, 2005; Kluge et al., 2002; Martins et al., 2007).

Certain well-studied Botryosphaeriaceae species, such as *B. dothidea*, *L. theobromae* and *N. parvum*, have been confirmed to infect a wide range of hosts (Slippers & Wingfield, 2007). Cross-inoculations in blueberry and macadamia leaves and stems showed no host specificity for several Botryosphaeriaceae species in both of the above-mentioned hosts. Nevertheless, blueberry was more susceptible, regardless of the isolates of Botryosphaeriaceae species, as blueberry leaves were colonized in a shorter time than macadamia leaves (Liddle et al., 2019). Macadamia leaves are highly suberized when compared with the waxy blueberry leaves (Liddle et al., 2019), which can influence pathogen penetration and colonization. Moreover, Botryosphaeriaceae species have several strategies to penetrate their hosts. Penetration by natural plant openings, such as lenticels, stomata or wounds, has been observed for these fungi in different hosts (Slippers & Wingfield, 2007). However, direct penetration by appressoria formation was observed in *B. dothidea* on apples (Kim et al., 1999).

A better understanding of the penetration mechanisms may guide strategies for disease control (Baggio et al., 2016). Additionally, the infection process of Botryosphaeriaceae species and the disease development at postharvest of fruit such as avocado, persimmon and guava are not well understood. The hypotheses of this study were that the Botryosphaeriaceae species *Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum* (i) have host specificity, (ii) have different levels of aggressiveness, depending on the host and (iii) have varying mechanisms of penetration according to the fungal species and hosts. Therefore, cross-inoculations and studies about the penetration mechanisms of *B. dothidea*, *L. pseudotheobromae* and *N. parvum* were performed on avocado, guava and persimmon. Pathogen penetration was analysed by scanning electronic

microscopy (SEM). The aggressiveness of Botryosphaeriaceae species on the tested fruit was compared by recording the incidence, incubation period and lesion size.

2 | MATERIAL AND METHODS

2.1 | Inoculation procedures and disease evaluation

Three isolates from three distinct Botryosphaeriaceae species were chosen for cross-inoculations in avocado, guava and persimmon. *Botryosphaeria dothidea* (A14) was isolated from avocado and characterized by Firmino et al. (2016). *Lasiodiplodia pseudotheobromae* (C1) was isolated and characterized for the first time from persimmon by Nogueira Júnior et al. (2017). *Neofusicoccum parvum* (N10) was isolated from guava and previously characterized by Nogueira Júnior et al. (2016). All isolates were taken from preservation cultures and grown on PDA (potato dextrose agar) plates. Isolates were induced to sporulate on WA (water agar) amended with sterilized pine needles as a substrate in order to obtain conidia for cross-inoculation experiments (Slippers et al., 2004). As conidia production in sterilized needles of *Pinus* was not enough for all experiments, conidia of the three fungal species were also produced in autoclaved avocado peels inoculated with mycelia discs under sterile conditions. Inoculations of the fruits below were performed using spore suspensions at 10^4 conidia mL⁻¹ in all experiments.

Fruit of avocado cv. Quintal, guava cv. Kumagai (white flesh) and persimmon cv. Rama Forte were inoculated at the harvest point. Data on fruit sugar content and firmness were measured using a digital refractometer (Pocket PAL-1, Atago) and a fruit penetrometer (PTR-100, Instrutherm), respectively, at the same day of inoculations. Before inoculation, fruit was immersed in a 0.5% hypochlorite solution for 1 min and then rinsed in sterile distilled water and air-dried. Five each of avocados, guavas and persimmons ($n = 5$ biological replicates) were inoculated per isolate and per inoculation method, that is, inoculations performed in wounded and non-wounded fruit. Additionally, five disinfested and non-wounded of each of avocado, guava and persimmon were maintained in humidity chambers at the same conditions as the control. A 50- μ l drop from the spore suspension was placed in a circle-marked area (~2 cm diameter) on the middle portion of the fruit. In the control fruit, a 50- μ l drop of sterile water was placed in a circle-marked area (~2 cm diameter) on the middle portion of the fruit. In total, six experiments were conducted for cross-infection evaluations of Botryosphaeriaceae species in avocado, guava and persimmon fruit. Four experiments were performed without wounding the fruit ($n = 4$ experimental replicates) and two in wounded fruit ($n = 2$ experimental replicates). All experiments were performed in a completely randomized design. In experiments using wounded fruit, a wound 2 mm deep was made with a sterile histological needle after the drop of spore suspension was placed on the fruit.

After inoculation, all fruits were placed in humidity chambers (41 × 29 × 13 cm) at $\geq 95\%$ relative humidity and 28°C on a 12-h light/12-h dark cycle. After 24 h, inoculated fruit was maintained in a growth chamber under the same environmental conditions; however, the relative humidity was decreased to 80%. The variables incidence (proportion of diseased fruit samples out of the total), incubation period (time to first symptom appearance after inoculation) and lesion size were evaluated daily on the marked area. Two perpendicular measurements of the lesion diameter were taken per fruit, and the average diameter of both measurements was considered as the lesion size (cm).

2.2 | Scanning electron microscopy

Penetration mechanisms for each combination fruit vs Botryosphaeriaceae species were identified by using SEM. SEM was performed on three non-inoculated sites (control) and three inoculation sites ($n = 3$ biological replicates) per fruit and Botryosphaeriaceae species. Fruit inoculated without wounding was taken out of the humidity chamber 8 h after inoculation and prepared for SEM analysis. A 50- μ l drop of Karnovsky fixation solution (Karnovsky, 1965 modified) was placed in the region of inoculation on each fruit. After 10 min, the drop was drained using filter paper, and the sample was fixed again by a second fixation technique using osmium tetroxide (OsO₄) vapour (Kim, 2008; May De Mio et al., 2006). In the second fixation, the inoculated region was cut and placed in a Petri dish containing filter paper humidified with OsO₄ solution 2% and cacodylate 0.1 M (v/v). All samples were maintained under OsO₄ vapour for 24 h, dried under ambient conditions and mounted on aluminium stubs using double-sided carbon tape. After stub assembly, the samples were sputtered with gold. Images were captured using a LEO VP 435 scanning electron microscope at an accelerating voltage of 20 kV. The experiment for observations on SEM was performed twice.

