Differential Interactions of a *Colletotrichum gloeosporioides* Isolate with Green and Red Pepper Fruits

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Differential interactions of *Colletotrichum gloeosporioides* isolate KG13 with green and red pepper fruits (*Capsicum annuum* were found when it was inoculated on unwounded and wounded fruits. The isolate produced the typically necrotic, sunken anthracnose symptom on unwounded and wounded green fruits, and wounded red ones, but not on unwounded red ones. Appressorial formation of the fungus on the surfaces of compatible green fruits was higher than on incompatible red ones up to 12 h after inoculation. More and longer infection pegs from appressoria were produced on green than on red fruits. When cuticular wax layers of gree n and red fruits were removed by dipping in chloroform, red ones only produced larger lesions and more conidia than water-dipped controls did. However, differences in lesion diameter and conidial production were not observed between green and red fruits wounded by pin-pricking. In addition, concentrations of wax extracted from the surface of green and red fruits affected conidial germination and appressorial formation of the fungus. These findings suggest that the isolate KG13 of *C. gloeosporioides* may react differentially to green and red pepper fruits, probably due to the physical and chemical differences in cuticular layers of the fruits.

KEY WORDS: Anthracnose; *Capsicum annuum; Colletotrichum gloeosporioides;* fungal infection; *Glomerella cingulata;* wax.

INTRODUCTION

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. [teleomorph: *Glomerella cingulata* (Stonem.) Spauld. & von Schrenk] is known to cause anthracnose disease on many plants. *Colletotrichum* spp. have been characterized by morphology, benomyl sensitivity and polymorphism in the ribosomal 18S and 28S transcriptional unit using 72 isolates from apple, peach, pecan and other hosts (2). Regardless of host origin, the isolates could be divided into pink and gray colony types, corresponding to *C. acutatum* and *C. gloeosporioides*, respectively. The research also demonstrated that the isolates of *C. gloeosporioides* from different hosts did not have host-specific characteristics. Thus, it is evident that *C. gloeosporioides* is a ubiquitous fungus in that it can infect many plant species regardless of its host origin.

Colletotrichum spp. are the causal agents of anthracnose in green (unripe) and red (ripe) peppers (*Capsicum annuum* L.) (5,6,11). *C. capsici, C. gloeosporioides* and *G. cingulata* have been isolated frequently from infected red peppers in Taiwan (5,6), although the first

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two species are the more important. Park and Kim (11) attempted to identify fungal species responsible for anthracnose on peppers in Korea. The causal agent, *C. gloeosporioides*, was the most prevalent species among *C. acutatum, C. coccodes, C. dematium, C. gloeosporioides* and *G. cingulata.* The predominant species of *C. gloeosporioides* has been classified into G and R strains by Kim *et al.* (3). The G strain infects both green and red fruits, whereas the R strain infects only red ones. Furthermore, Park *et al.* (12) successfully differentiated *C. gloeosporioides* into the G and R strains by isozyme patterns of esterase, leucine amino peptidase, phosphatase and glutamic oxaloacetic transaminase.

The occurrence of anthracnose on green pepper fruits has been found recently in Korea (9). An isolate of *C. gloeosporioides* was obtained to produce typical anthracnose symptoms on green fruits only (9). In our microscope study (8), we observed that successful invasion and colonization of the isolate into the epidermal cells through epicuticular layers occurred on green fruits, but not on red ones. Therefore, these studies were conducted to (i) evaluate anthracnose development on green and red pepper fruits infected by the isolate of *C. gloeosporioides*; (ii) examine conidial germination, appressorial formation, and infection peg formation of the isolate on the fruits; and (iii) determine the fungal infection on the fruits from which cuticular wax layers were removed by dipping in chloroform. Companion research was conducted to determine the effects of wax extract from the surfaces of the fruits on conidial germination and appressorial formation.

MATERIALS AND METHODS

Fungal inoculum, pepper fruits and disease assessment

A monoconidial isolate KG13 of *C. gloeosporioides* was obtained from a culture provided by I.S. Oh, Chungnam Provincial Rural Development Administration, Taejon, Korea. The isolate was grown on potato dextrose agar (Difco Laboratories, USA) for 5 days at 28°C. The cultures were overlaid with 10 ml of sterile distilled water, after which conidia were obtained by filtration of the water extract through four layers of sterile cheesecloth to remove mycelial debris. Conidia were washed three times in sterile distilled water by centrifugation and adjusted to 1×10^6 conidia/ml with sterile distilled water.

