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Research article

Role of cuticle in *Colletotrichum* infection of chili fruit

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Abstract

Importance of the work: Anthracnose is a major fungal disease that causes fruit rots in *Capsicum* in the tropics and subtropics worldwide. *Capsicum annuum*, the most economically important species, lacks resistance. However, non-wound inoculation of some genotypes has shown differential resistance at different fruit maturity stages, indicating the importance of the fruit cuticle in resistance.

Objectives: To investigate the physical and biochemical characteristics of chili fruit cuticular wax in chili genotypes that have differential host reactions to *Colletotrichum truncatum*.

Materials & Methods: Four *Capsicum annuum* genotypes at two fruit maturity stages (mature green and ripe red), were non-wound inoculated with *Colletotrichum truncatum* isolate '158ci'. The fruit cuticle thickness and wettability were measured and wax profiles were analyzed. Spore germination and appressoria formation of *C. truncatum* were assessed on cuticular waxes extracted from different genotypes.

Results: Differential anthracnose reactions occurred between fruit stages and chili genotypes. Cuticle thickness and wettability did not correlate with the anthracnose resistance. Wax profiles were similar at both fruit stages for CA758, CA857 and CA1113, whereas CA965 had the lowest triterpenoids/sterols at both fruit stages. Spore germination levels on extracted cuticular waxes from most genotypes were above 80%, while they were the lowest and slowest on CA965. Appressoria formation on all chili genotypes was above 67% higher than the control, indicating that the wax promoted the formation of appressoria.

Main finding: There was no correlation between host resistance reaction to infection by *C. truncatum* and the composition of cuticular wax of the chili genotypes, indicating that other host mechanisms influence resistance.

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Introduction

Chili anthracnose is caused by at least 30 species of *Colletotrichum* with *C. truncatum* (formerly *C. capsici*) along with *C. scovillei* (formerly *C. acutatum*) and *C. siamense* (formerly *C. gloeosporioides*) being the most important species affecting chili fruit production in the tropics and sub-tropics worldwide (Mongkolporn, 2018; Mongkolporn and Taylor, 2018; De Silva et al., 2019).

Capsicum annuum L. is the world's most important *Capsicum* species as a commercially grown chili and is well known for lack of resistance to anthracnose. In contrast, genotypes of the closely related *Capsicum* species, *Ca. chinense* and *Ca. baccatum* have been shown to be resistant to several *Colletotrichum* species that are pathogenic on *Ca. annuum* (Mongkolporn et al., 2010; De Silva et al., 2021). Therefore, breeding for anthracnose resistance has relied on the transfer of resistance genes from these closely related *Capsicum* species to *Ca. annuum* through interspecific crossing and the embryo rescue of hybrid lines (Voorrips et al., 2004; Lin et al., 2007; Sun et al., 2015; Mahasuk et al., 2016; Kim et al., 2017; Mishra et al., 2017; Suwor et al., 2017; Zhao et al., 2020).

Screening for anthracnose resistance has usually been based on differential anthracnose reactions in *Capsicum* spp. germplasm using a wounding bioassay where a needle is used to penetrate the cuticle and epidermis before applying a spore suspension of inoculum (Montri et al., 2009; Mongkolporn et al., 2010). More recently, non-wound inoculation techniques have identified resistant genotypes of *Ca. annuum* and *Ca. chinense* at different fruit maturity stages, after inoculation with different *Colletotrichum* species (De Silva et al., 2021). These bioassays used mature green and ripe red fruit inoculated with spore suspensions of eight *Colletotrichum* species that cause chili anthracnose in Asia.

Cuticle resistance may be a result of: 1) the chemical and physical properties of the cuticular wax, with the cuticle thickness and wettability providing a pre-existing structural defense; 2) induced defense of R-genes that mobilize pathogenesis-related (PR) proteins such as cutinases, glucanases, chitinases and proteinases to target the site of appressoria formation and infection; or 3) both structural and induced defenses (Serrano et al., 2014; Auyong et al., 2015).

The cuticle is an extracellular layer covering the epidermal cells of the aerial plant organs to protect the plants from water loss and abiotic or biotic stresses (Serrano et al., 2014). The cuticle comprises two layers of polymer cutin and cuticular waxes that produce the hydrophobic or hydrophilic properties of the cuticle. The cuticular wax is the outermost layer and most of its compounds are derived from very long chain fatty acids including alkanes, aldehydes, alcohols, ketones and esters (Yeats and Rose, 2013).

