

## **METABOLISM OF EPICATECHIN BY LACCASE OF *Colletotrichum gloeosporioides***

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During avocado fruit ripening, levels of the flavonoid epicatechin decrease and the metabolism of antifungal compounds is regulated, while the quiescent *Colletotrichum gloeosporioides* infections are activated. Epicatechin levels also decrease when *C. gloeosporioides* grows in the presence of epicatechin in culture medium. Extracts of laccase enzyme obtained from diseased tissue and culture medium fully metabolized the epicatechin substrate within 4 and 20 h, respectively. Isolates of *C. gloeosporioides* with reduced laccase activity and no capability to metabolize epicatechin showed reduced pathogenicity on mature fruits. On the contrary, Mexican isolates with increasing capabilities to metabolize epicatechin showed early symptoms of disease in unripe fruits. The present results suggest that biotransformation of epicatechin by *C. gloeosporioides* in ripening fruits is followed by the decline of the performed antifungal diene compounds, resulting in the activation of quiescent infections.

Key words: pathogenicity, induced susceptibility, decay

## **METABOLISMO DE EPICATEQUINA POR LACASA DE *Colletotrichum gloeosporioides*.**

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Durante el proceso de maduración del fruto de aguacate, decrecen los niveles del flavonoide epicatequina y se regula el metabolismo de compuestos antifúngicos y se activan infecciones quiescentes de *Colletotrichum gloeosporioides*. Los niveles de epicatequina también decrecen cuando *C. gloeosporioides* crece en presencia de epicatequina en medio de cultivo. Extractos de la enzima lacasa obtenidos a partir de tejido enfermo y medio de cultivo, metabolizaron completamente el sustrato de epicatequina en 4 y 20 horas, respectivamente. Los aislamientos de *C. gloeosporioides* con poca actividad de lacasa y sin capacidad para metabolizar epicatequina mostraron

poca patogenicidad en frutos maduros. Por el contrario, los aislados Mexicanos con gran capacidad para metabolizar epicatequina mostraron síntomas anticipados de la enfermedad en frutos inmaduros. Estos resultados sugieren que la biotransformación de la epicatequina por parte de *C. gloeosporioides* en frutos en proceso de maduración es seguida por la reducción de compuestos dienos antifúngicos, dando como resultado la activación de infecciones quiescentes.

Palabras clave: patogenicidad, susceptibilidad, podredumbre

*C. gloeosporioides* germinates on avocado (*Persea americana*) fruit peel and forms appresoria. The infection hyphae then penetrate into the epidermal cells of the avocado exocarp but remind quiescent until the fruit ripens. The resistance of unripe avocado fruits to fungal attack during quiescence has been reported to depend on the presence of preformed antifungal compounds (Prusky et al, 1982) and the consequent lack of secretion of fungal pathogenicity factors occurring during quiescent infections (Prusky et al, 2001). In unripe-resistant fruits enhanced resistance to the pathogen is characterized by the activation of synthesis of the antifungal diene (AFD). In ripe-susceptible fruits, however, the decline in concentration of AFD has been attributed to its metabolism through the oxidative activity of lipoxygenase (Karni et al, 1989). The activity of lipoxygenase is modulated by the level of its inhibitor the flavan-3-ol-epicatechin (Prusky, 1996), a natural antioxidant that is generated in the phenylpropanoid pathway, and that declines in concentration during fruit ripening. Decline in epicatechin levels leads to increase in lipoxygenase activity, along with metabolism of the AFD (Ardi et al, 1998). Thus, epicatechin is an important factor that modulates avocado fruit resistance to fungal attack. Some fungal pathogens can avoid the contact with antifungal compounds by detoxification, and so enable fungal attack. The objectives of this work were to determine the contribution in decay development of *Colletotrichum* spp. in avocado fruits, and to describe the decline in concentration of the flavonoid epicatechin by *C. gloeosporioides* laccase and the importance on this process for the pathogenicity of *C. gloeosporioides* in ripening avocado fruits.

## **MATERIAL AND METHODS**

Avocado fruits were inoculated with the isolate Cg-14 and with Mexican isolates by placing a conidial suspension ( $10\mu\text{L } 10^6$  conidia per mL) at six longitudinal spaced spots, three on each side of the fruit. Following inoculation, fruits were incubated at 22°C, 95% RH until fruit ripening and symptoms development were observed (Figure 1). The average diameter of decay of 10 fruits was reported.

Epicatechin was extracted from avocado pericarp. Pericarp tissue was homogenized in 95% ethanol, the extract was filtered and the ethanol sample was dried and fractionated (Guetsky et al, 2005). The pooled organic phases were dried with anhydrous  $\text{MgSO}_4$  and evaporated to dryness. The samples were dissolved in ethyl acetate/dichloromethane (1:3) and subjected to flash chromatography. The epicatechin levels were calculated by comparison with a

commercial epicatechin standard. Concentrations were expressed as micrograms per gram fresh weight of fruit peel.

Purification of laccase secreted to the culture media by *C. gloeosporioides* was carried out following the inoculation of spores on MS medium, incubated, shaking at 150 rpm for 5 days, then harvested by filtration and washing. The washed mycelia were resuspended in laccase inducing medium (containing epicatechin). The hyphae were filtrated through a membrane and the filtrated was concentrated by ultrafiltration.

