



# Extracellular enzymes of *Colletotrichum fructicola* isolates associated to Apple bitter rot and Glomerella leaf spot

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## ABSTRACT

*Colletotrichum fructicola* causes two important diseases on apple in Southern Brazil, bitter rot (ABR) and Glomerella leaf spot (GLS). In this pathosystem, the *Colletotrichum* ability to cause different symptoms could be related to differences of extracellular enzymes produced by the fungi. Thus, the objectives of this study were to compare the production of these enzymes between ABR- and GLS-isolate *in vitro* and to evaluate their involvement on infected apple leaves with *C. fructicola*. In agar plate enzymatic assay, ABR- showed significantly higher amyolytic and pectolytic activity than GLS-isolate. In contrast, for lipolytic and proteolytic no significant differences were observed between isolates. In culture broth, ABR-isolate also had higher activity of pectin lyase (PNL), polygalacturonase (PG) and laccase (LAC). Notably, LAC was significantly five-fold higher in ABR- than GLS-isolate. On the other hand, in infected apple leaves no significant difference was observed between isolates for PNL, PG and LAC. Although differences in extracellular enzymes of ABR- and GLS-isolate have not been observed *in vivo*, these results contributed to highlight the importance to investigate such enzymes in depth.

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## 1. Introduction

*Colletotrichum fructicola* Prihastuti, Cai & Hyde (formerly *Colletotrichum gloeosporioides*) is the main species causing two important diseases on apple (*Malus domestica* Borkh.) in Southern Brazil, i.e. the bitter rot (ABR) and Glomerella leaf spot (GLS) (Velho et al. 2015; Rockenbach et al. 2016).

ABR is a fruit disease that occurs in countries where apple trees are cultivated (Alaniz et al. 2015; Munir et al. 2016). It causes important losses especially during favourable environmental conditions, rainy and warm climate (Wang et al. 2015). Typical symptoms of ABR include light brown, slightly sunken lesions that enlarge over time becoming dark brown and water soaked (Alaniz et al. 2015; Velho et al. 2015).

On the other hand, GLS is an emerging leaf disease that is still limited to a few countries, such as the USA (González et al. 2006), Brazil (Velho et al. 2015), China (Wang et al. 2012) and recently in Uruguay (Casanova et al. 2017). Symptoms of GLS appear as reddish-purple spots 2 days after infection. The spots coalesce evolving to irregular necrotic lesions about 10 days later, when leaves turn yellow and fall off (Araújo and Stadnik 2013;

Velho et al. 2016). In fruits, GLS causes small slightly sunken lesions, which seldom evolve into rot (Velho et al. 2015).

It has been observed that some *Colletotrichum* isolates exhibit a particular organ specialisation being able to cause either, ABR or GLS (Velho et al. 2015, 2016; Rockenbach et al. 2016). Causes for such differences remain unknown. In previous studies, it was suggested that genotypes causing GLS probably were originated from ABR-isolates (González et al. 2006), favoured mainly by the high production of susceptible apple “Gala” acting as selection force (Rockenbach et al. 2016). However, such ability in infect fruits and leaves could be related to a production of fungal extracellular enzymes that degrade the plant cuticle and cell wall components. So far, no study has been conducted to clarify the production of these enzymes by *Colletotrichum fructicola* isolates causing ABR and GLS.

*Colletotrichum fructicola* is a hemibiotrophic fungus, which combines an initial short biotrophic phase, followed by highly destructive necrotrophic development killing extended tissue areas of host plants (O’Connell et al. 2012). For successful pre-penetration, it is

necessary a combination of mechanical pressure and secretion of some cuticle- and cell wall degrading enzymes, such as, cutinases, pectinases, hemicellulases and cellulases which are liberated sequentially (Huang 2001, Kubicek et al. 2014). Secretions of such enzymes are highly responsive to host and are perceived before penetration. In addition to these enzymes, amylases, lipases and proteases are also secreted to perform degradation of plasma-membrane components, providing nutrients to help fungus spread through plant tissue (Liao et al. 2012). These enzymes act facilitating hyphal penetration, release carbon sources, or modify chemical signals produced by the host (Huang 2001, ten Have et al. 2002).

Pectinases are important in colonisation of plant tissue by *Colletotrichum* and are secreted by the pathogen to hydrolyse pectin during infection (Shih et al. 2000, ten Have et al. 2002; Kubicek et al. 2014). Pectin is a major component of the primary cell wall and middle lamella (Herbert et al. 2004; Ramos et al. 2010). It is a critical component for plant tissue organisation, and most fungal pathogens need to degrade it when encountering plant cell physical barrier (Reignault et al. 2008). Polygalacturonases (PG), pectate lyases (PL) and pectin lyases (PNL) are the main pectinases secreted by *Colletotrichum*. While PG cleaves  $\alpha$ -1, 4 glycosidic bonds between two galacturonic acid residues, PL and PNL are responsible to degrade pectin polymers directly by  $\beta$ -elimination mechanism (Gregori et al. 2008). Together these enzymes play an important role in the pathogenicity, as previously reported for *C. gloeosporioides* (Yakoby et al. 2001), *C. magna* (Wattad et al. 1995), *C. truncatum* (Ramos et al. 2010) and *C. lindemuthianum* (Herbert et al. 2004).