2.3 | Data analysis

Generalized linear mixed models were fitted to logit-transformed disease incidence at the fifth and seventh day postpathogen inoculation. Wound, isolates and type of fruit were set as fixed factors and experimental replicates as random effect. Odds ratios and estimated probabilities of incidence of fruit rotting were calculated according to wounded or non-wounded fruit, to Botryosphaeriaceae species and type of fruit. Data analysis was performed using the lmer and lsmeans packages of R 3.6.0 software (R Core Team, 2019).

Nonlinear regression analyses were used to estimate the relationship between fruit rot incidence and time for all treatments (type of fruit, Botryosphaeriaceae species and inoculation procedure). Monomolecular model

$$Y(t) = Y_{max} - (Y_{max} - Y_0) * \exp(-r * t)$$

was fitted to the fruit rot incidence. In the models, $y(t)$ is the disease incidence in proportion, y_{\max} is the curve asymptote, y_0 is a constant of integration (equal to the value of y at $t = 0$), r is the disease progress rate, and t is time in days postinoculation (Campbell & Madden, 1990).

Data analysis for the variable incubation period was performed by applying a linear mixed model and estimations by the restricted maximum-likelihood method using the lmer package of R 3.6.0 software (R Core Team, 2019). Wounded or non-wounded fruit samples, Botryosphaeriaceae species and type of fruit were considered as main effects, and the experiment replications were considered as a random effect. A Tukey pairwise comparison test was used to compare fruit and Botryosphaeriaceae species ($p < .05$).

The area under the disease progress curve of lesion growth (AUDPC) was calculated with the daily average diameter of both measurements of the lesion size (cm). The AUDPC was estimated by trapezoidal integration (Berger, 1988) according to the following formula:

$$\text{AUDPC} = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)$$

where y_i is the lesion size at the i^{th} evaluation, t_i is time in days postinoculation at the i^{th} evaluation, and n is the total number of evaluations.

Data on AUDPC were calculated for wounded fruit for 2–5 days postinoculation (dpi) in the first experiment and from 3 to 6 dpi for the second experiment. Statistical analyses were performed by each type of fruit separately as avocado, guava and persimmon have different fruit sizes. Data were analysed by applying a linear mixed model and estimations by the restricted maximum-likelihood method using the lmer package of R 3.6.0 software (R Core Team, 2019). Botryosphaeriaceae species and type of fruit were considered as main effects, and an experimental replication was considered as a random effect. A Tukey pairwise comparison test was used to compare the AUDPC between Botryosphaeriaceae species ($p < .05$).

3 | RESULTS

Avocado, guava and persimmon fruit inoculated with Botryosphaeriaceae species showed typical symptoms of rot. Non-inoculated fruits did not show symptoms of rot. All fruit was covered by grey-to-whitish mycelia (Figure 1a–c). Especially in avocado, many pycnidia were formed for all three inoculated Botryosphaeriaceae species. After scratching pycnidia with a scalpel, it was possible to visualize cirrus on their surface (Figure 1c). A mass of conidia involved by mucilage could be observed in the