Cuticular wax represents a barrier to pathogen invasion and its composition varies substantially with plant species, cultivars and growth stages (Leide et al., 2007; Yeats and Rose, 2013). However, *Colletotrichum* spp. can penetrate the cuticle using enzymatic degradation and mechanical rupture (Yeats and Rose, 2013; Auyong et al., 2015), with the latter involving the formation of appressoria from germinating spores, which develop penetration pegs (Ranathunge et al., 2012). Various cuticular wax constituents, such as alcohols, fatty acids, alkanes and triterpenoids, have been reported to interact with pathogens. For example, some fatty acids, alcohols and alkanes promoted fungal spore germination and appressorium formation of *Magnaporthe grisea* (syn. *M. oryzae*; Hegde and Kolattukudy, 1997), *Colletotrichum gloeosporioides* (Podila et al., 1993) and *Austropuccinia psidii* (dos Santos et al., 2019). Alkanes and triterpenoids from pear fruit were reported to inhibit *Alternaria alternata* spore germination and mycelial growth (Yin et al., 2011; Li et al., 2014).

In *Capsicum*, the major waxes of 50 genotypes were found to be composed of fatty acids, alcohols, alkanes and triterpenoids/sterols, with different wax profiles between genotypes (Parsons et al., 2013). These wax compounds have been reported to have interactions with pathogens. The aims of the current study were: 1) to use a non-wound bioassay to assess the host reactions of four chili genotypes at two fruit maturity stages; 2) to evaluate the structure of the cuticle and the composition of the fruit cuticular waxes of these chili genotypes; and 3) to correlate the cuticular waxes to the host reaction after infection by *C. truncatum*.

Materials and Methods

Anthracnose assessment on chili fruit

Four chili genotypes of *Capsicum annuum* L. were selected from a preliminary anthracnose resistance screening trial based on differential anthracnose reactions at different fruit maturity stages to *C. truncatum*. The selection was based on the consistency of the anthracnose reactions at the different fruit stages inoculated with *C. truntacum* isolate '158ci'. Each chili plant was grown in a black 30cm plastic pot. The chili plants were placed at 50 cm × 80 cm spacing in a bird-proofed, wire-meshed house with a transparent glass roof at the

Department of Horticulture, Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom, Thailand from March to August 2018. Five plants of each chili genotype were presented in a randomized complete block experimental design. Five chili fruit each at mature green (approximately 25–35 d after flowering; DAF) and ripe (approximately 37–46 DAF) maturity stages were collected for anthracnose assessment. The chili fruit characteristics of the four genotypes are provided in Table S1.

C. truncatum isolate '158ci' was selected because this pathotype was shown to differentially interact with *Capsicum* spp. according to the fruit maturity stage (Montri et al., 2009) and has been used in the chili breeding program at Kasetsart University. The '158ci' was cultured on half potato dextrose agar (Difco, USA.) and incubated for 7 d at 28°C with a 12 hr photoperiod until sporulation. Spore suspension was prepared at 5.0×10^6 spores/mL for drop inoculation on non-wounded fruit. The harvested chili fruit was dipped in deionized water followed by a dip in sterilized water. Each fruit was then inoculated with a 10 µL drop of the prepared spore suspension onto the fruit center (Kanchana-udomkan et al., 2004). The inoculated fruit was incubated in a moist chamber for 3 d. The experiment was repeated and produced similar results (Table S2)

Due to the different fruit sizes between the chili genotypes, anthracnose symptoms were assessed as the lesion percentage (% lesion) proportional to the whole fruit size (Montri et al., 2009) from 3–15 d after inoculation. A modified area under the disease progress curve (AUDPC) was calculated following the formula developed by Simko and Piepho (2012), which was called the area under the disease progress stairs, to exhibit quantitative anthracnose severity:

$$AUDPC = \left[\sum_{i=1}^{n-1} \frac{y_i+y_{i+1}}{2} \times (t_{i+1} - t_i)\right] + \left[\frac{y_1+y_n}{2} \times \frac{D}{n-1}\right] \quad (1)$$

where y_i is the % lesion size proportional to fruit size, t_i is number of days at the i^{th} observation, n is the total number of observations and D is the difference between the total number of days and the first day of observation.

Physical characteristics of chili fruit cuticle

Cuticle thickness

Fresh chili fruit samples at the mature green and ripe stages from each genotype were transversely cut in half after harvest. Cross sections were performed with an ultrathin razor blade (Gillette; USA) to achieve thin cross-sectioned tissues with 1–2 cell layers. The cuticle thickness was measured under a light microscope following Domínguez et al. (2008). The sliced tissue was placed on a glass microscope slide with deionized water and covered with a cover slip. Under a light microscope at 10× magnification, the cuticle thickness of the chili fruit section was measured at three positions (far left, center and far right), as shown in Fig. 1) using a Dino-Lite digital microscope accompanied with a Dino Capture 2.0 software (AnMo Electronics Corp; Taiwan). Five fruit (replicates) were measured for each chili genotype.

Cuticle wettability

Fresh chili fruit samples were cleaned after harvest by dipping the fruit once in deionized water for 30 s. The fruit was dried at room temperature then fixed horizontally on glass slides with plasticine to ensure the fruit surface was level. A 10 µL drop of deionized water was placed on the surface of each fruit and the water droplet side view image was taken with a digital camera. The contact angle between the water droplet and the fruit surface was measured following Zhao and Jiang (2016), as shown in Fig. 2. A representative contact angle of each sample was averaged from the angles measured from both sides. Five fruit (replicates) were measured for each chili genotype.

