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Polygalacturonase isozymes produced during infection of the grape berry by *Botrytis cinerea*

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Summary

This paper focuses on polygalacturonase activity during the early stages of infection of the grape berry by the fungus *Botrytis cinerea*. While a weak polygalacturonase activity was measured in healthy, ripe Semillon grape berries, in infected berries the activity increased rapidly with infection. Liquid isoelectrofocalisation was used to separate polygalacturonase isozymes in healthy and infected berries. In healthy berries, we found 4 isozymes of polygalacturonase with pI values of 4.2, 4.9, 6.0 and 8.0. In infected berries, we have separated up to 7 isozymes of polygalacturonase in infected berries. The first isozymes detected had acidic pIs; basic isozymes of polygalacturonase with considerable activity were separated. The most active isozyme had a pI of 8.8. At the onset of infection by *B. cinerea*, polygalacturonase activity consists of a large number of isozymes, most of which are identical with the isozymes previously described.

K e y w o r d s : *Botrytis cinerea*, grey mould, grape berry, polygalacturonase, isozyme.

Introduction

Botrytis cinerea is a widespread fungus infecting many plant species and causing soft rot. It is responsible for the grey mould disease of grapevines (Verhoeff 1992; Elad and Shtienberg 1995). In grape berries, this leads to extensive biochemical modifications with unfavourable effects on fermentation (Donèche 1992). Cell wall degrading enzymes play an important role in the infection process. Pectic enzymes and, in particular, polygalacturonase (PG) are the first cell wall degrading enzymes secreted by many micro organisms. Polygalacturonase is reported to be constitutively expressed in the ungerminated conidia of *B. cinerea* (Verhoeff and Liem 1978). Polygalacturonase catalyses the hydrolysis of α-(1,4) glycosidic bonds between two unesterified galacturonic acid units. The two types of polygalacturonases, endoPG (EC 3.2.1.15) and exoPG (EC 3.2.1.67), denote random or terminal cleavage activity, respectively. Different isozymes of polygalacturonase produced *in vitro* by *B. cinerea* have already been purified and characterised (Marcus and Schejter 1983; Leone *et al.* 1990; Johnston and Williamson 1992; Tobias *et al.* 1993; Lee *et al.* 1997). The sequential *in vitro* production of polygalacturonase and its regulation by different inductors have also been studied (Zalewska-Sobcsak and Urbanek 1975; Van Den Heuvel and Waterreus 1985; Leone and Van Den Heuvel 1987). Recently, 6 endo-polygalacturonases coding genes were cloned and partially characterised (Wubben *et al.* 1999). However, little research has been done on *in vivo* polygalacturonase production in infected tissue (Tobias *et al.* 1995). To date, there is no rigorous demonstration that polygalacturonases are a virulence factor in fruit infection by *B. cinerea*. However, they are known to participate in tissue maceration symptomatic of soft rot (Staples and Mayer 1995). Like many other fruits, grape berries are able to synthesise polygalacturonase during ripening (Cabanne and Donèche 2001). The role of this enzyme in fruit softening is not yet clearly defined. However, polygalacturonase activity seems to be both, necessary and sufficient for pectin depolymerisation in the late stages of fruit ripening (Hadfield and Bennett 1998).

In this study, we describe the sequential appearance of polygalacturonase isozymes in the grape berry before and during early stages of infection by *B. cinerea*.

Material and Methods

P l a n t m a t e r i a l : *Vitis vinifera,* cv. Semillon grape berries growing in a Sauternes vineyard (Bordeaux, France) were used. Fully ripe *Botrytis cinerea*-infected berries were harvested and immediately frozen at -30 °C. Homogeneity of samples was previously checked by berry diameter and density (Barnavon 1999).

Infection by *B. cinerea* often occurs by spore germination. The resulting hyphae penetrates through the berry skin (Pucheu-Planté and Mercier 1983). The lesion starts with a brown spot, which is easy to distinguish on white grape berries. The spot area then increases slowly until the whole berry is brown. Before freezing, berries were quickly classified in three previously described stages (Donèche 1987): Stage 1: Healthy berries without spot; Stage 2: Berries just infected with spots (diameter <5 mm); Stage 3: Infected berries with spots (diameter >5 mm) covering up to 50 % of the berry surface.