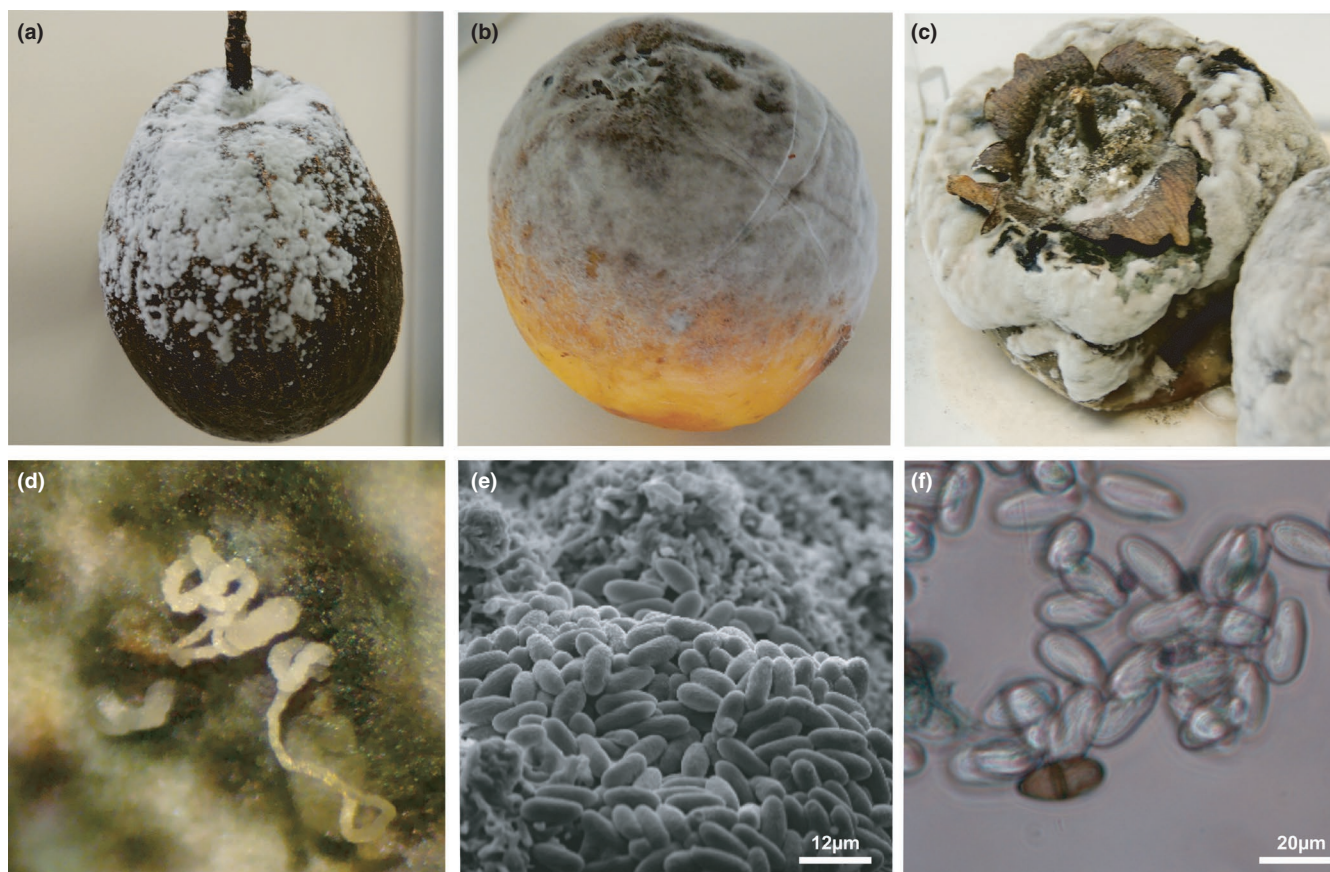


FIGURE 1 Symptoms of fruit rot caused by Botryosphaeriaceae species in avocado (a), guava (b) and persimmon fruit (c), eight days after inoculation. A cirrus of conidia of *Lasiodiplodia pseudotheobromae* formed in avocado fruit (d). Scanning electronic microscopy of a cirrus surface shows higher amount of conidia (e). Mature conidia of *Lasiodiplodia pseudotheobromae* in brown colour (f)

TABLE 1 Sugar content, firmness and standard errors for avocado, guava and persimmon fruit used in the experiments. Sugar content and firmness were measured on the day of inoculations

Fruit	Sugar content (%) ±SE*	Firmness (N) ±SE*
Avocado	6.78 ± 1.40	1.83 ± 0.51
Guava	8.84 ± 0.19	3.82 ± 0.94
Persimmon	12.63 ± 0.63	1.27 ± 0.53

*Number of sampled fruit: n = 6.

cirri by SEM (Figure 1e). Mature conidia of *L. pseudotheobromae* were usually brown (Figure 1f). The sugar content of inoculated fruit was on average 6.78%, 8.84% and 12.63% for avocado, guava and persimmon fruit, respectively (Table 1). The firmness of the fruit on the day of inoculation was 17.95 N, 37.47 N and 12.45 N for avocado, guava and persimmon fruit, respectively.

3.1 | Penetration mechanisms

In all avocado samples inoculated with all three Botryosphaeriaceae species, a penetration via the lenticels could be visualized (Figure 2a,d,g). For all three tested isolates, a germ tube, or two germ tubes in the case of bipolar germination, developed in the direction of the lenticel opening. Penetration through stomata, or microcracks, was not observed for any tested isolates. Additionally, there was no hint of direct penetration as no appressoria formation was verified.

Penetration through the stomata was observed for *B. dothidea* and *N. parvum* in guava (Figure 2b,h). In the two experiments conducted for SEM analysis, a clear penetration through the stomata was not observed for *L. pseudotheobromae* (Figure 2e). Neither appressoria formation nor penetration through microcracks was observed for any tested fungal species on guava.

Appressoria-like structures were visualized on persimmon inoculated with *B. dothidea* (Figure 2c). In some samples, the germ tube entered the wax layer on the persimmon epidermis. However, direct penetration without appressoria formation could not be concluded (Figure 2f,i). In both experiments, no penetration through opening structures or microcracks was observed.

3.2 | Host specificity

Host specificity of *B. dothidea*, *L. pseudotheobromae* and *N. parvum* for avocado, guava and persimmon fruit was not observed in cross-inoculation experiments. All three species were able to cause fruit rot in the three wounded and non-wounded fruits (Figure 3). The incidence of fruit rot on the last day of evaluation was significantly higher for wounded fruit ($p < .01$). The estimated probabilities for wounded and non-wounded fruit were higher than the actual probabilities calculated from the actual values of incidence (Table 2). However, no significant differences were verified between Botryosphaeriaceae species and fruit. The parameter r estimated by

the monomolecular model for fruit rot incidence data ranged from 0.05 to 0.87 (Table 3, Figure 3). There were no significant differences between wounded and non-wounded fruit in the estimated parameters r ($p = .06$), y_0 ($p = .78$) and y_{max} ($p = .49$).

3.3 | Isolate aggressiveness

There were significant interactions between the factors Botryosphaeriaceae species and fruit regarding the variable incubation period (Table 4). A significant difference between the incubation period of *L. pseudotheobromae* and *N. parvum* was observed on guava fruit (Figure 4). In general, the longest incubation period was verified for *N. parvum* inoculated on guava fruit for an average of 4.52 days, and the shortest incubation period was verified for *B. dothidea* inoculated on avocado, an average of 2.81 days (Figure 4). The incubation period was not different for wounded and non-wounded fruit ($p = .28$).

The first experiment evaluated was conducted from 2 to 5 dpi. After 5 dpi, most of the fruit was completely rotted and covered by dense mycelia (Figure 5). Large lesions were observed in the second experiment in the measurements recorded from 3 to 6 dpi (Figure 5). At 2 dpi, most of the fruit did not show symptoms in both experiments (Figure 3). Avocado fruit showed the largest lesions, with average maximum lesion diameters of 12 cm (Figure 5). The average maximum lesion diameter recorded for guava and persimmon was 7 cm. Since avocado fruit was larger, values of AUDPC were compared within fruit (Figure 6). The AUDPC of the rot caused by Botryosphaeriaceae species in avocado was similar for the three species evaluated, with values between 8.3 and 13.9. In diseased guava and persimmon, the lowest AUDPC was recorded in fruit inoculated with *B. dothidea*, whereas *L. pseudotheobromae* (8.6 and 7.9) and *N. parvum* (8.8 and 7.9) had similar values of AUDPC (Figure 6).