Extraction of laccase from *C. gloeosporioides* mycelia was carried out after growing the fungus in inducing liquid media containing epicatechin. Mycelia were grounded in liquid nitrogen and the enzyme was extracted.

Activity assays for laccase was tested according to Guetsky et al (2005) by enzymatic activity and/or HPLC analysis of the reminding amount of phenol substrates.

Isolates of *C. gloeosporioides* showing reduced pathogenicity were isolated from a healthy part of the exocarp tissue of ripe, decayed cv Fuerte avocado fruits, obtained from an orchard in Israel. *Colletotrichum* spp were confirmed as *C. gloeosporioides* by means of PCR.

Single spores of *C. gloeosporioides* isolates obtained from avocado orchards in Michoacan, Mexico showing symptoms of anthracnose in unripe or in ripening fruits were compared.

## RESULTS

When *C. gloeosporioides* hyphae were transferred from primary growth cultures to secondary growth cultures containing epicatechin, the concentration of epicatechin level declined by 50% during the first 5h of incubation and a 50% reduction of the remaining level was observed 17 h later. Five hours after transfer of the fungus to the secondary growth culture containing epicatechin, activity of laccase was detectable, but the highest activity was observed 45 h later.

Epicatechin levels in the pericarp of healthy cv. Fuerte avocado fruits decreased from 998 to 153  $\mu\text{g g}^{-1}$  fresh weight during fruit ripening. However, extracts of healthy ripe pericarp did not contain any laccase activity and did not affect the decline in epicatechin concentration in vitro.

Out of 30 isolates of *C. gloeosporioides* obtained from healthy, ripe, avocado tissue, only two isolates, Cg-W7 and Cg-W10, showed no decay development when symptoms of decay of the wild-type *C. gloeosporioides* Cg-14 isolate were expressed. The reduced pathogenicity isolates did not differ from the wild type in germination rates, germ tube elongation, or appresorium formation on fruit exocarp. However, the laccase activity in culture media 36 h after inoculation with reduced pathogenic isolates was only 20% of that produced by the wild type. During the same period, the wild-type *C. gloeosporioides* metabolized 96% of the epicatechin substrate, whereas the reduced pathogenic isolates reduced the epicatechin concentration by only 8 to 15% of its initial value. Mexican isolates CgM-3 and CgM-6, showing early symptoms of anthracnose in unripe fruits (Figure 2), could metabolize epicatechin almost twofold faster than isolates CgM-4 and CgM-5, which cause anthracnose symptoms on ripe fruits only (Table 1)

## DISCUSSION

Decreases in the level of the performed AFD was suggested as the main factor modulating quiescence of *C. gloeosporioides* during ripening of avocado fruits. Faster epicatechin metabolism during ripening of avocado fruits increased susceptibility to decay development by *C. gloeosporioides* and shortened the quiescent period (Prusky et al, 1988). The capability of *C. gloeosporioides* laccases to reduce epicatechin concentration in the decayed tissue suggests that laccase secretion by *C. gloeosporioides* may be a factor contributing to the activation of quiescent infections (Wang and Nuss, 1988). Fungal laccases play a significant role in plant pathogenicity by detoxification of antifungal compounds and tannins in the host environment (Adrian et al, 1998). Different experiment suggested the involvement of fungal laccase in the activation of quiescent infections: i. secretion of laccase was detected during fruit decay development accompanied by highest laccase activity; ii. purified laccase from decay tissue rapidly metabolized epicatechin in vitro, while no laccase activity was detected in healthy fruits; iii. *C. gloeosporioides* isolates that showed reduced capability to metabolize epicatechin were not able to colonize ripe fruits; iv. Mexican isolates that metabolized epicatechin more rapidly showed early decay symptoms in unripe fruits. Still the mechanism affecting the differential secretion of laccase by the pathogenic versus the reduced pathogenicity isolates remains unknown. The results however support the importance of laccase activity as a factor that induces susceptibility in ripening fruits by modulating the level of epicatechin, the decline of the AFD, and consequently, the activation of quiescent infections by *C. gloeosporioides* in ripening fruits.

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Table 1. Metabolism of the epicatechin by Mexican isolates of *Colletotrichum gloeosporioides* with symptoms of disease expressed during the period of avocado fruit development or after fruit harvesting.

Metabolismo de la epicatequina por aislamientos mexicanos de *Colletotrichum gloeosporioides* con síntomas de enfermedad, expresados durante el desarrollo del fruto o en poscosecha.

isolate	cultivar	Stage of attack	Epicatechin metabolized after 24 h	
			$\mu\text{g/mL}$	%
CgM-3	Fuerte	Young fruits	$21.2 \pm 1.7$	84.9
CgM-6	Hass	Young fruits	$17.4 \pm 0.9$	69.4
CgM-4	Criollo	Ripe fruits	$7.5 \pm 1.0$	30.1
CgM-5	Hass	Ripe fruits	$9.2 \pm 0.1$	36.8

Figure 1. Pathogenic tests with Mexican isolates of *C. gloeosporioides*



Figure 2. Early symptoms of anthracnose in unripe Mexican fruits