Well-developed green and red pepper fruits were harvested from pepper plants (cv. 'Nokwang') grown in commercial greenhouses at Kwangju, Korea. Fruits were treated in 10% Clorox® for 3 min, washed in sterile distilled water several times, and dried with sterile paper towels. The fruits for inoculations following wounding or not, were placed in plastic containers (25 ×16 × 6 cm) with plastic mesh screens. Four layers of paper towels with 100 ml of sterile distilled water were placed in the containers to maintain 100% r.h. The fruits were dot-marked on their center and pin-pricked for wound inoculation as described by Park and Kim (11). Twenty μl of the conidial suspensions was dropped near the dot. Control fruits received 20 μl of sterile distilled water. Containers were then covered and kept in darkness at 28°C. The lids of the containers were removed 48 h after inoculation (HAI); thereafter fruits were incubated for 7 additional days under the same conditions until evaluation. These methods of preparing fruit and inoculum, and the procedures of inoculation and incubation, were used throughout all experiments.

Lesion diameter and number of conidia on the inoculated fruits were evaluated as parameters of anthracnose development. The disease parameters were recorded

periodically up to 9 days after inoculation (DAI). To count conidia, 5-mm^2 pieces of fruit tissue were cut from lesions and placed in 1 or 5 ml of sterile water. After vortexing for 1 min, the number of conidia was determined using a haemocytometer. Twenty fruits per treatment were evaluated for lesion diameter and five for number of conidia. These experiments were done three times.

Fungal behavior on the surface of pepper fruits

Five green and red fruits each were used for evaluation of conidial germination, appressorial formation, and infection peg formation of the isolate KG13 of *C. gloeosporioides*. Fruits were inoculated with 10 μl of 5×10^5 conidia/ml. Pieces (5 mm^2) of inoculated fruit tissues were freehand-sectioned with sterile razor blades and immediately stained with drops of lactophenol cotton blue solution. The tissues were periodically sectioned and observed under a microscope for 24 HAI. More than 100 conidia per sample were observed. Glass slides with drops of the conidial suspension served as controls. Percent germination and appressorial formation were determined, as the number of germinated conidia and appressoria divided by the total number of conidia observed $\times$ 100, respectively. Percent infection peg formation also was determined, as the number of infection pegs divided by the total number of conidia with appressoria observed $\times$ 100. The length of infection pegs from appressoria to penetration of cuticular layers of fruits was measured under a microscope. These experiments were done three times.

Chloroform treatments

Green and red fruits were dipped in 99.8% chloroform for 5 sec to remove cuticular wax layer and placed in plastic containers (25 $\times$ 16 $\times$ 6 cm) with four layers of paper towels and 100 ml sterile water. Fruits dipped in sterile distilled water served as controls. Wounded fruits were also prepared by pin-pricking (11) to enable access to direct fleshy tissues by conidia of *C. gloeosporioides*. These fruits were inoculated with 20 μl of 1×10^6 conidia/ml. Disease development expressed as lesion diameter and number of conidia was evaluated 7 DAI. The number of conidia was determined as described previously. The experiments were done three times with ten replicates each for lesion diameter and five each for number of conidia.

Effect of wax on conidial germination and appressorial formation

Wax from the surface of green and red peppers was isolated by dipping fruits in 500 ml chloroform for 30 sec. The treated chloroform solution was then extracted with 250 ml of distilled water for removal of water-soluble substances. The chloroform phase containing wax was vacuum-evaporated in a rotary evaporator. The wax residue was resolved again with chloroform.

Conidial germination and appressorial formation of *C. gloeosporioides* isolate KG13 affected by the wax from the fruits were examined with a cover glass-coating method (13). Wax was applied at 0.01, 0.1, 1, 10 and 100 μg concentrations in 1-cm-diam areas on cover glasses (Marienfeld, USA), surrounded by parafilm (Whatman, USA), and then air-dried. Chloroform without wax served as control. One hundred μl of 2×10^4 conidia/ml was applied to the glass surfaces coated with the wax. The treated glasses were put in petri dishes with a moist filter paper and then incubated for 21 h in darkness at 28°C. After the cover glasses were placed on a haemocytometer, germinated conidia and appressoria were

stained with drops of lactophenol cotton blue solution and observed under a microscope. More than 100 conidia per sample were counted for evaluation of conidial germination and appressorial formation. These experiments were done four times with three replicates each.