4 | DISCUSSION

Differences in the mechanisms of penetration by Botryosphaeriaceae species were observed between avocado, guava and persimmon fruit. In avocado and guava, fungi were able to penetrate through natural openings such as lenticels and stomata in guavas. Appressoria-like structures observed in persimmon fruit were formed by *B. dothidea* during the penetration stage (Figure 2). *L. pseudotheobromae* and *N. parvum* seemed to penetrate persimmon fruit directly, with no appressoria formation. Appressoria might have a mechanical function, exerting physical forces in order to penetrate through the fruit epidermis, as observed for *Magnaporthe grisea* (Money, 1995) and *Colletotrichum graminicola* (Ludwig et al., 2014). Ludwig et al. (2014) measured the internal turgor pressure of appressorium at approximately 5.4 MPa, which provides enough force to penetrate the leaf epidermis directly. However, appressoria formation can be an energy-consuming process, especially during the early stages of infection, and appressoria formation may be stimulated in the absence

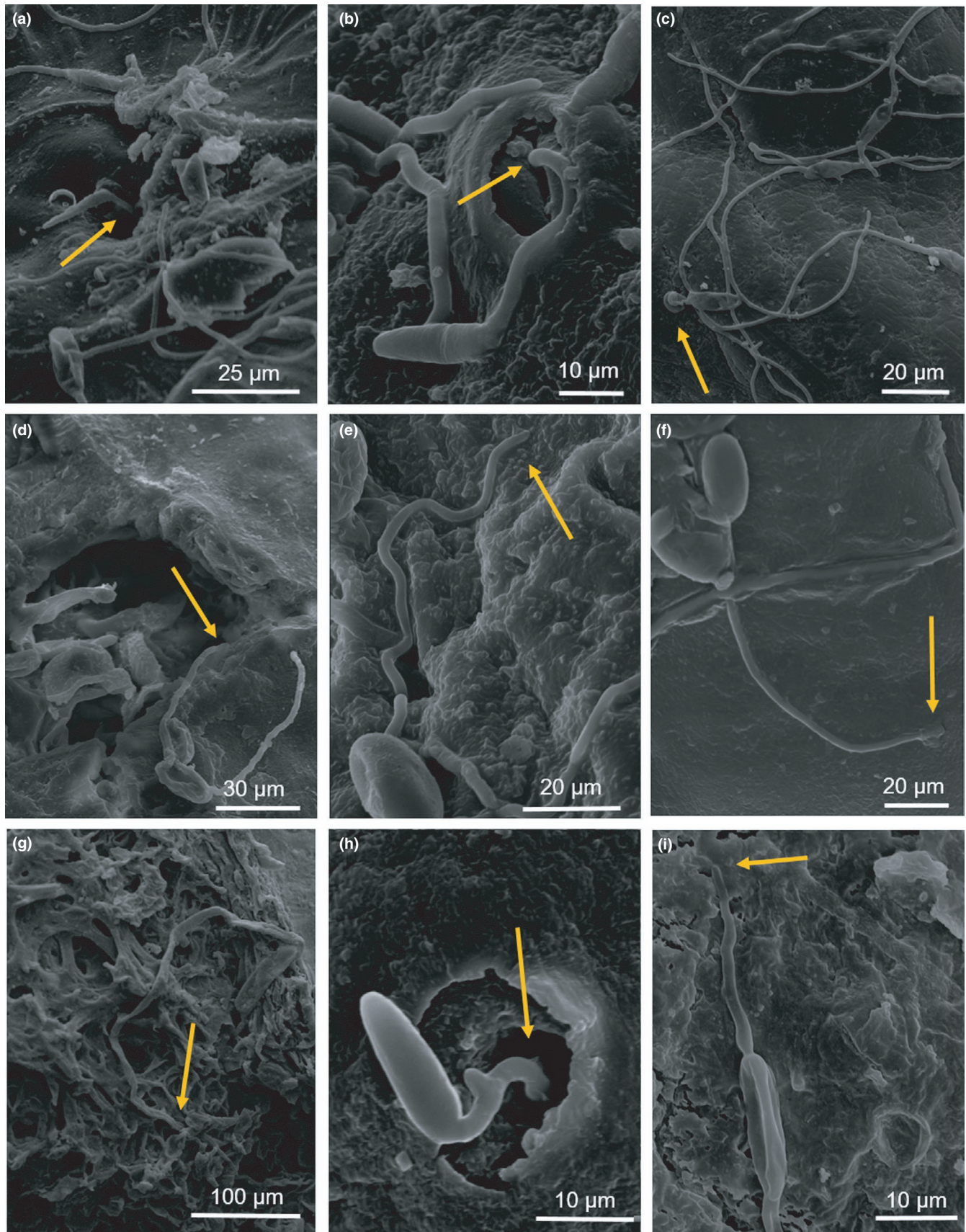


FIGURE 2 Penetration mechanisms of *Botryosphaeria dothidea* (a–c), *Lasiodiplodia pseudotheobromae* (d–f) and *Neofusicoccum parvum* (g–i) in avocado (a, d, g), guava (b, e, h) and persimmon (c, f, i) fruit. Arrow indicates penetration site

FIGURE 3 Fruit rot incidence (proportion) in avocado, guava and persimmon fruit caused by *Botryosphaeria dothidea* (a, b) *Lasiodiplodia pseudotheobromae* (c, d) and *Neofusicoccum parvum* (e, f) in function of time on fruit that were non-wounded (a, c, e) and wounded during the inoculation of the pathogens (b, d, f). Data of non-wounded fruit correspond to four experiments (n = 20 fruit) and data of wounded fruit correspond to two experiments (n = 10 fruit). Lines represent the monomolecular model fitted to observed disease incidence