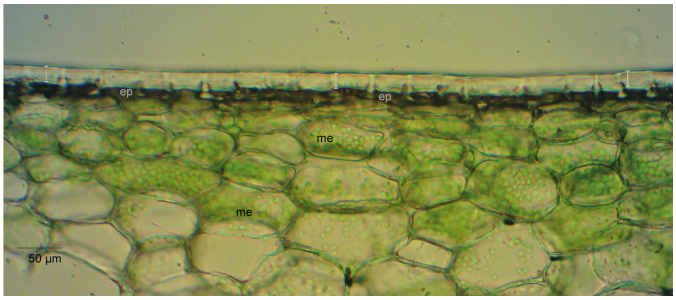

Fig. 1 Anatomy of cross-sectioned chili fruit pericarp delineating three measurement positions (white bars) of cuticle thickness under a light microscope (10×), where ep = epidermis and me = mesophyll

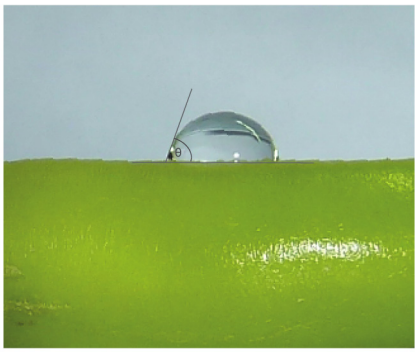

Fig. 2 Measurement of contact angle (θ) of water droplet on chili fruit surface

The experiments of cuticle thickness and wettability were repeated and produced similar results (Table S3).

Cuticle surface structure

The cuticle surface structure of chili fruit samples was examined using a scanning electron microscope (SEM; JSM-IT500HR; JEOL Ltd.; Japan), modified from Wu et al. (2017). Three chili fruit each at mature green and ripe stages were collected from each chili genotype. The harvested fruit samples were washed in deionized water and dried. A 5×5×1 mm piece of chili fruit pericarp was excised from the middle of the fruit and fixed in 2.5% glutaraldehyde (volume per volume in phosphate buffer, pH 7.2) at 4 °C overnight. The fixed tissue was rinsed in 0.2 M phosphate buffer, pH 7.2, for 15 min, 3 times. The chili tissue was then dehydrated in ethanol in a serial concentration of 30%, 50%, 70%, 80%, 90% and 2×100% for 15 min at each step. The tissue was dried in a critical point drier (K850, Emitech Ltd; UK) using CO_2 as the intermedium. The dry sample was placed on an aluminum stub and coated with gold particles. The coated surface was examined using an SEM (JSM-IT500HR; JEOL Ltd.; Japan) at 500× magnification.

Identification of cuticular wax compounds

The cuticular wax of chili fruit samples at mature green and ripe stages was extracted in chloroform (GC grade) with hexadecane as the internal standard (Parsons et al., 2013). Then, the wax extract was evaporated under nitrogen gas and derivatized with *N,O*,-bis (trimethylsilyl) trifluoroacetamide (Sigma-Aldrich; Switzerland) and pyridine (Sigma-Aldrich; Switzerland). Wax compounds were identified using a gas chromatography mass spectrophotometer (GC-MS; Shimadza, GC 2010 QP; Japan) using a silica capillary column HP-5MS-UI (60 m × 0.25 mm internal diameter × 0.25 µm film; Agilent Technology; USA) as the stationary phase. The wax sample was manually injected into the GC-MS injector port under splitless mode at a 1.0 mL/min helium flow rate. The injector temperature was 220 °C. The oven temperature was held at 80 °C for 2 min, then raised at 10 °C/min to 200 °C, held for 2 min and raised at 3 °C/min to 320 °C and held for 30 min. Wax constituents were identified based on mass spectral matching with the NIST-98/Wiley 07 library and quantified by comparing their GC peak areas to an external standard (hexadecane). Chili fruit was flattened to measure the surface area using a leaf area meter (Li-3100C; Li-Cor Inc.; USA). Wax amounts were normalized to the chili sample surface areas.

Colletotrichum spore germination and appressorium formation on isolated cuticular wax

The cuticular wax extracted from each chili genotype was coated on a glass slide, modified from Liu et al. (2011) and Tang et al. (2017). A sample (10 µL) of the cuticular wax dissolved in chloroform was spread on a glass slide restricted to a 7 mm diameter circle. Control treatments comprised water agar on a glass slide (control 1), a glass slide coated with chloroform (control 2) and a non-coated glass slide (control 3). All the prepared glass slides were kept in a fume hood at room temperature for 10 min to let the chloroform evaporate before dropping a spore suspension sample on each glass slide (Zhu et al., 2020).