Data analysis

Statistical analyses were conducted using the Statistical Analysis System (15). Data for number of conidia were transformed to $\log_{10}(x+1)$ before statistical analysis. Analysis of variance was determined using the general linear model procedure and means were separated by least significant difference. Relationships among dependent variables were examined using the correlation procedure. Regression analysis was done to compare the curves of conidial germination or appressorial formation on various concentrations of wax extract from green and red pepper fruits. Variables for concentration of wax were transformed to $\log_{10}(x+0.001)$ before regression analysis.

RESULTS

Fig. 1. Anthracnose symptoms caused by *Colletotrichum gloeosporioides* isolate KG13 on green and red pepper fruits unwounded or wounded by pin-pricking 7 days after inoculation. Control fruits inoculated with sterile water produced no disease symptoms. (1) Inoculated on green fruits wounded by pin-pricking; (2) inoculated on unwounded green fruits; (3) inoculated on red fruits wounded by pin-pricking; and (4) inoculated on unwounded red fruits.

Anthracnose development on pepper fruits

The isolate KG13 of *C. gloeosporioides* produced typically necrotic, sunken anthracnose symptoms on both unwounded and wounded green pepper fruits, and wounded

red ones, but not on unwounded red ones 7 DAI (Fig. 1). However, on some occasions, a small brownish discoloration was observed on unwounded red fruits 5–9 DAIs. Anthracnose disease on pepper fruits as measured by lesion diameter and number of conidia produced from lesions increased over time after inoculation (Fig. 2). Initial symptoms occurred on fruits 3 DAI and severe sunken symptoms were observed 7 and 9 DAIs (Fig. 2A). Unwounded red fruits had significantly ($P = 0.05$) smaller lesions than the other treatments. Conidial production on unwounded red fruits was significantly ($P = 0.05$) lower than the other treatments (Fig. 2B). Lesion diameter was positively correlated with number of conidia ($r = 0.809$, $P < 0.001$). Water-inoculated pepper fruits as a control did not have any disease symptoms or conidia.

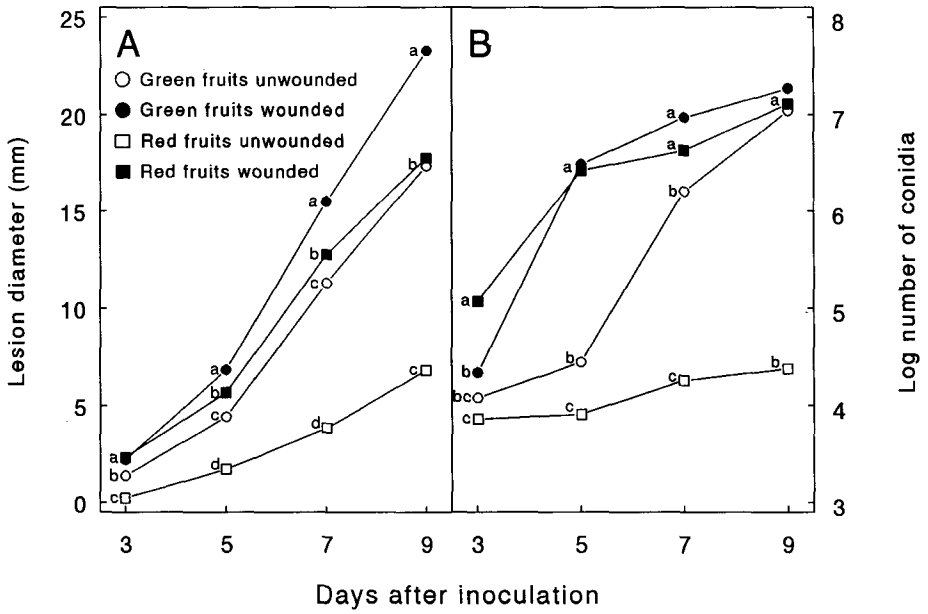

Fig. 2. (A) Anthracnose lesion diameter and (B) conidial production caused by *Colletotrichum gloeosporioides* isolate KG13 on unwounded green and red pepper fruits or fruits wounded by pin-pricking. Control fruits inoculated with sterile water produced no disease symptoms. Each value for lesion diameter and number of conidia represents a mean of 60 and 15 observations, respectively. Means marked with the same letter do not differ significantly ($P = 0.05$).