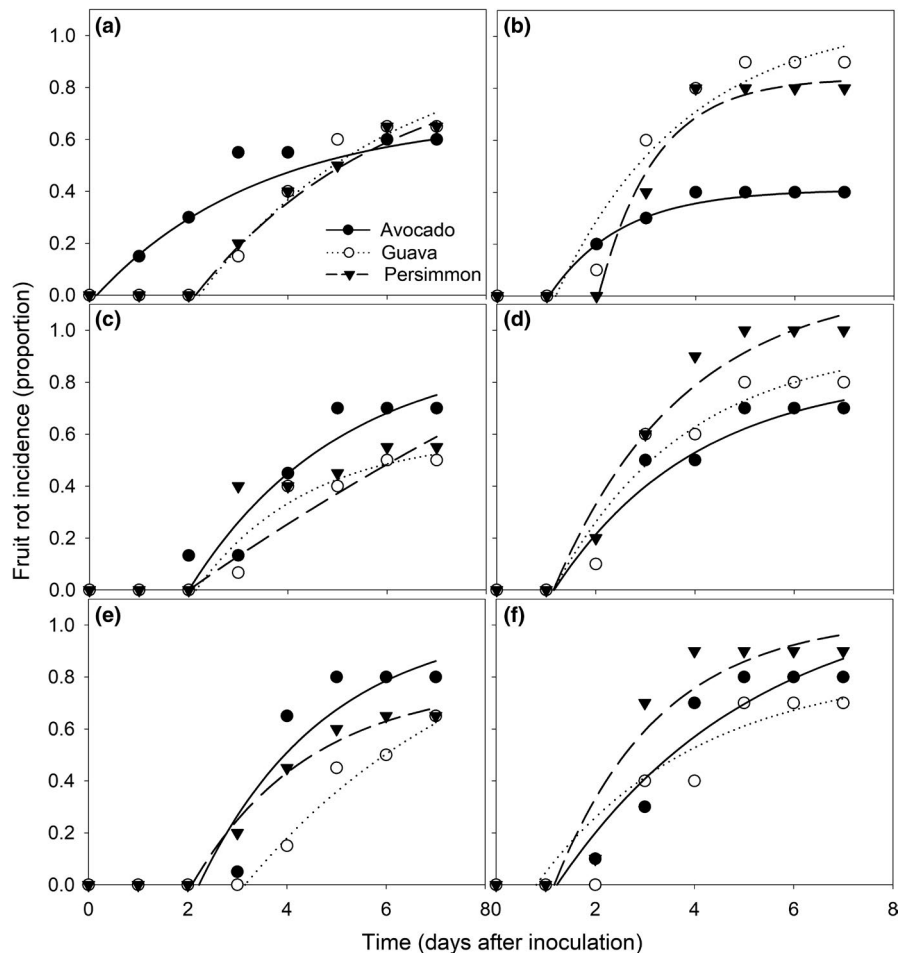


TABLE 2 Actual and estimated probabilities of fruit rot incidence on the fifth and seventh days postpathogen inoculation caused by *Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum* in wounded (data from four experiments) and non-wounded (data from two experiments) fruit (avocado, guava and persimmon)

Inoculation procedure	Experiment replicates	N inoculated fruit	Incidence (Probability)		
			Estimated ^a ±SE	Actual ^b ±SE	p-value
Wounded fruit	4	180	0.88 ± 0.05	0.77 ± 0.04	≤.001
Non-wounded fruit	2	90	0.65 ± 0.10	0.62 ± 0.04	

Note: Experimental replicates were applied as random effects.

^aEstimated probability based on logit transformation from generalized linear mixed model estimates.

^bActual probability based on calculated proportions from samples used in this study on the fifth and seventh days postpathogen inoculation.

of natural openings or wounds (Kim et al., 1999), as observed for *B. dothidea* on persimmon fruit (Figure 2). Appressoria-like structures from *Magnaporthe oryzae* are formed in hypha tips, whereas appressoria are formed in the end of the germinate tube. These structures have shown lower turgor and lower penetration efficiency than typical appressoria (Kong et al., 2013). The penetration is a pre-phase in pathogen infection. After this stage, the pathogen needs to establish parasitic relationships with the host for the success of infection (Agrios, 2005).

In general, it is not necessary for the pathogen to form appressoria-like structure during the penetration process on avocado and guava. In fact, the pathogen presents tropism towards

the lenticels (Guan et al., 2015), which confirms the occurrence of penetration into natural openings of guava and avocado. In apples, natural openings in the fruit exocarp are an important factor that increases fruit susceptibility (Guan et al., 2015). Kim et al. (1999) did not observe appressoria formation of *B. dothidea* on apples when spores were close to natural openings. *B. dothidea* germ tubes could easily enter through the lenticel (Kim et al., 1999). Additionally, Marsberg et al. (2017) identified the presence of genes of carbohydrate active enzymes (CAZymes) in the *B. dothidea* genome. As observed for *Zymoseptoria tritici*, CAZymes can degrade plant cell wall components such as cellulose, hemicellulose, xylan, xyloglucan and pectin. CAZymes might be necessary for direct penetration and

TABLE 3 Estimated parameters (\pm standard error) of the monomolecular model fitted to the data of incidence of fruit rot caused by *Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum* as a function of time on wounded or non-wounded fruit of avocado, guava and persimmon