Additionally, the role of extracellular enzymes in pathogenesis is also related to the fungal lifestyle (ten Have et al. 2002). For example, PG is the first cell-wall-degrading enzyme synthesised by fungi and acts mainly during the biotrophic phase, and less in the later stage of infection (Bailey et al. 1992). On the other hand, PNL is important especially during necrotrophic phase, but is also involved in the switch from biotrophic to necrotrophic phase (Shih et al. 2000). Specific conditions in the host tissue, such as, temperature and pH, are also important factors for regulation of gene expression (Prusky et al. 2001; Drori et al. 2003) and subsequent enzyme production (Yakoby et al. 2001).

Laccase (LAC), also known as p-diphenol oxidase, is another extracellular enzyme widely found in fungi, and has a variety of biological activities, such as, lignin degradation (Baldrian 2006), detoxification of antifungal compounds, suppression of plant defence responses (Chi et al. 2009), fungal virulence and appressorial melanisation (Guetsky et al. 2005; Lin et al. 2012). LAC has an important role in pathogenicity by detoxifying epicatechin in avocado and capsaicin in chilli pepper infected by *C. gloeosporioides* (Guetsky et al. 2005; Liao et al. 2012).

In this context, the successful establishment of fungal pathogens and their ability to degrade fruits and leaves depends on a series of events leading to production of extracellular enzymes. Thus, the objectives of this study were to compare the production of these enzymes in ABR- and GLS-isolate *in vitro* and to evaluate their involvement on infected apple leaves with *C. fructicola*.

## 2. Material and methods

### 2.1. Biological material

*Colletotrichum fructicola* isolates MANE40 and MANE147 were originated from fruits and leaves with ABR- and GLS symptoms, respectively (Velho et al. 2015). Fungus was grown on potato dextrose agar medium (PDA-Himedia Labs) at  $\pm 25^{\circ}\text{C}$  and 12 h photoperiod under white fluorescent light for 15 days.

Ripened apple fruits of cv. Gala and susceptible seedlings were used in the experiments. Seedlings were produced from "Gala"  $\times$  "Fuji" cross seeds according to Araújo and Stadnik (2013). Plants were grown in individual pots under greenhouse conditions at  $25 \pm 5^{\circ}\text{C}$  until having c. 12 expanded leaves.

### 2.2. Agar plate enzymatic assay

In order to evaluate amylolytic activity, fungus was grown in nutrient agar medium supplemented with 0.2% of soluble starch (pH 6.0). Five days after incubation at  $\pm 25^{\circ}\text{C}$  and 12 h photoperiod, plates were flooded with 1 mL of iodine solution and a white zone formed in the colony indicated the fungus ability to degrade starch (Hankin and Anagnostakis 1975). Fungal growth and starch degradation area were calculated using the formula:  $A = \pi r^2$ .

For lipolytic activity, fungus was grown in culture medium supplemented with 10 g L<sup>-1</sup> peptone, 5 g L<sup>-1</sup> NaCl, 0.1 g L<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O and 1% Tween® 20 (pH 7.4) (Hankin and Anagnostakis 1975). For proteolytic activity, cultures were grown on agar medium supplemented with 0.4% of soluble skim milk (Liao et al. 2012). Five days after incubation, lipolytic and proteolytic activities were evaluated by the size (mm) of halos formed around the colony measured with digital caliper.

For pectolytic enzymes production, cultures were grown in medium composed of 10 g apple-pectin, 2 g yeast extract, 15 g agar and mineral solution containing 2 g L<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 6 g L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g L<sup>-1</sup> FeSO<sub>4</sub>·7H<sub>2</sub>O, 1 mg L<sup>-1</sup> CaCl<sub>2</sub>, 10 µg L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 10 µg L<sup>-1</sup> MnSO<sub>4</sub>, 70 µg L<sup>-1</sup> ZnSO<sub>4</sub>, 50 µg L<sup>-1</sup> CuSO<sub>4</sub> and 10 µg L<sup>-1</sup> MoO<sub>3</sub>. In order to detect pectate lyase (PL EC 4.2.2.2) pH medium was adjusted to 7.0 and, for polygalacturonase (PG EC 3.2.1.15) it was adjusted to 5.0 (Hankin and Anagnostakis 1975). Five days after incubation, pectolytic enzymes were determined by measuring the size of halos (mm) around the colonies after flooding them with 10 g L<sup>-1</sup> of Hexadecyltrimethylammonium bromide (CTAB) solution for 10 min at room temperature.