Fungal behavior on the surface of pepper fruits

The infection processes of *C. gloeosporioides* including conidial germination, appressorial formation and infection peg formation on fruit surfaces are necessary to produce susceptible symptoms; therefore, the fungal behavior was examined with the isolate KG13 (Fig. 3). Conidia began to germinate 2 HAI and were near maximum (94%) 6 HAI with no difference in germination of conidia on green and red fruits (Fig. 3A). Conidia did not germinate on the surfaces of glass slides until 24 HAI. Appressoria began to form

on the fruits 2 HAI and maximized 24 HAI (Fig. 3B). Appressoria formed on green fruits significantly ($P = 0.05$) more than on red fruits from 3 to 12 HAIs, but not for 1.5, 2, 18 and 24 HAIs. Infection pegs from appressoria were observed 12 HAI and continued to form 24 HAI (Fig. 3C). The infection pegs were formed significantly ($P = 0.05$) more on green than on red fruits 12 and 18 HAI, but not 24 HAI. When the length of infection pegs from appressoria was measured, it was significantly ($P = 0.05$) longer on green than on red fruits 24 HAI (Fig. 3D).

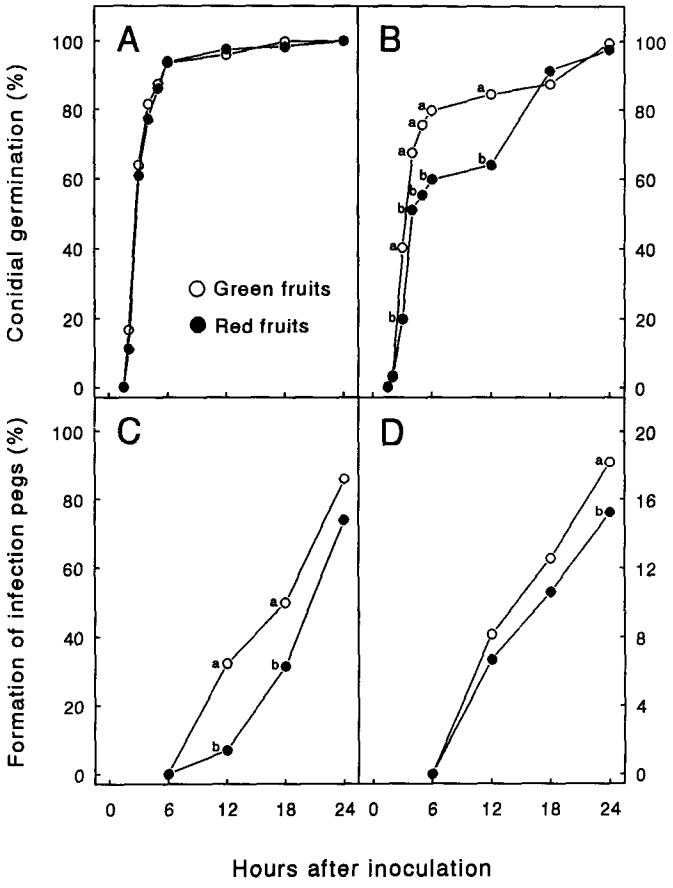

Fig. 3. (A) Conidial germination, (B) appressorial formation, (C) formation of infection pegs, and (D) length of infection pegs of *Colletotrichum gloeosporioides* isolate KG13 on green and red pepper fruits. No conidial germination was observed on the surfaces of glass slides as control up to 24 h after inoculation. Each value represents a mean of 15 observations. Means marked with the same letter do not differ significantly ($P = 0.05$); means without letters indicate no significant differences between green and red pepper fruits.