In the same way three more stages are defined until the berries become shriveled and desiccated (Donèche 1987; Darrieumerlou *et al.* 2001).

C r u d e p r o t e i n e x t r a c t p r e p a r a t i o n : According to previous work on grape enzymes, extracts were performed with chelating and anti-oxidant agents to prevent oxidation of phenolic compounds. In addition, the presence of anionic polymers prevented precipitation of proteins (Meynhardt *et al.* 1974; Zhou *et al.* 2000). All steps were

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performed at +4 °C. Fifty frozen berries were homogenized with an Ultra-Turrax (IKA, Staufen, Germany) for 10 min with 100 ml of cold sodium phosphate buffer (0.1 M, pH 7) containing 5.8 mM diethyldithiocarbamic acid, 2.5 mM ethylenediaminetetraacetic acid, 5.7 mM ascorbic acid and 8 % (w/v) polyethylene glycol (PEG 3350) (MEYNHARDT *et al.* 1974; ZHOU *et al.* 2000). The homogenate was then centrifuged for 10 min at 18,500 *g*. The supernatant was quickly adjusted to pH 6.4 with NaOH (0.1 N), if necessary, and stirred slowly for 30 min after adding 59 % (w/v) PEG. Then, the suspension was centrifuged for 20 min at 18,500 *g*. The precipitate was suspended in 8 ml of phosphate citrate buffer (0.1 M, pH 3.4). Insoluble material was removed by centrifugation at 18,500 *g* for 20 min. Low molecular compounds were eliminated by gel filtration on small columns (PD-10 Desalting columns, Pharmacia, Orsay, France) with sodium phosphate (0.1 M, pH 7) buffer as eluant. The eluted proteic fraction was then used for analysis. The whole extraction process was done in triplicate.

Polygalacturonase activity assay: Polygalacturonase activity was determined quantitatively by the 2-cyanoacetamid method (GROSS 1982) based on the spectrophotometric determination of reducing groups released from sodium polygalacturonate as substrate. The assay mixture consisted of 750 µl of sodium acetate buffer (0.1 M, pH 4.5) containing 0.2 % (w/v) polygalacturonic acid and 250 µl of proteic extract. Incubation was performed for 2 h at 30 °C. D-galacturonic acid was used as standard to establish the calibration curve. Previous data indicate that although the molar responses of uronides with various degrees of polymerisation may differ very slightly in terms of chromophore yield, the assay is suitable for estimating both, endo- and exo-polygalacturonase activities (GROSS 1982). Activity was expressed in nanokatals and represents the average of three assays.

Isoelectrofocalisation (IEF): This was performed using the Rotofor liquid phase preparative isoelectric focusing column (Biorad, Marnes La Coquette, France) with a pH range of 3-10 obtained by addition of 2 % (v/v) ampholytes (Bio-Lyte® 3/10, Biorad). The IEF device was operated at 4 °C using 10 µm pore polyester separators defining the 20 fractions in a 18 ml chamber; ion exchange membranes were in the IEF orientation in electrolyte assemblies. Focusing was conducted for 3-5 h at 12 W constant power until voltage remained constant. The fractions were collected and their pH values determined. Polygalacturonase activity was measured after dilution with 1 V of acetate buffer (0.1 M, pH 4.5) and pH adjustment between 4 and 5 if necessary.

Each profile obtained by averaging the triplicate assays was determined after two runs under the same conditions. Only polygalacturonase isozymes detected during both runs were presented in IEF profiles.