Inoculation procedure	Botryosphaeriaceae species	Fruit	Parameters		
			r (\pm SE)	y_0 (\pm SE)	y_{\max} (\pm SE)
Non-wounded fruit	<i>Botryosphaeria dothidea</i>	Avocado	0.30 \pm 0.42	-0.03 \pm 0.17	0.69 \pm 0.44
		Guava	0.26 \pm 0.27	-0.77 \pm 0.69	0.99 \pm 0.60
		Persimmon	0.26 \pm 0.30	-0.69 \pm 0.71	0.93 \pm 0.60
Non-wounded fruit	<i>Lasiodiplodia pseudotheobromae</i>	Avocado	0.33 \pm 0.30	-0.87 \pm 0.86	0.93 \pm 0.40
		Guava	0.45 \pm 0.54	-0.96 \pm 1.51	0.59 \pm 0.29
		Persimmon	0.05 \pm 0.25	-0.28 \pm 0.31	2.67 \pm 11.83
Non-wounded fruit	<i>Neofusicoccum parvum</i>	Avocado	0.39 \pm 0.22	-1.41 \pm 0.93	1.02 \pm 0.27
		Guava	0.20 \pm 0.25	-1.02 \pm 0.92	1.16 \pm 0.99
		Persimmon	0.42 \pm 0.21	-1.09 \pm 0.69	0.78 \pm 0.16
Wounded fruit	<i>Botryosphaeria dothidea</i>	Avocado	0.69 \pm 1.32	-0.43 \pm 1.22	0.46 \pm 0.21
		Guava	0.38 \pm 0.19	-0.61 \pm 0.36	1.08 \pm 0.22
		Persimmon	0.87 \pm 0.46	-4.11 \pm 4.52	0.84 \pm 0.11
Wounded fruit	<i>Lasiodiplodia pseudotheobromae</i>	Avocado	0.35 \pm 0.33	-0.42 \pm 0.46	0.84 \pm 0.35
		Guava	0.38 \pm 0.36	-0.52 \pm 0.59	0.95 \pm 0.38
		Persimmon	0.38 \pm 0.70	-0.65 \pm 0.42	1.19 \pm 0.27
Wounded fruit	<i>Neofusicoccum parvum</i>	Avocado	0.25 \pm 0.20	-0.41 \pm 0.31	1.14 \pm 0.50
		Guava	0.31 \pm 0.37	-0.27 \pm 0.41	0.84 \pm 0.42
		Persimmon	0.47 \pm 0.19	-0.76 \pm 0.39	1.03 \pm 0.15

Factor	Incubation period			
	df_n^a	df_d^a	F-value	p-value
Inoculation procedure	1	5.57	1.39	.28
Botryosphaeriaceae species	2	155.68	4.21	.01
Fruit	2	156.85	4.01	.02
Inoculation procedure \times Botryosphaeria species	2	155.68	0.07	.93
Inoculation procedure \times fruit	2	156.85	1.52	.22
Botryosphaeriaceae species \times fruit	4	156.18	5.59	\leq .001
Inoculation procedure \times Botryosphaeriaceae species \times fruit	4	156.18	0.95	.43

^aNumerator degrees of freedom (df_n) and denominator degrees of freedom (df_d); degrees of freedom calculated using the Satterthwaite formula for a mixed model; F-value for testing effect and probability (significance) level of F-value (p-value).

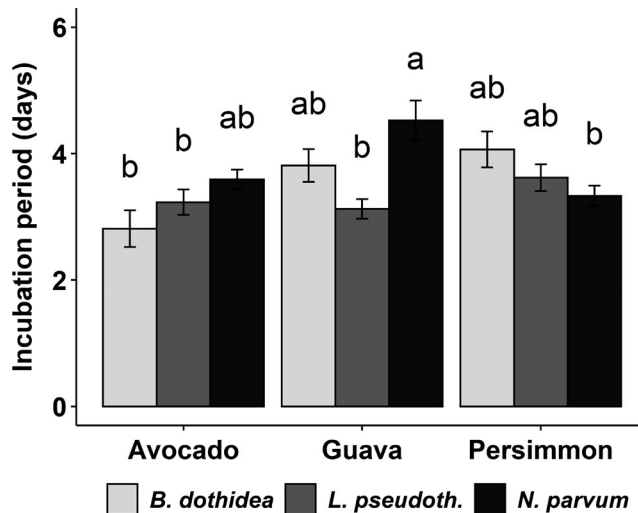
TABLE 4 Effect of inoculation procedure (wounded and non-wounded fruit), Botryosphaeriaceae species (*Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum*), fruit (avocado, guava and persimmon) and their interaction data of incubation period based on a mixed model analysis of variance

used to avoid host defences by *B. dothidea* (Marsberg et al., 2017). Another study demonstrated that 70% of the tested strains from Botryosphaeriaceae species were able to produce several extracellular enzymes, such as cellulases, laccases, xylanases, pectinases, pectin lyases, amylases, lipases and proteases (Esteves et al., 2014).

It can be hypothesized that *N. parvum* and *L. pseudotheobromae* penetrate microcracks caused by handling during harvest, transport or storage, as observed for *Rhizopus stolonifer* on nectarine (Nguyen-The et al., 1989). However, infections by Botryosphaeriaceae species in non-wounded tissue or regions where no lenticels are present could be attributed to direct penetration with or without

the presence of appressoria-like structures by cuticle-degrading enzymes (Kim et al., 1999). In mango, only *N. ribis* and *N. parvum* were able to infect non-wounded fruit directly by mechanisms of cell wall degradation (Li et al., 2020). These species may produce several extracellular enzymes able to degrade cell walls (Esteves et al., 2014), which are important for this type of direct penetration. Additionally, direct penetration by *R. stolonifer* on nectarine was associated with the production of esterase enzymes, such as cutinases, pectinases, polygalacturonases, pectin methyl esterases and other pectolytic enzymes. *R. stolonifer* was able to penetrate directly when an external source of nutrients was provided (Baggio et al., 2016). For

FIGURE 4 Incubation period of *Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum* on avocado, guava and persimmon. A linear mixed model was fitted to the data of incubation period applying experiment replications as random effect (wounded and non-wounded experiments were analysed together as the factor wound had no effect on incubation period). Data were compared by analysis of variance (ANOVA) and by multiple comparison applying Tukey test. Means sharing the same letter are not significantly different ($p < .05$). Bars indicate standard error ($n = 18$ fruit)



Botryosphaeriaceae species, Sammonds et al. (2019) observed that high germination rates slow germ tube growth on a surface rich in cellulose, indicating that this compound may work as source of nutrients. Additionally, these authors identified that hydrophobic or hydrophilic surfaces of fruit of varying hardness were not related to germination or growth of Botryosphaeriaceae species (Sammonds et al., 2019). Furthermore, we did not recognize differences in germ tube growth in this study.