### 2.3. Enzyme activity of cultured broth

Enzyme activities were carried out by growing the cultures in a basal medium composed of 10 g L<sup>-1</sup> apple-pectin, 4 g L<sup>-1</sup> asparagine monohydrate, 0.5 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g L<sup>-1</sup> H<sub>2</sub>KPO<sub>4</sub>, 0.6 g L<sup>-1</sup> HK<sub>2</sub>PO<sub>4</sub>, 0.4 mg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.09 mg L<sup>-1</sup> MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.07 mg L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 0.02 mg L<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 1 mg L<sup>-1</sup> FeCl<sub>3</sub>, 3.5 mg L<sup>-1</sup> ZnCl<sub>2</sub> and 0.1 mg L<sup>-1</sup> of thiamine hydrochloride (pH 3.5) (Ramos et al. 2010). One 5-mm agar plug from *C. fructicola* isolate was added to each flask and incubated without agitation at ±25°C and 12 h photoperiod under fluorescent light. Mycelium was harvested after 10 days by filtration through a Whatman n°1 filter paper using a Buchner funnel. Dry weight of mycelium was determined after drying it to a constant weight at 70°C. The culture supernatants were used as enzyme extracts.

Pectin lyase (PNL EC 4.2.2.10) activity was assayed by the thiobarbituric acid (TBA) method (Ramos et al. 2010). The reaction mixture contained 0.5 mL of 1.2%

pectin in 0.05 M Tris-HCl buffer (pH 8.0) and 0.5 mL of enzyme extract. Samples were prepared in test tubes and incubated at 30°C for 1 h. After incubation, 0.75 mL of 1 N HCl and 1.25 mL of 0.04 M TBA were added and kept at 100°C for 20 min. After cooling, absorbance was measured at 550 nm using the SpectraMax Paradigm® microplate reader.

Polygalacturonase activity (PG) was assayed using the dinitrosalicylic acid (DNS) method (Tewari et al. 2005). The reaction mixture contained 0.2 mL of 0.2 M acetate buffer (pH 4.5), 0.3 mL of 1% polygalacturonic acid and 1 mL of enzyme extract. Samples were incubated at 37°C for 30 min. The reaction was stopped by adding DNS solution and then kept in water boiled for 5 min. After cooling, the absorbance was measured at 530 nm.

Laccase (LAC EC 1.10.3.2) activity was determined with 100 µL of 0.5 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 800 µL of 0.1 M sodium acetate buffer (pH 3.5) and 100 µL of enzyme extract at 30°C. Oxidation of ABTS was measured by the increase in absorbance at 420 nm ( $\epsilon_{420} = 36 \text{ mM}^{-1} \text{ cm}^{-1}$ ) (Levin et al. 2007).

Enzymatic activities of PNL, PG and LAC were expressed in International Units (U) as the amount of enzyme needed to oxidise 1 µmol of substrate per min. Protein concentration was determined using the Bradford method (Bradford 1976) and bovine serum albumin (BSA) served as standard.

### 2.4. Cross infection assay

For inoculation, susceptible apple seedlings were sprayed with a suspension of  $1 \times 10^6$  conidia mL<sup>-1</sup> using an atomiser connected to an air compressor (58 psi) and delivering approximately 4.5 mL plant<sup>-1</sup>. Plants sprayed with sterile distilled water served as control. All plants were kept at ±25°C and 100% relative humidity in the dark for 48 h and after transferred to the greenhouse again.

Necrotic leaf severity was assessed on 10th day after inoculation. For this, upper (1st to 4th) and lower (5th to 10th) leaves were detached, scanned and the percentage of infected area was determined for each group using the software Quant® (Velho et al. 2016).

Fruit lesion diameter was assessed according to Velho et al. (2015). "Gala" apple fruits were inoculated with two 10 µL drops ( $1 \times 10^6$  conidia mL<sup>-1</sup>)

onto wounds, incubated in a moist chamber and stored at 25°C and 12 h photoperiod for 10 days. Diameter of the lesions was measured on each fruit, in two perpendicular directions using a digital caliper.

## 2.5. Leaf enzyme assay

Fungal extracellular enzymes were performed at 0, 6, 12, 24 and 48 h after inoculation (HAI). For each sampling time, 1st and 2nd fully expanded leaves were collected, snap-frozen and stored at -80°C until use.

For enzyme extraction, apple leaves were homogenised using a pre-chilled mortar and pestle, with 100 mM sodium acetate buffer (pH 5.5) in the proportion of 1:3 (w v<sup>-1</sup>). The suspension was centrifuged at 20,000 × g for 30 min at 4°C and the supernatant was collected and placed on ice for further determination of protein content and enzyme activities. PNL, PG and LAC activities were determined as previously described in section enzyme activity of cultured broth.