Chloroform treatments

Anthracnose lesion diameter and conidia production were influenced by pepper fruits ($P< 0.0001$) and treatments ($P< 0.0001$). Significant interactions between pepper fruits and treatments also were detected for lesion diameter ($P< 0.0001$) and conidial production ($P< 0.0001$). Red fruits produced significantly ($P< 0.001$) smaller lesions and less conidia than green ones did 7 DAI when inoculated on the fruits dipped in chloroform (Fig. 4). Differences of lesion diameter and conidial production on green and red fruits, however, were not observed after inoculation of fruits wounded by pin-pricking. Red fruits had significantly ($P< 0.001$) smaller lesions and less conidia than green ones did when unwound-inoculated. Significant ($P< 0.001$) differences among unwounded, wax-removed, and wounded red fruits were observed in both lesion diameter and number of conidia. Wounded green fruits had significantly ($P< 0.05$) larger lesions compared with unwounded and wax-removed green ones. Lesion diameter was positively correlated with number of conidia ($r = 0.782$, $P< 0.001$).

Effect of wax on conidial germination and appressorial formation

When effects of wax extract from pepper fruits on fungal behavior of the isolate KG13 were examined, quadratic relationships were described between conidial germination or appressorial formation and concentrations of wax extract from green and red fruits (Fig. 5). Conidial germination and appressorial formation were lowest on the concentration of 0.1–0.01 μg of wax from green and red fruits, but were enhanced on higher concentrations of wax. Differences in appressorial formation between green and red fruits were not detected at concentrations of wax (Fig. 5).

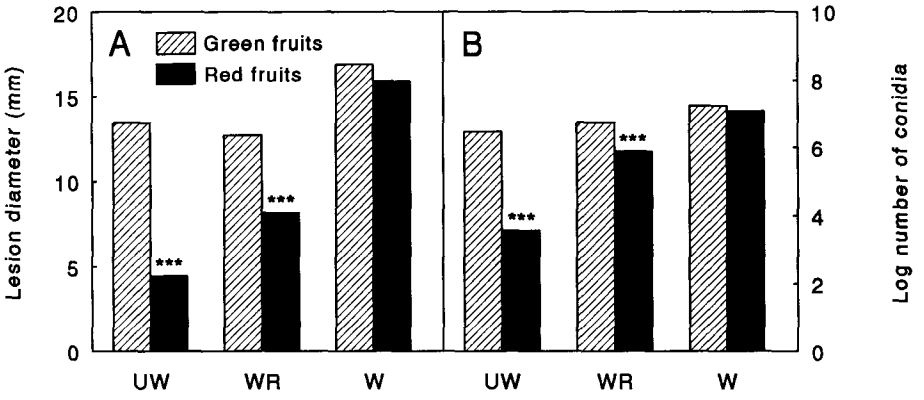

Fig. 4. (A) Anthracnose lesion diameter and (B) conidial production caused by *Colletotrichum gloeosporioides* isolate KG13 on green and red pepper fruits left unwounded (UW), wax-removed (WR) by dipping in chloroform for 5 sec, or wounded (W) by pin-pricking 7 days after inoculation. Control fruits inoculated with sterile water produced no disease symptoms. Each value for lesion diameter and number of conidia represents a mean of 40 and 12 observations, respectively. ***indicates a significant difference at P=0.001 between green and red fruits within a treatment.

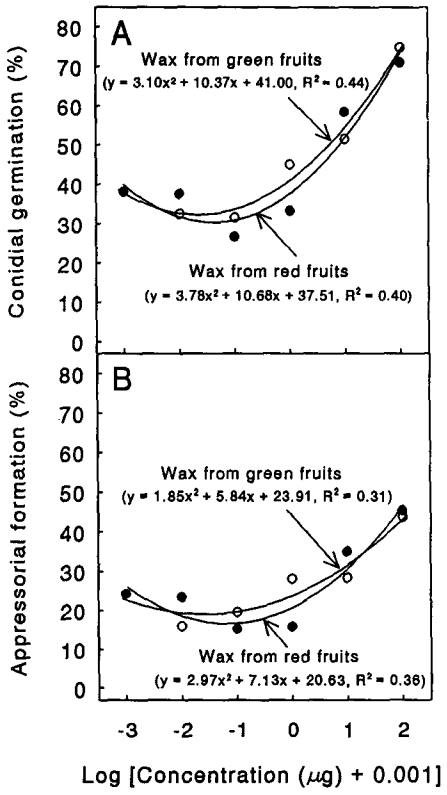

Fig. 5. Relationship between concentration of wax extract from green (white dots) and red (black dots) pepper fruits, and (A) conidial germination and (B) appressorial formation of *Colletotrichum gloeosporioides* isolate KG13. Conidial germination and appressorial formation were examined on cover glasses coated with various concentrations of wax extract from green and red pepper fruits 21 h after treatment. Chloroform without wax applied on cover glasses served as control. Each value represents a mean of 12 observations.