Grey-to-whitish lesions were visualized in all cross-inoculations of *B. dothidea*, *N. parvum* and *L. pseudotheobromae* on avocado, guava and persimmon fruit. These typical symptoms of fruit rot diseases were similar in all three host species after artificial inoculation (Figure 1). Avocado, guava and persimmon fruit are from

three distinct botanical families. Avocado is from the Lauraceae family (Bergh & Ellstrand, 1986), guava is from the Myrtaceae family (Piccinin et al., 2016), and persimmon is from the Ebenaceae family (Butt et al., 2015). In the literature, *B. dothidea* do not present any host preference when causing trunk diseases (Marsberg et al., 2017). Moreover, Botryosphaeriaceae species isolated from diseased native

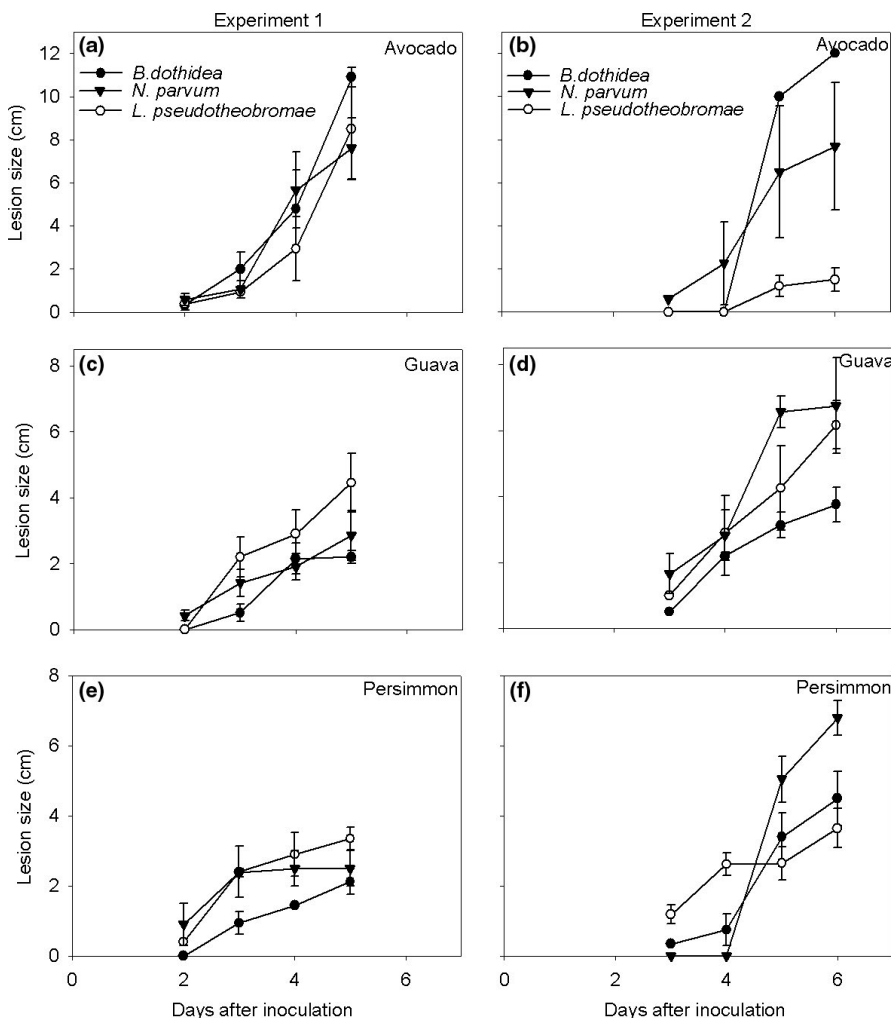


FIGURE 5 Lesion growth in function of time of *Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum* inoculated on avocado, guava and persimmon. Data represent the diameter of lesion size (cm) for the first (a, c, e) and second experiment (b, d, f) on wounded fruit ($n = 6$ fruit)