## 2.6. Experimental design and statistical analyses

The experiments were arranged in a completely randomised design using six replicates for each treatment and were repeated twice. Data were subjected

to analysis of variance (ANOVA) in order to determine the significance of differences. Means were compared by Student's t test ( $p \leq 0.05$ ) using SAS programme (v.9.1 SAS Institute, Inc., Cary, NC).

## 3. Results

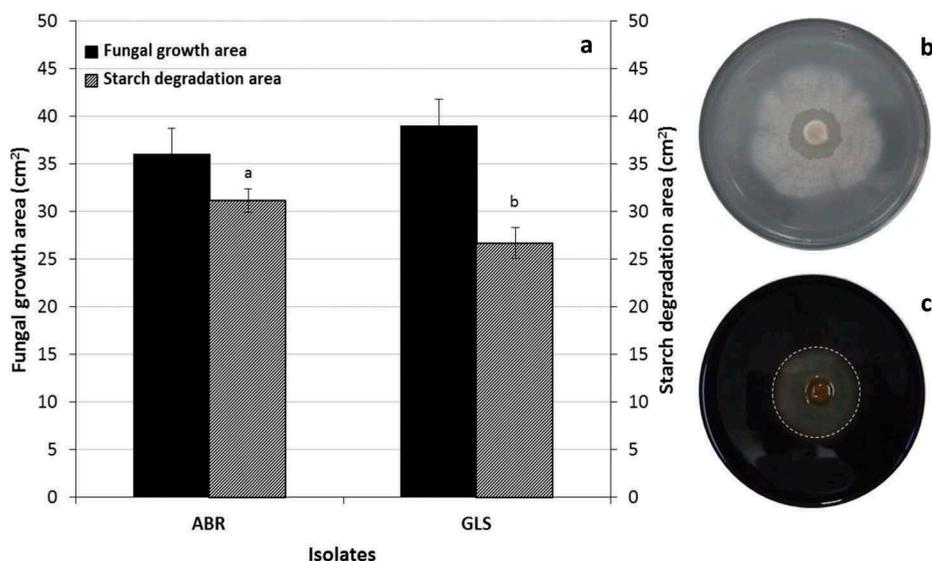
### 3.1. Agar plate enzymatic assay

ABR- and GLS-isolate successfully grow on agar plates and showed an average growth area around 37.5 cm<sup>2</sup> (Figure 1(a,b)). Both exhibited amylolytic, lipolytic, proteolytic and pectolytic activity at 5 days after incubation.

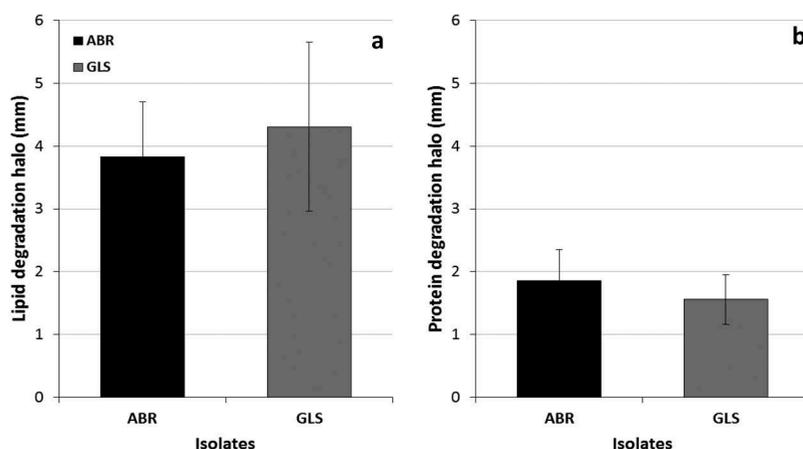
ABR-isolate showed significantly higher amylolytic (Figure 1(a)) and pectolytic (Figure 3(a,b)) activity when compared to GLS-isolate. In contrast, for lipolytic (Figure 2(a)) and proteolytic (Figure 2(b)) no significant differences were observed between isolates.

Amylolytic activity of ABR- and GLS-isolate was successfully detected using iodine solution and the white zone inside the colony of ABR was approximately 1.2-fold higher than GLS-isolate (Figure 1(a, c)). For lipolytic and proteolytic activity both isolates produced small degradation halos, with an average of 3.9 and 1.7 mm, respectively (Figure 2(a,b)).