Results and Discussion

Polygalacturonase activity of crude enzyme extracts of healthy and infected berries was determined at the beginning of infection (Fig. 1). In ripe, healthy berries the endogenous polygalacturonase activity was weakly expressed (0.08 nkat berry^{-1}). Then, from the very onset of infection, there was a marked increase in activity until stage 3 (0.4 nkat berry^{-1}). Thereafter, the activity decreased rapidly to a level close to that of ripe uninfected berries (data not shown). The activity measured during infection might be due to the synthesis of polygalacturonase either by the grape, or the fungus, or both. However, infection by *B. cinerea* is known to be very harmful for the fruit and is accompanied by the death of cells adjacent to the infected walls (PUCHEU-PLANTÉ and MERCIER 1983; DONÈCHE 1992). Thus polygalacturonase activity produced by *B. cinerea* was probably responsible for the increase observed during the early stages. It seems possible that the endogenous activity of the healthy berry disappears from the first stages of infection.

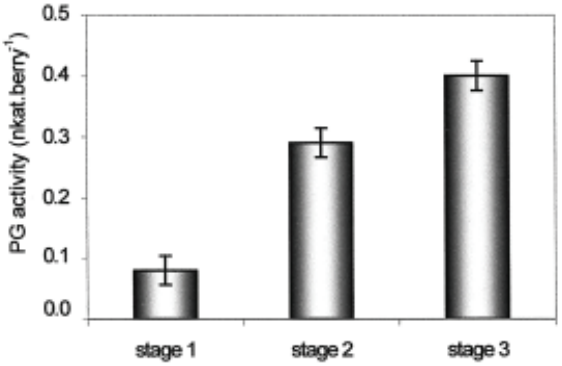

Fig. 1: Development of polygalacturonase activity at the beginning of infection of Semillon berries by *Botrytis cinerea*. Plotted values are means ± s.e. (n=3 replicates).

Analysis of polygalacturonase isozymes of early stage infection was performed by liquid phase isoelectrofocalisation (Figs 2-4). The profile obtained after IEF revealed the presence of at least 4 polygalacturonase isozymes in ripe, healthy Semillon berries (Fig. 2). Their pI were 4.2, 4.9, 6.0, and 8.0, indicating that three of them were acidic isozymes. Most fruits produce several polygalacturonase isozymes during ripening (DELLA PENNA *et al.* 1990; PATHAK and SANWALL 1998). Previous findings obtained with other grapevine cultivars indicate a polygalacturonase isozyme in berries with a pI value of 5.3 (TYURINA 1977). Results from our laboratory (unpubl.) indicate a polygalacturonase isozyme with a pI value of 5.3 in berries of Cabernet-Sauvignon similar to the study of TYURINA (1977). This possibly indicates that these pIs are a varietal character. Therefore further research is needed on this topic.

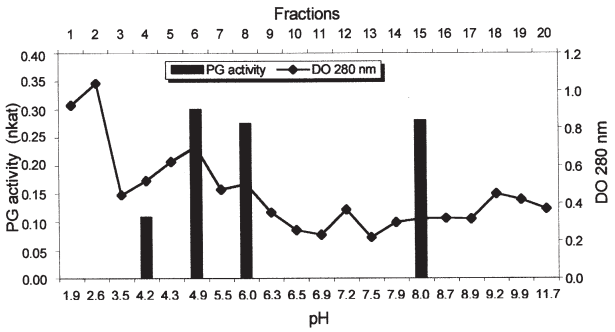

Fig. 2: Separation of polygalacturonase isozymes of healthy ripe berries by liquid IEF (stage 1).

At the beginning of infection, 5 isozymes were detected (3.2, 4.9, 5.5, 8.8 and 12.0) (Fig. 3). Their pI values were different from those of isozymes detected in healthy berries, except the isozyme of pI 4.9, so they were probably neosynthetized. Most isozymes in healthy berries seemed to disappear rapidly at the beginning of infection. The activity in this stage (2) may be due to polygalacturonase production by *B. cinerea*. We cannot definitely decide whether the pI 4.9 isozyme is a fungal or a plant polygalacturonase. Previous work on polygalacturonase isozymes indicated that a polygalacturonase isozyme (pI value 4.9) is produced early by many strains of *B. cinerea in vitro* (Van Der Cruyssen and Kamoen 1992). Furthermore, 4 PGs have been characterized *in vitro*, two of which have a pI value of 4.9 and the others two pI values of 8.8 and 3.5 (Johnston and Williamson 1992).