Spore suspension of *C. truncatum* '158ci' was prepared at 1.0×10^6 spores/mL, of which 10 µL (approximately 10,000 spores) droplets were transferred onto the wax on the prepared glass slides. The inoculated slides were incubated in a plastic box at high humidity and were placed in the dark. The fungal spores were dyed with lactophenol cotton blue and assessed for percentage germination and appressoria formation at 3 hr, 6 hr and 9 hr under a light microscope (Olympus Cx31; Olympus Optical Co. Ltd.; Japan) at 40×. The spore germination and appressoria formation was repeated at a lower spore concentration of 0.5×10^6 spores/mL and produced similar results (Table S5).

Statistical analyses

Analysis of variance and mean comparison

Analysis of variance was performed using the IBM SPSS Statistics software (version 28.0; IBM Corp.; USA). Means between chili genotypes were compared using Tukey's honestly significant difference test, while means between chili fruit maturity stages used an independent sample t test.

Correlation of fruit physical characteristics and anthracnose reactions

The anthracnose reactions were analyzed for % lesion (lesion size proportional to fruit size), AUDPC correlating to the cuticle thickness and wettability (contact angle) using Pearson's correlation coefficient (r). The genotypes without anthracnose infections were excluded from the correlation analysis.

Principal component analysis

Principal component analysis (PCA) was performed with the data from 99 types of the identified cuticular wax compounds using the MetaboAnalyst 5.0 program (Pang et al., 2021). Prior to performing the PCA, the cuticular wax data were normalized by median, transformed by $\log_{10}$ and standardized by mean centering. The PCA score plot was performed from three biological replicates according to the wax identification method.

Results

Differential anthracnose reactions in Capsicum annuum genotypes at two fruit maturity stages

Among the four chili genotypes, CA758 did not show anthracnose symptoms at both fruit maturity stages, CA857 had only ripe fruit infected, whereas in contrast, CA965 had only the mature green fruit infected, and CA1113 had both fruit stages infected (Table 1). There were no differences in lesion size to fruit size percentage (%L) between the infected genotypes of the same fruit maturity. The %L values were in the range 17–19% in the mature green fruit and 9–21% in the ripe fruit. The chili genotypes with infected mature green fruit had AUDPC values in the range 33–56, while the genotypes with infected ripe fruit had AUDPC values in the range 32–74. The anthracnose symptoms were visualized as early as 7 d onward after inoculation with averages in the range 10–13 d.

Physical properties of chili fruit cuticle

The cuticle thickness of all chili genotypes was in the range 14–17 µm (Table 2) without any clear differences between the chili genotypes and fruit stages. The cuticle wettability (measured as the contact angle) for all genotypes was in the range 69–88°, where a lower angle indicated more wetting. All chili genotypes had a hydrophilic fruit cuticle. The ripe fruit of CA1113 became more hydrophilic (Table 2).

The morphology of the chili fruit wax examined using the SEM instrumentation displayed three different patterns: parallel curve, non-parallel curve and rough (Fig. 3). CA758 and CA857 had the parallel curved surface pattern, whereby the fruit surface was wavy and had longitudinal bands forming in parallel with smaller ridges in between those bands in the same direction. CA1113 had the non-parallel curve pattern, whereby the wavy surface had longitudinal bands intervened by shallow ridges in a non-parallel direction to the main bands. The curves on the chili surface did not form in

Table 1 Anthracnose reactions assessed as % lesion size to fruit size and area under the disease progress curve (AUDPC) on mature green (G) and ripe (R) fruit of four chili genotypes

Genotype	% Lesion			AUDPC		
	Green	Ripe	*p* value	Green	Ripe	*p* value
CA758	0	0	na	0	0	na
CA857	0	21.3±14.8	na	0	74.0±71.1	na
CA965	19.1±4.4	0	na	33.1±14.7	0	na
CA1113	17.1±7.1	8.9±11.8	0.221	56.4±30.3	32.3±62.6	0.461
p value	0.599	0.181	-	0.160	0.353	-
Coefficient of variation (%)	31.2	94.0	-	57.3	125.8	-

Values expressed as mean ± SD; *p* value greater than 0.05 indicates no statistical difference based on independent sample t test between chili genotypes compared within same column and between fruit stages of same chili genotype, respectively, and chili genotypes not infected were excluded from statistical analysis; na = not applicable

Table 2 Values (mean ± SD) of cuticle thickness and wettability (measured as contact angle) of chili fruit

Genotype	Thickness (µm)			Contact angle (°)		
	Green	Ripe	*p* Value	Green	Ripe	*p* value
CA758	16.6±0.5	17.3±0.8	0.115	86.5±2.5	75.9±10.4	0.058
CA857	15.6±2.0	16.1±2.6	0.720	87.5±4.2	83.3±10.9	0.493
CA965	15.8±2.2	15.7±2.7	0.934	85.4±2.1	83.4±4.2	0.371
CA1113	14.1±1.5	15.3±1.3	0.187	84.5±12.1^{A}	69.0±7.4^{B}	0.041
p value	0.147	0.426	-	0.916	0.052	-
Coefficient of variation (%)	11.5	12.1	-	7.3	12.9	-

p value greater than 0.05 indicates no statistical difference based on Tukey's honestly significant difference test between chili genotypes within same column and based on independent sample t test between fruit stages of the same chili genotype, respectively.