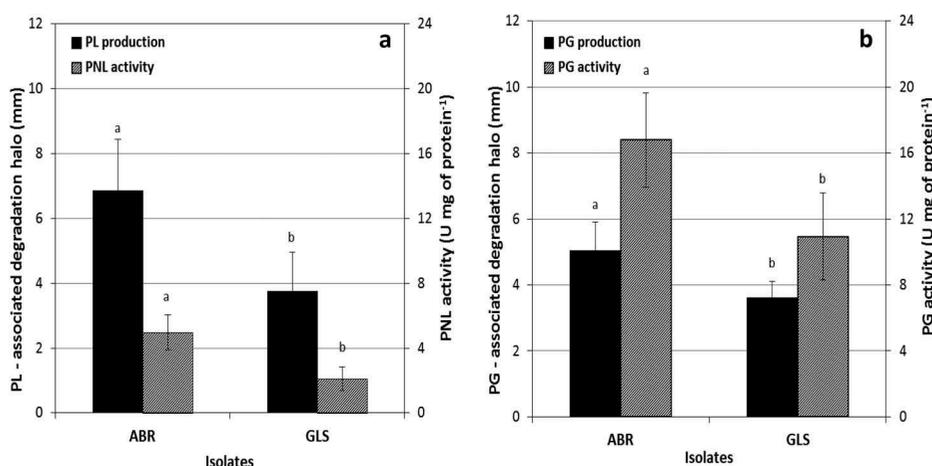
In relation to pectolytic enzymes, PL and PG produced degradation halos significantly 2 and 1.5-fold higher in ABR- than for GLS-isolate (Figure 3(a,b)).



**Figure 1.** Fungal growth and starch degradation area of apple bitter rot (ABR) and *Glomerella leaf spot* (GLS) isolate at 5 days after incubation (a). *Colletotrichum fructicola* colony without reaction (b) and with starch degradation area after revelation with iodine solution (c). Dotted lines delimit starch degradation zone. Different letters indicate significant differences between isolates according to Student's t test ( $p \leq 0.05$ ). Bars represent standard deviation.



**Figure 2.** Lipid (a) and protein (b) degradation halo of apple bitter rot (ABR) and *Glomerella leaf spot* (GLS) isolate at 5 days after incubation. Bars represent standard deviation.



**Figure 3.** Pectate lyase (PL)-associated degradation halo, Pectin lyase (PNL) activity (a), Polygalaturonase (PG)-associated degradation halo and PG activity (b) of apple bitter rot (ABR) and *Glomerella leaf spot* (GLS) isolate at 5 days after incubation. Different letters indicate significant differences between isolates according to Student's *t* test ( $p \leq 0.05$ ). Bars represent standard deviation.

### 3.2. Enzyme activity of cultured broth

All isolates were able to grow in basal medium and produced around 2.5 mg mL<sup>-1</sup> of dried mycelium after a 10-day incubation period (data not shown).

ABR-isolate showed high activity of the three enzymes evaluated, mainly LAC. PNL and PG activities were significantly 2 and 1.5-fold higher in cultured broth with ABR- than GLS-isolate, respectively (Figure 3(a,b)), while for LAC, ABR- was significantly five-fold higher than GLS-isolate showing values of 10 and 2.1 U mg of protein<sup>-1</sup>, respectively (Figure 4).

### 3.3. Cross infection assay

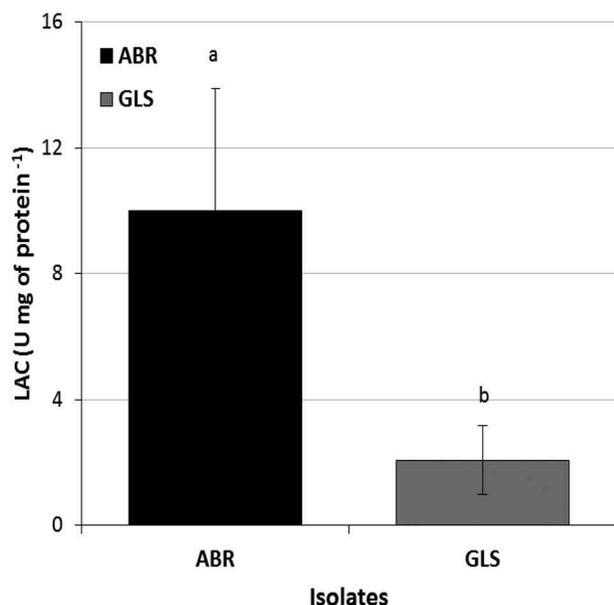
Symptoms on apple leaves appeared as reddish-purple spots 2 days after infection with GLS-isolate. The spots coalesced, evolved into irregular necrotic lesions and at

10 days after inoculation the disease severity reached 15.7 and 13.1% on upper and lower leaves, respectively (Figures 5(a) and 6(c)). Plants inoculated with ABR-isolate and control (water-treated) did not exhibit any symptom (Figures 5(a) and 6(a)).