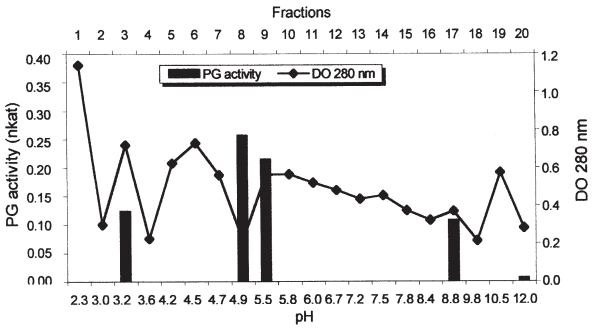

Fig. 3: Separation of polygalacturonase isozymes by liquid IEF during infection of Semillon berries by *B. cinerea* (stage 2).

In the following stage (3) of infection, IEF revealed the presence of at least 7 isozymes (Fig. 4). Their pI were 2.3, 3.2, 6.5, 7.8, 8.0, 8.8 and 12.0. The polygalacturonases with pI 3.2 and 8.8 were already present in the previous stage of infection. In stage 3, the isozyme of pI 8.8 had the highest catalytic activity. This number of isozymes is in agreement with IEF PAGE profiles obtained from 6 different isolates of *B. cinerea* (Magro *et al.* 1980). The isozyme of pI 8.0 has already been reported in different strains of *B. cinerea* inoculated together with an isozyme of pI 8.8 (Di Lenna and Fielding 1983; Sharrock *et al.* 2000). Isozymes of pI 4.9 and 5.5 were not detected in stage 3; between stages 2 and 3, they may have disappeared or their activity level may have dropped below the detection threshold.

Therefore, the pI values of polygalacturonase isozymes produced by *B. cinerea* in plant tissue are close to those described previously for *in vitro* experiments. This agrees with the results of Tobias *et al.* (1993, 1995) who found the same polygalacturonase isozymes produced by *B. cinerea in vitro* and *in vivo* on infected apples.

Conclusion

This study confirms most of the previous findings on polygalacturonase isozymes of *B. cinerea* and points out two important characteristics of polygalacturonase.

The first is the multiplicity of polygalacturonase types. This could confer flexibility and a greater adaptive capacity

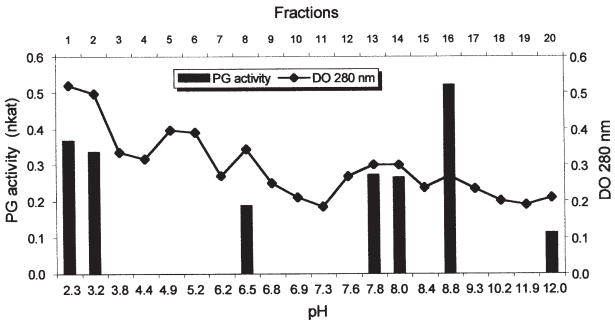

Fig. 4: Separation of polygalacturonase isozymes by liquid IEF during infection of Semillon berries by *B. cinerea* (stage 3).

to the pathogen. The sequential pattern of polygalacturonase during infection can be interpreted as the successive induction and repression of enzyme synthesis for the complete degradation of the pectic polymer. The phenomenon of polygalacturonase multiplicity is also common in other micro-organisms producing pectic enzymes (Cervone *et al.* 1986; Blanco *et al.* 1999).

The second is the variability of polygalacturonase isozymes between isolates and fungal growth conditions. Previous studies have shown a great variability for polygalacturonase isozymes between strains of *B. cinerea*. The present study demonstrates some polygalacturonase isozymes similar to others described previously. Like Leone (1992), we assume that polygalacturonase isozymes in *B. cinerea* strains are relatively stable, perhaps because the isozymes described here are those which are most commonly synthesised by this fungus. Indeed, in various studies the cultural conditions for polygalacturonase studies are often the same; they consist of a liquid mineral medium supplemented with pectin, which is probably the most closely related to conditions existing in the apoplast during *in vivo* infection of a fruit by *B. cinerea*.

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