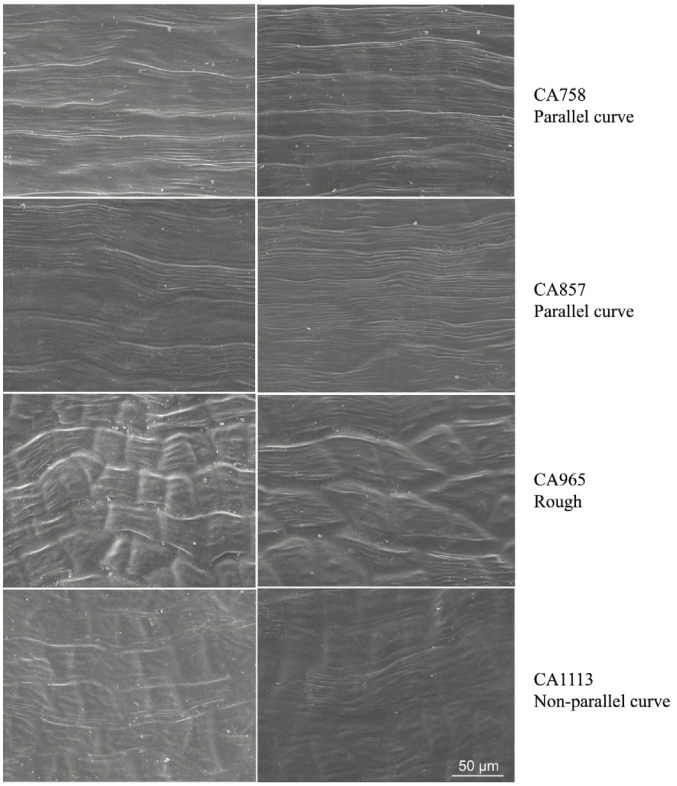

Fig. 3 Chili fruit surface of four chili genotypes at mature green (left) and ripe (right) taken using a scanning electron microscope

parallel and the surface looked smoother than for the other patterns. CA965 had the rough surface pattern, where the roughness was the outcome of deep holes between the parallel bands. In all chili genotypes, the surface patterns did not change with fruit maturity but were smoother in the more mature fruit.

There was a slight negative correlation between the AUDPC and the cuticle thickness (p = 0.037), while the other interactions showed no correlation (Table 3).

Table 3 Correlation coefficient (r) between anthracnose reactions and physical cuticular characteristics (thickness and wettability), where two measurements of anthracnose reactions are % lesion size proportional to fruit size and AUDPC (area under the disease progress curve)

	% Lesion	AUDPC	Thickness	Contact angle
% Lesion	1	0.906**	-0.384	0.056
AUDPC		1	-0.481*	-0.033
Thickness			1	-0.188
Contact angle				1

*, ** = correlations significant at $p < 0.05$ and < 0.01, respectively

Cuticular wax profiles of different chili genotypes and maturity stages

In total, 99 cuticular wax compounds were identified from the four *Ca. annuum* genotypes (Table S6). The compounds were grouped into seven wax classes: acids, alcohols, alkanes, amides, esters, ketones and triterpenoids/sterols. The alkanes and triterpenoids/sterols were the two major wax classes, accounting for 36–54% and 9–49%, respectively, of the total wax compounds (Fig. 4, Table S4). CA965 had the lowest triterpenoids/sterols. The wax classes with the second largest proportion (less than 19%) were alcohols, ketones and acids, while the smallest proportion (less than 5%) was found in amides and esters. Different chili genotypes had different wax profiles. Mature green and ripe fruit of the same chili genotype had similar wax profiles, except for CA965, which had a substantial increase in the alcohols in the ripe fruit.

Colletotrichum spore germination and appressorium formation

Among the *Ca. annuum* genotypes, at 3 hr, 9–63% of the spores germinated (Table 4). The chili genotype CA857 had