DISCUSSION

Differential interactions of *C. gloeosporioides* between green and red pepper fruits were found in the isolate KG13 originating from anthracnose lesions of green fruits. The isolate caused typical sunken anthracnose on green fruits, but only small brownish discoloration on red ones. The differential infections of the isolate KG13 on pepper fruits may be due to cuticular layers of the fruits that play a significant role in preventing the infection and colonization by *C. gloeosporioides*. This possibility is supported by the phenomenon that inoculated red fruits incompatible with the fungal isolate produced the

sunken susceptible symptom when wound-inoculated by pin-pricking. The importance of the cuticular layers of the pepper fruits to the fungal infection also could be found in a report (7) that cuticle thickness is negatively correlated with anthracnose lesion expansion and conidial production on the fruits. Differences in infection of green and red fruits by *C. gloeosporioides* also may be explained by differences in biochemical properties of pepper fruits. In biochemical analyses of peppers, Ko (4) has demonstrated that green fruits had higher activities in peroxidase and polyphenoloxidase than red ones, but lower levels of total phenolics, amino acids and carbohydrates, some of which have been related to host resistance. Thus, the differential infections by the KG13 isolate on green and red fruits may be due to the outer cuticular layers and/or biochemical properties of the fruits.

The initial infection processes of *C. gloeosporioides* include conidial germination and production of appressoria on the host surfaces. After appressoria successfully penetrated into the host layers *via* infection pegs, infection hyphae could be formed to establish colonization inside tissues (1). Manandhar *et al.* (5) observed that conidial germination and appressorial formation of an isolate of *C. gloeosporioides* obtained from infected red pepper fruits did not differ between green and red ones. In the case of avocado, *C. gloeosporioides* produced short infection pegs from appressoria in unripe fruits and then remained quiescent until the fruits ripened (14). Well-developed infection pegs could be seen with ripening of the fruits. In our study, a difference in conidial germination on green and red fruits was not detected; however, significantly more appressoria and infection pegs on green fruits were recognized 3–12 and 12–18 HAI, respectively. Since conidia initially used for inoculum became mycelia with new conidia that began to germinate and form new appressoria 18 HAI, no difference in appressorial formation on green and red fruits18 and 24 HAI were found. This may suggest that the initial penetration and infection by the isolate KG13 of *C. gloeosporioides* may determine the susceptibility of green ones. O'Neill and Saunders (10) observed a similar phenomenon in the *C. trifolii* and alfalfa system. They found that appressoria matured on both resistant and susceptible cotyledons but infection pegs did not develop on resistant ones. In addition, the longer infection pegs on green fruits 24 HAI observed in this study may indicate successful infection and colonization in the compatible host cells, that eventually resulted in sunken anthracnose symptoms 7–9 DAI.

The cuticular wax layer of pepper fruits is the first barrier to infection by *C. gloeosporioides*. In an investigation of chloroform effects on red pepper fruits (7), the chloroform treatment of the fruits for 3 sec enhanced anthracnose development. Similarly, we have also found larger lesions on incompatible red fruits from which cuticular wax layers were partially removed by dipping in chloroform for 5 sec. The chloroform treatment, however, failed to enhance anthracnose development on compatible green fruits. These results may indicate that the cuticular wax layers of incompatible red fruits play a role in preventing infection and colonization by *C. gloeosporioides*. Podila *et al.* (13) reported that the surface wax from avocado fruits quantitatively induced germination and appressorial formation of *C. gloeosporioides*; however, the quadratic relationships between wax from pepper fruits and conidial germination or appressorial formation were observed in this *C. gloeosporioides*–pepper system. In our study, it was also recognized that conidia of the isolate KG13 germinated well and produced more appressoria on the surface wax from both red and green fruits at the wax concentration higher than 1 μg, which is close to the actual concentration on an inoculated site, based on the extracted amount. From these

studies, it is suggested that the isolate KG13 of *C. gloeosporioides* may react differentially to green and red pepper fruits, probably due to the physical and chemical differences in cuticular wax layers of the fruits.

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