First symptoms on wounded apple fruits appeared at 4 days after incubation. Fruits inoculated with ABR-isolate exhibited diameter of lesions 3.5-fold higher than GLS-isolate (Figures 5(b) and 6(b,d)) and, after 10 days frequently produced acervuli with abundant conidial masses over the lesions (Figure 6(b)).

### 3.4. Leaf enzyme assay

No significant changes in PNL, PG and LAC activities were observed throughout the time in susceptible



**Figure 4.** Laccase (LAC) activity of apple bitter rot (ABR) and Glomerella leaf spot (GLS) isolate at 5 days after incubation in culture broth. Different letters indicate significant differences between isolates according to Student's t test ( $p \leq 0.05$ ). Bars represent standard deviation.

apple leaves inoculated with both isolates and control (water-treated) (Figure 7(a-c)).

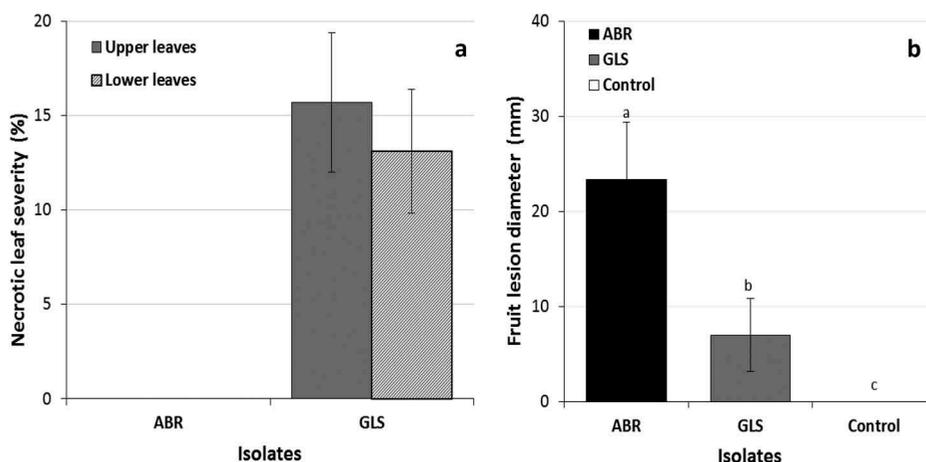
#### 4. Discussion

*Colletotrichum fructicola* has different strategies to colonise and interact with their hosts and the production of extracellular enzymes *in vitro* clearly demonstrates differences between ABR- and GLS-isolate. In overall, ABR-isolate showed significantly

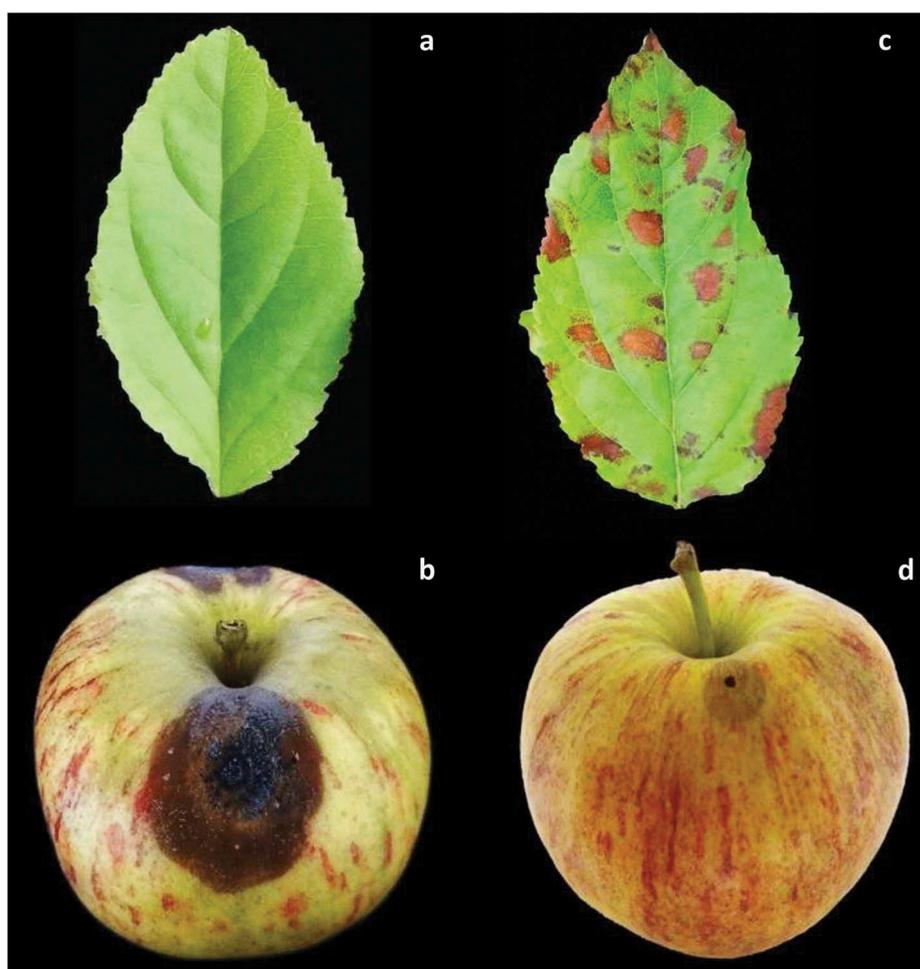
higher extracellular enzymes activity when compared to GLS-isolate. However, during *C. fructicola* leaf infection no significant differences between isolates were detected for all enzymes tested.