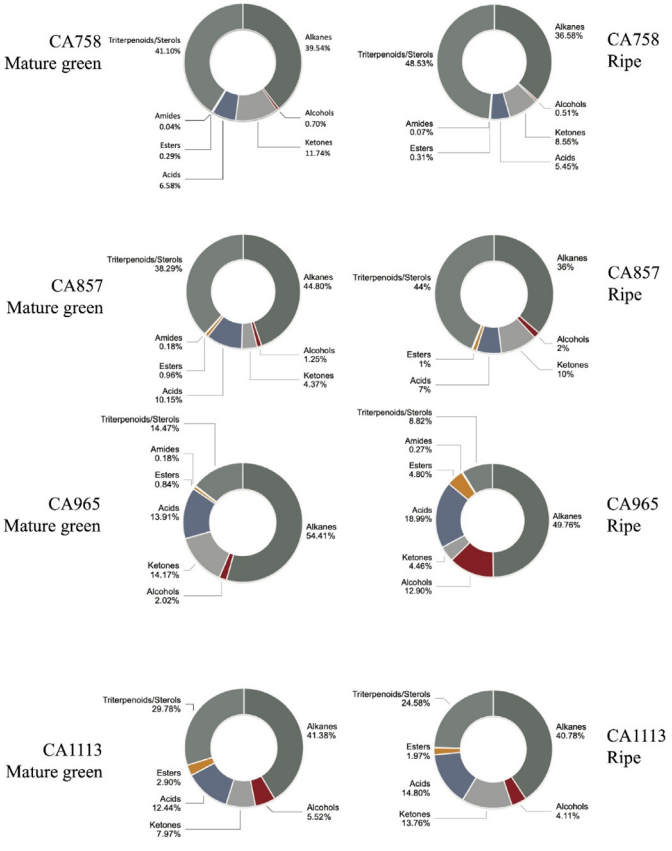

Fig. 4 Cuticular wax profiles of four *Capsicum annuum* genotypes (CA758, CA857, CA965 and CA1113) at mature green and ripe fruit stages

Table 4 *Colletotrichum* spore germination and appressorium formation based on number of germinating spores on glass slides coated with cuticular wax isolated from mature green and ripe chili fruit and spore suspension prepared at 1.0×10^6 spores/mL

Time (hr)	Genotype	% Spore germination			% Appressorium		
		Green	Ripe	*p* value	Green	Ripe	*p* value
3	CA758	43.2±9.5^{c}	45.7± 6.9^{c}	0.742	65.0±14.3^{a}	69.8±4.1^{ab}	0.594
	CA857	62.2±7.4^{b}	63.3±14.5^{b}	0.918	74.5±4.6^{a}	89.7±8.7^{a}	0.058
	CA965	20.7±1.9^{Ad}	8.9±3.2^{Bd}	0.007	72.6±1.5^{a}	55.4±10.2^{b}	0.050
	CA1113	23.7±3.7^{Bd}	36.3±3.5^{Ac}	0.012	33.5±5.7^{Ab}	19.7±5.3^{Bc}	0.032
	Water agar	90.2±2.1^{a}	90.2±2.1^{a}	-	45.2±1.1^{b}	45.2±1.1^{c}	-
	Chloroform	6.6±1.5^{e}	6.6±1.5^{d}	-	76.3±16.4^{a}	76.3±16.4^{ab}	-
	Non coated	6.6±2.1^{e}	6.6±2.1^{d}	-	79.2±7.7^{a}	79.2±7.7^{ab}	-
6	CA758	79.0±4.3^{ab}	67.2±9.3^{b}	0.106	93.2±4.4^{a}	91.6±4.1^{a}	0.650
	CA857	81.7±3.1^{ab}	83.7±2.4^{a}	0.417	93.5±1.9^{a}	95.4±5.0^{a}	0.606
	CA965	49.5±10.8^{Abc}	20.9±6.8^{Bc}	0.018	90.6±3.3^{a}	78.9±19.4^{ab}	0.365
	CA1113	65.2±28.8^{ab}	83.7±6.0^{a}	0.380	93.1±3.4^{Aa}	56.2±9.1^{Bb}	0.003
	Water agar	95.1±1.4^{a}	95.1±1.4^{a}	-	42.8±1.2^{b}	42.8±1.2^{c}	-
	Chloroform	16.0±1.7^{d}	16.0±1.7^{c}	-	95.0±2.8^{a}	95.0±2.8^{a}	-
	Non coated	17.1±4.5^{cd}	17.1±4.5^{c}	-	80.3±22.8^{a}	80.3±22.8^{ab}	-
9	CA758	83.0±0.9^{ab}	81.2±4.2^{a}	0.566	94.8±1.0^{ab}	96.4±0.8^{a}	0.116
	CA857	84.3±3.3^{ab}	89.1±0.8^{a}	0.077	96.1±0.6^{ab}	98.5±1.0^{a}	0.055
	CA965	67.0±21^{Ab}	34.1±6.6^{Bb}	0.024	97.1±1.8^{a}	89.6±3.8^{ab}	0.224
	CA1113	84.5±2.0^{ab}	91.2±4.6^{a}	0.095	93.6±2.2^{Aab}	67.9±8.3^{Bc}	0.007
	Water agar	96.0±0.7^{a}	96.0±0.7^{a}	-	42.4±1.3^{d}	42.4±1.3^{d}	-
	Chloroform	33.0±11.5^{c}	33.0±11.5^{b}	-	76.4±14.6^{c}	76.4±14.6^{bc}	-
	Non coated	20.9±3.0^{c}	20.9±3.0^{b}	-	80.5±3.0^{bc}	80.5±3.0^{abc}	-