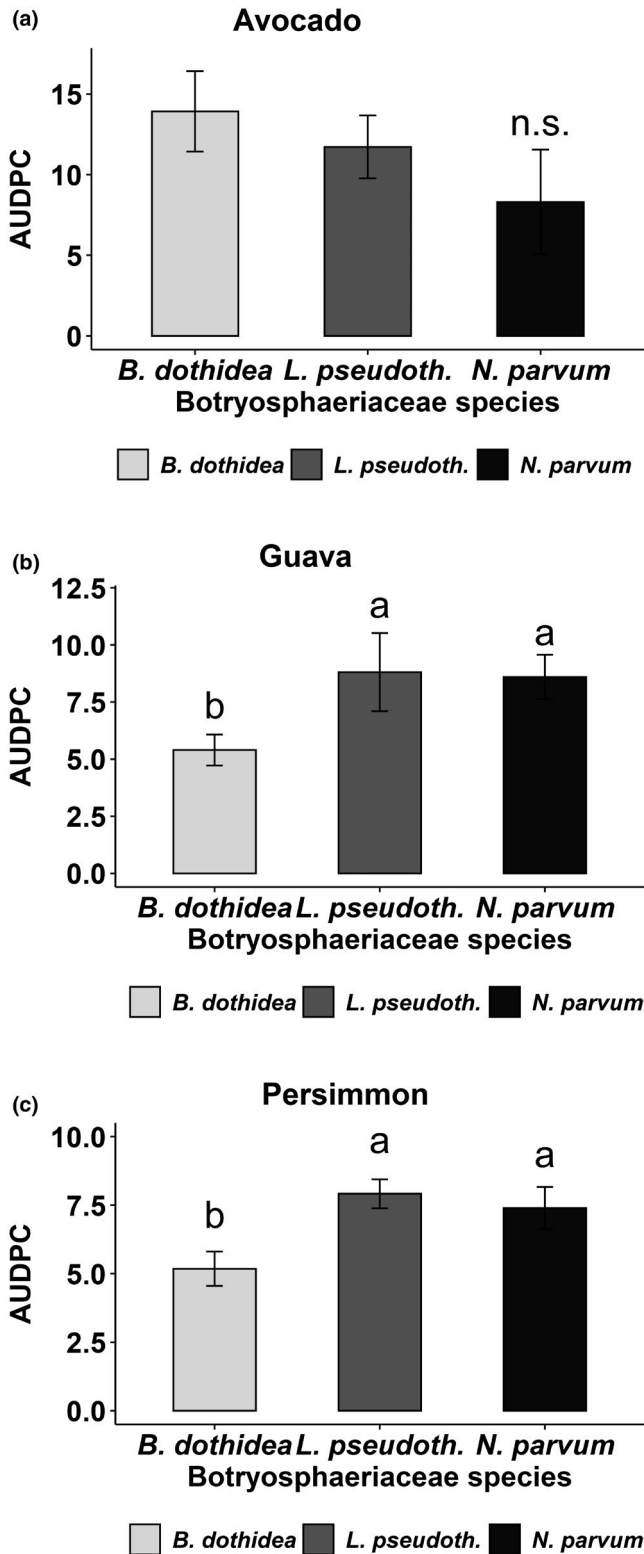


FIGURE 6 Area under the disease progress curve (AUDPC) of *Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Neofusicoccum parvum* inoculated on avocado (a), guava (b) and persimmon (c). A linear mixed model was fitted to the data of AUDPC for each fruit applying experiment replications as random effect. Botryosphaeriaceae species were compared within fruit by analysis of variance (ANOVA) and by multiple comparison applying Tukey test. Means sharing the same uppercase or lowercase letters are not significantly different ($p < .05$). Bars indicate standard error ($n = 6$ fruit)

penetration, it was able to infect and colonize the host tissue effectively as no significant differences were observed for the length of the incubation period between wounded and non-wounded fruit (Table 4). Variations in the incubation period and the AUDPC variables of the Botryosphaeriaceae species indicate differences in pathogen aggressiveness. The shortest incubation period was recorded for *B. dothidea* infecting avocado (Figure 4). *B. dothidea* was previously isolated from avocado fruit (Firmino et al., 2016) and had shown high AUDPC values on avocado, which did not differ from those of other species on this fruit (Figures 5 and 6). However, *B. dothidea* showed the lowest values of AUDPC on guava and persimmon, differing from those of *L. pseudotheobromae* and *N. parvum*. In general, the *B. dothidea* isolate was less aggressive. Besides, *B. dothidea*, *L. pseudotheobromae* and *N. parvum* showed significant differences in the length of incubation period on guava fruit (Figure 4). Curiously, the longest incubation period was recorded for *N. parvum* on guava, and *N. parvum* was isolated from guava fruit (Nogueira Júnior et al., 2016). In our study, there was no correlation with the incubation period or the AUDPC of guava fruits. Botryosphaeriaceae species have been shown to be cosmopolitan and to infect different plant organs of tropical and subtropical fruit. It seems that all three tested isolates are well adapted to the three types of fruit.

5 | CONCLUSIONS

The three Botryosphaeriaceae species tested had no host specificity and showed slight differences in variables related to aggressiveness such as the incubation period. Besides host specificity and aggressiveness, Botryosphaeriaceae species were demonstrated to have distinct penetration mechanisms. Botryosphaeriaceae species were able to penetrate through natural openings such as lenticels and stomata in avocado and guava. An appressoria-like structure was observed for *B. dothidea* in the absence of natural openings in persimmon. These results add information about the penetration mechanisms of Botryosphaeriaceae species on avocado, guava and persimmon, which has practical implications for postharvest disease management. Penetration through natural opening structures or direct penetration has implications for the possibility of infection wherever conidia land on the fruit surface, and environmental conditions are conducive to infection. Strategies to reduce the inoculum and possible latent infections from fields, which may produce inoculum for secondary infections during the postharvest period,

and ornamental trees in the Western Balkan region and inoculated in the stems of 21 hosts showed a broad host range and were able to cross-infect taxonomically unrelated trees (Zlatković et al., 2018).

Wounding fruit before inoculation increased the number of infected fruit samples as penetration was favoured by direct contact with nutrients (Tables 2 and 3). In this case, the pathogen does not need to cross the barrier of the epidermis (Figure 3). After

must be prioritized for more effective disease control. Additionally, fruit must be handled carefully, especially during harvest, transport and storage, in order to avoid wounds and the consequent increase in postharvest disease incidence. Knowledge about the pathogenic mechanism of penetration may aid the development of more effective postharvest disease control strategies, such as the improvement of fruit handling or the use of a protective barrier like waxes. Wax application may reduce postharvest diseases by formation of a physical barrier that reduces wounds or avoids penetration via natural openings, by modification of the fruit atmosphere, and by its antifungal activity (Gonçalves et al., 2010), consequently decreasing pathogen penetration and the disease progress rate. Knowledge about pathogen aggressiveness combined with information about pathogen prevalence may help in decision-making about the best regions for fruit cultivation and improvement of control in regions of high disease risk.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Electron Microscopy Laboratory Prof. Elliot Watanabe Kitajima ESALQ—USP and the National Council for Scientific and Technological Development (CNPq, Brazil) for a granted scholarship (B.L.N.). BLN is thankful to Prof. Marcel Bellato Spósito and Prof. Lillian Amorim for their comments and support to the work and manuscript. Open access funding enabled and organized by ProjektDEAL.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Barbara Ludwig Navarro contributed to project conceptualization, experimentation, data analysis, preparation of original draft and review and editing; **Juan Pablo Edwards Molina** contributed to experimentation, and review and editing; **Antonio Fernandes Nogueira Júnior** contributed to project conceptualization, experimentation, data analysis, and review and editing.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/jph.13055>.

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How to cite this article: Navarro, B. L., Edwards Molina, J. P., & Nogueira Júnior, A. F. (2021). Penetration by Botryosphaeriaceae species in avocado, guava and persimmon fruit during postharvest. *Journal of Phytopathology*, *00*, 1–12. <https://doi.org/10.1111/jph.13055>