In this study, amylolytic, pectolytic, lipolytic and proteolytic activities were successfully detected using solid medium, but differences between isolates were found only for *C. fructicola* ability to secrete amylases and pectinases. During infection, *Colletotrichum* species produce a range of these enzymes that affect organisation of the host cells providing nutrients for their growth and development (Gregori et al. 2008). In this case, lipids and proteins from degradation of host cell are considered an important source of nutrients during fungal colonisation (Bailey et al. 1992; Huang 2001; Kubicek et al. 2014).

ABR- showed significantly higher amylolytic activity than GLS-isolate. Most pathogens utilise starch to perform their metabolic activities, and the degradation occurs directly by the action of amylases (Saranraj and Stella 2013). The end product of starch breakdown, i.e. glucose is used directly by the pathogens to promote their growth (Kubicek et al. 2014), but the direct role of amylases in pathogenicity of *Colletotrichum* is not fully understood. In this case, ABR-isolate probably needs to break down a higher amount of starch, uses more glucose for its growth, and subsequently to secrete other enzymes required for efficient colonisation. Furthermore, amylase is a pH sensitive enzyme (Saranraj and Stella 2013). It has been reported that fungal amylases are active in



**Figure 5.** Necrotic leaf severity (a) and fruit lesion diameter (b) at 10 days after inoculation with apple bitter rot (ABR) and Glomerella leaf spot (GLS) isolate. Different letters indicate significant differences between isolates according to Student's t test ( $p \leq 0.05$ ). Bars represent standard deviation.



**Figure 6.** Leaves and fruits of apple cv. Gala inoculated with ABR- (a and b) and GLS-isolate (c and d), respectively, at 10 days after inoculation.

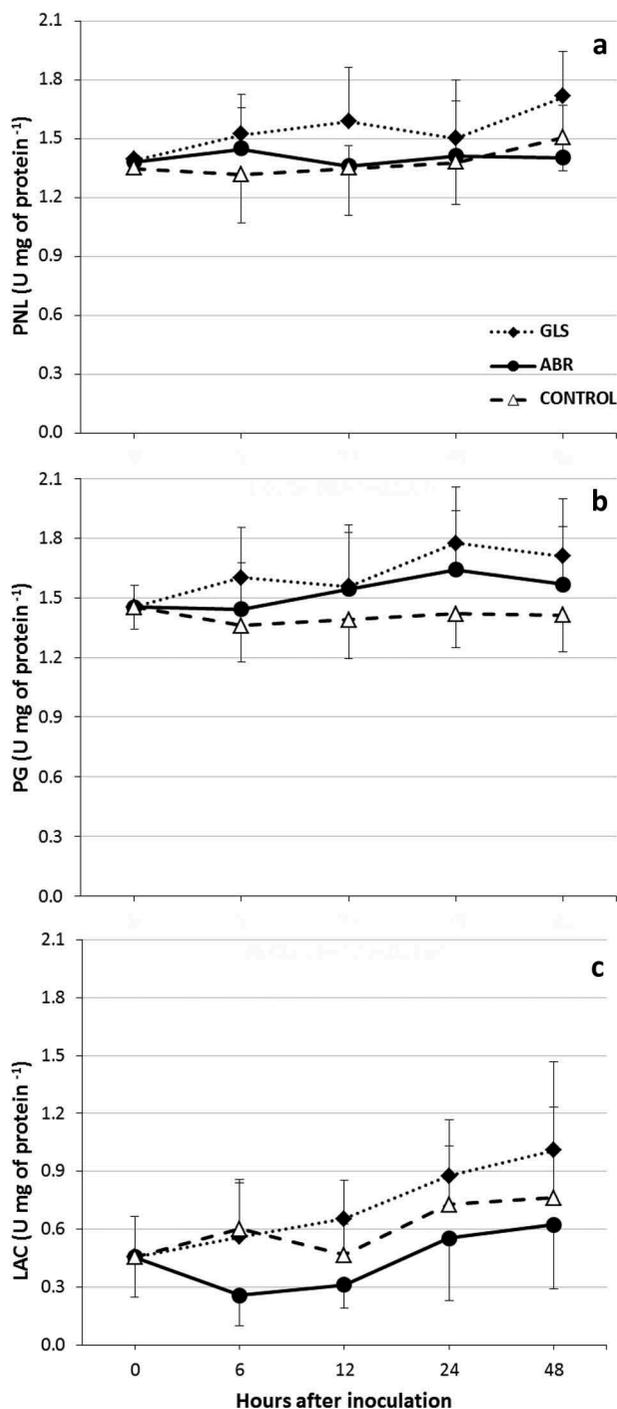
acidic medium, but interestingly in our study, amylases of both isolates were active in pH 6.0.