Means with same uppercase superscript in a row are not significantly (*p* greater than 0.05) different based on independent sample t test.
Means with same lowercase superscript in a column are not significantly (*p* greater than 0.05) different based on Tukey's honestly significant difference test.

the highest spore germination (approximately 60%), CA758 had the second highest (approximately 40%) and CA965 had the lowest (9%). Among the three controls, water agar was the best for promoting spore germination (90%), while chloroform and non-coated were equally poorer at promoting spore germination (7%). The spores germinated more at 6 hr and 9 hr accordingly, whereby the spore counts ended at 9 hr. The chili genotypes CA758, CA857 and CA1113 had similarly high germination rates (above 80% at both fruit stages), which was similar to the water agar control (96%). CA965 reached maximum spore germination at 67% in mature green fruit and at 34% in ripe fruit. Chloroform and non-coated had spore germination rates of only 33% and 21%, respectively.

In the chili genotypes CA758, CA857 and CA965, over 90% of the germinating spores formed appressoria, while for CA1113, 70% of the germinating spores formed appressoria. Among the controls, water agar had the lowest appressorium formation of 40%, and the other controls had 76% appressorium formation. Fig. 5 shows germinating spores and appressoria.

Principal component analysis

PCA provided an overview of the various separation patterns into groups of the 99 identified compounds from the cuticular wax of four chili genotypes and two maturity fruit stages. Two principal components significantly accounted for 62.4% of the original variance, with PC1 explaining 36.9% and PC2 explaining 25.5% of the total variance. Along both PCs, all samples with different fruit stages within the same chili genotype were grouped together. Therefore, four individual groups according to chili genotype were formed (Fig. 6). The PCA indicated that the cuticular wax compounds were associated with the chili genotype; thus, they were less associated with the anthracnose reactions.

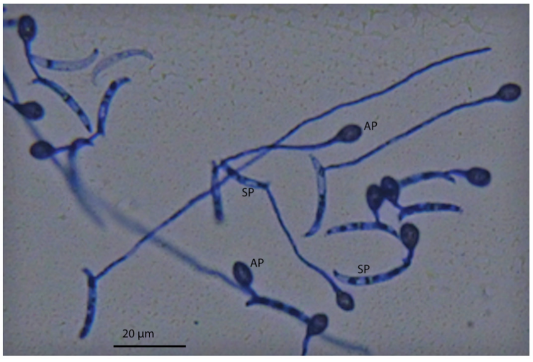

Fig. 5 Germinating spores (SP) and appressoria (AP) of *Colletotrichum truncatum* '158ci' at 6 hr, where spores dyed with lactophenol cotton blue

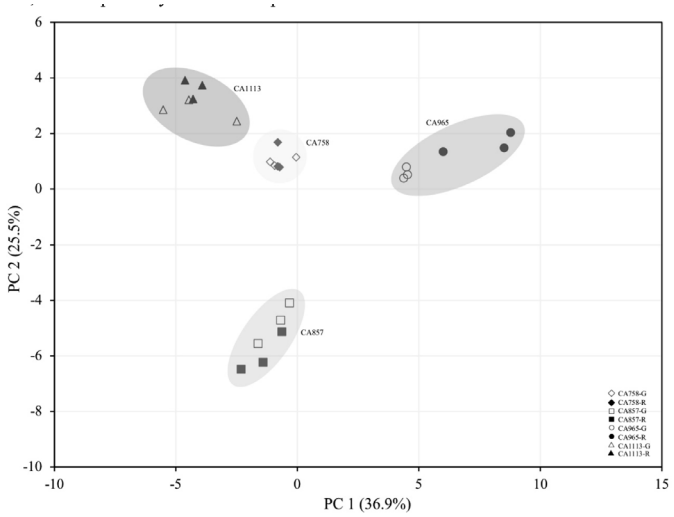

Fig. 6 Principal component (PC) analysis of 99 wax compounds from cuticular wax of four *Capsicum annumm* genotypes at two fruit maturity stages, mature green (G) and ripe (R)

Discussion

Anthracnose reaction

Development of anthracnose symptoms on chili fruit of *Ca. annuum* genotypes CA758, CA857, CA965 and CA1113 inoculated with *C. truncatum* isolate '158ci' was dependent on the fruit maturity stage. Previous genetic study of resistance to the isolate '158ci' based on wound inoculation bioassays (Mahasuk et al., 2009) showed that resistance in the different fruit stages was controlled by different genes. Therefore, the differential anthracnose reactions at the different chili fruit maturity stages appeared to be genetically controlled, regardless of wound or non-wound inoculation.

In addition, De Silva et al. (2021) showed differential host reactions between mature green and ripe fruit of *Ca. annuum* Bangchang and *Ca. chinense* PBC932 with several isolates of *C. scovillei* and *C. siamense* with non-wound inoculation.

The pathogen's ability to infect non-wounded fruit is more important in assessing the pathogenicity of *Colletotrichum* pathogens than infection through wound inoculation. Many studies that assessed the pathogenicity of *Colletotrichum* pathogens on fruit used a bioassay based on wounding the cuticle and epidermis prior to inoculation to provide a biased measure of pathogenicity (Nasehi et al., 2016; Diao et al., 2017). All *Colletotrichum* species produced appressoria formed on the cuticle as the primary means of infection (De Silva et al., 2017). Wounding dismisses the important function of the appressoria and cuticle waxes in the infection stage. Even *Colletotrichum* spp. that have a primary lifestyle as endophytes can be quite virulent in a bioassay, where the tissue is wounded prior to inoculation. The lack of infection in non-wounded fruit is often dismissed, which is important in understanding the disease cycle of these pathogens and host resistance.

Physical role of fruit cuticle in anthracnose reactions

The physical properties of the chili fruit cuticle, including thickness and wettability, did not seem to correlate with the anthracnose reactions, despite differential anthracnose reactions at different fruit maturity stages. Although the thickness had a slight effect on the AUDPC, it did not affect other anthracnose parameters. The thickness in all chili genotypes in the range 14–17 μm may not have been large enough to physically prevent infection.

Likewise, a study by Manandhar et al. (1995) investigated the cuticle thickness in relation to anthracnose incidence in chili. During fruit development of one genotype, the cuticle of more mature fruit was significantly thicker than that of the immature fruit, with the mature fruit showing more infections than the younger fruit. That study suggested that chili genotypes more significantly influenced the anthracnose infections than the cuticle thickness. Fruit of all chili genotypes became slightly wetter when more mature, indicating there was no relationship between the wettability and the differential anthracnose reactions between fruit stages and between genotypes. Surface wettability is a factor influencing fungal spore attachment, which subsequently induces spore germination and appressoria formation. Chaky et al. (2001) reported that on rigid surfaces with high hydrophobicity (contact angles above 55°), *C. graminicola* spores had high germination (above 75%). The more hydrophilic surfaces (below 50°) critically inhibited the spore germination (to below 20%). The surface wettability values of the chili genotypes in the current study were above 55° and did not affect fungal spore germination, indicating that the differential anthracnose reactions found in different chili genotypes were affected by mechanisms other than the fruit cuticle wettability.

Wax compositions in relation to anthracnose reactions

The similar wax profiles of the three chili genotypes CA758, CA857 and CA1113 with different anthracnose reactions suggested that the wax compositions did not influence

anthracnose resistance. In addition, the similar wax profiles between fruit stages of these three genotypes supported the observations that the wax composition had no influence on the anthracnose reactions in chili.

Interestingly, chili genotype CA965 produced the slowest and lowest spore germination, which could have been affected by different physical or chemical cuticle characteristics. CA965 had a rougher cuticle surface and significantly lower triterpenoids/sterol in the wax profiles compared to the other *Ca. annuum* genotypes, although the alkanes were still high. Wax extracted from pear fruit was shown to have antifungal activity (Yin et al., 2011; Li et al., 2014; Tang et al., 2017) and inhibited *in vitro Alternaria* spore germination and growth. Those studies found that the major waxes extracted from pear fruit were alkanes, triterpenoids and fatty acids; however, the efficacy of each wax to inhibit spore germination was not assessed.

Another issue of the wax profiles of CA965 was that the ripe fruit had significantly higher wax alcohols, predominantly of the shorter chain type C4–C20, than the mature green fruit. Differential anthracnose reactions were also detected in CA965, whereby the ripe fruit did not show symptoms. Very long chain alcohols extracted from avocado fruit cuticular wax induced *Colletotrichum* appressoria formation (Podila et al., 1993); however, appressoria induction was not solely dependent on the fatty alcohols but also on host specificity. In addition, that study proved that only long chain alcohols (C24 or longer) showed ability to induce the appressoria. Therefore, the higher alcohol content in the CA965 ripe fruit may not have affected *Colletotrichum* spore growth.

In addition, spore concentration did not have any influence on the *Colletotrichum* spore germination and appressoria formation. A repeat spore and appressorium study was executed using a lower spore concentration at 0.5×10^6 spores/mL and it delivered similar results (Table S5).

Conclusion

The four chili tested genotypes possessed similar physical structure (thickness and wettability) and wax composition of the fruit cuticle. The fruit cuticular waxes from all chili genotypes similarly promoted the *in vitro* formation of *C. truncatum* appressoria. All the similar properties derived from the chili genotypes with differential anthracnose reactions on two fruit maturity stages indicated that the fruit cuticle played a small role in the *Colletotrichum* infection and suggested other host mechanisms were involved in anthracnose resistance.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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