Pectolytic enzymes (PL, PNL and PG) were also higher in ABR- when compared to GLS-isolate, suggesting the possible involvement of these enzymes in fruit pathogenesis. Probably, the alkaline environment where they were subjected (pH 7.0 in agar plate and 8.0 in culture broth) also influenced PL and PNL production. The *Colletotrichum* ability to change the environmental pH from acidic to neutral has been known for decades (Kubicek et al. 2014). This species produces ammonia in culture medium and in infected tissues causing alkalinisation of the medium and consequently favours enzymatic secretion (Prusky et al. 2001; Drori et al. 2003). Thus, secretion of some extracellular enzymes, i.e. PL, is caused by a pH-dependent mechanism that occurs

naturally during fungal growth and consequently improves the infection process (Yakoby et al. 2000).

The expression of pathogenicity genes encoding pectolytic enzymes, i.e. *pnIA*, *pelB* are also secreted only at pHs higher than 6.0 (Yakoby et al. 2000, Drori et al. 2003; Prusky et al. 2013). Besides the pH, efficacy of pectolytic enzymes to degrade cell walls can also depend upon the structure of host cell (Kubicek et al. 2014) and the different isoforms of PNLs and PGs during colonisation of infected tissues (Shih et al. 2000; Yakoby et al. 2001). In general, *Colletotrichum* isolates deficient in such enzymes seem to be less virulent as demonstrated in studies with gene disruption (Wattad et al. 1995).

ABR- and GLS-isolate have clear differences and this could be used as a tool for understanding the importance some enzymes in this pathosystem. Such



**Figure 7.** Time course of pectin lyase (a), polygalacturonase (b) and laccase (c) activity in susceptible apple leaves inoculated with apple bitter rot (ABR) and *Glomerella* leaf spot (GLS) isolate. Different letters indicate significant differences between isolates for each sampling time according to Student's *t* test ( $p \leq 0.05$ ). Bars represent standard deviation.

differences can be observed when we evaluated the evolution of lesions on apple fruits. ABR-isolate was capable to cause a higher diameter of the lesion,

whereas in fruits infected with GLS-isolate the lesions did not exceed 7 mm.

ABR- also exhibited significantly higher LAC activity when compared to GLS-isolate. It is known that products of pectin breakdown act as a second inducer of LAC, (Guetsky et al. 2005). In addition, LACs have been known for its influence on enzymatic degradation of lignin, fungal virulence (Lin et al. 2012) and help fungi to eliminate toxic plant metabolites (Guetsky et al. 2005). These metabolites, such as, polyphenols, phytoalexins and other fungitoxic substances present or induced, differ markedly during leaf and fruit development (Terry et al. 2004). Generally, highest LAC activity is found during fruit decay development. Therefore, a higher activity of such enzyme may be an adaptative advantage for specialised fruit isolates, like ABR-isolate.

Even though differences between isolates have been found *in vitro* for PNL, PG and LAC activities, ABR- and GLS-isolate produced small amounts of these enzymes and no significant difference were observed on infected apple leaves. Although these enzymes are markedly important in some anthracnose diseases (Guetsky et al. 2005), their role in pathogens that cause leaf diseases has not been established so far. In fact, causes of the lower activity found *in vivo* could be associated to the alkaline environment needed for gene expression and subsequently secretion of some extracellular enzymes. Another possibility could be related to the presence of preformed or induced antifungal compounds in leaf tissues which inhibit fungal extracellular enzymes (Prusky et al. 2001). To clarify such differences between ABR- and GLS-isolate, for future research it would be interesting to evaluate gene expression of extracellular enzymes involved in virulence.

Overall, the secretion of extracellular enzymes may lead to a greater fungal adaptability on distinct host plant tissues. Therefore, this might explain the differences between isolates that infect fruits and leaves. This study provides novel information about the differences between production of extracellular enzymes of ABR- and GLS-isolate *in vitro*. Although it was expected to find differences between enzymes on infected apple leaves, these results contributed to highlight the importance to investigate such enzymes in depth.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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