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INHIBITION OF FUNGAL POLYGALACTURONASES BY EXTRACTS OF *Eucalyptus grandis* WITH DIFFERENT DISEASE SUSCEPTIBILITIES

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ABSTRACT

Eucalyptus grandis is an important exotic forest plantation species in many parts of the world. Diseases caused by fungal pathogens are an important constraint to the productivity of *Eucalyptus* plantations. Fungi secrete cell wall degrading enzymes (CWDEs) during the process of colonising plants. Endopolygalacturonases (endoPGs) constitute some of the important CWDEs. The aim of this study was to detect and compare the levels of polygalacturonases produced by a group of different *Eucalyptus* pathogens. A further objective was to determine the inhibition levels of polygalacturonase-inhibiting proteins (PGIPs) associated with a disease tolerant and a susceptible *Eucalyptus* clone on endoPGs from the fungal pathogens. Extracts of the polygalacturonases were collected from selected isolates of the *Eucalyptus* canker pathogens *Cryphonectria cubensis, Coniothyrium zuluense, Botryosphaeria dothidea* and the root pathogen *Phytophthora cinnamomi*. Polygalacturonase production and inhibition by PGIPs was measured by reducing sugar assays. We found that *B. dothidea* and *C. zuluense* produced more endoPGs than *C. cubensis* and *P. cinnamomi*. In most cases PGIPs from the generally disease tolerant clone were equally as effective as those from the susceptible clone, in their capacity to inhibit the endoPGs. An exception was for *C. zuluense*, where PGIPs from the resistant clone were considerably more effective than those from the susceptible clone. We conclude the PGIP extracts from *Eucalyptus* selectively inhibit endoPGs from different species. The tolerance of the resistant clone to *C. zuluense* could be linked to the ability of its PGIPs to inhibit endoPGs from *C. zuluense*.

Key words: *Eucalyptus grandis*, polygalacturonases, PGIPs and host defence.

INTRODUCTION

Pectic polysaccharides are a major component of primary cell walls of most plants (Carpita and Gibeaut, 1993). The breakdown of pectin is one of the first functions of fungal pathogens during infection (Albersheim and Anderson, 1971; Favaron *et al.*, 1997; Walton, 1994, 1997). This is achieved by secretion of pectin degrading enzymes such as polygalacturonases, especially endoPGs. There are also reports that specifically associate the production of endoPGs by pathogens with pathogenesis (Ten Have *et al.*, 1998; Walton, 1997).

Plants possess systems that prevent the enzymatic breakdown of pectin. Susceptibility of pectin to degradation by endoPGs can be affected by the formation of calcium bridges across pectin chains (Favaron *et al.*, 1997). Formation of such bridges result in decreased rates of degradation of pectin by endoPGs. Furthermore, plants possess proteins that inhibit the action of these enzymes and these are known as polygalacturonase-inhibiting proteins (PGIPs). These proteins reduce the rate of hyphal penetration into plants and allow plants to mobilise other defence systems to halt disease development (Cervone *et al.*, 1989; De Lorenzo and Cervone, 1997; Stotz *et al.*, 2000).

EndoPGs have been reported to have dual and opposing roles in fungal pathogenesis. Firstly, they are utilised by fungi as agents involved in disease development and secondly, they are thought to act as potential defence signal molecules. The early production of these endoPGs is consistent with both roles (Cervone *et al.*, 1997). It has been shown that PGIPs favour the formation of elicitor-active oligogalacturonides from pectin degradation by endoPGs (Cervone *et al.*, 1989).

PGIPs possess structural characteristics of leucine rich repeats (LRRs) similar to those of signal transduction molecules (De Lorenzo *et al.*, 1994; Jones and Jones, 1997). They can be induced by salicylic acid, wounding and fungal pathogens. This illustrates the importance of PGIPs in general host defence responses to stress (Bergmann *et al.*, 1994; Nuss *et al.*, 1996; Favaron *et al.*, 1997). The host defence responses include production of chitinases and glucanases, phytoalexin production, lignification and hypersensitive responses (Cervone *et al.*, 1997).

The economic importance of *Eucalyptus grandis* in commercial plantation forestry worldwide has justified studies on pathogens that damage this tree. Some species of the most important stem and root pathogens of *Eucalyptus* plantations include *Cryphonectria cubensis, Botryosphaeria dothidea, Phytophthora cinnamomi* and *Coniothyrium zuluense* (Van Heerden *et al.*, 2001; Van Zyl, 1999; Smith *et al.*, 1994, 1996; Linde *et al.*, 1999). *C. cubensis* causes Cryphonectria canker in tropical and subtropical environments, *B. dothidea* causes die back and cankers in plantations in temperate areas, *C. zuluense* causes a stem canker disease in the tropics and subtropics and *P. cinnamomi* is a well known root pathogen on *Eucalyptus* in many parts of the world (Van Heerden *et al.*, 2001; Fischer *et al.*, 1993; Van Zyl, 1999; Smith *et al.*, 1994, 1996; Linde *et al.*, 1999).

The role of cell-wall degrading enzymes, such as polygalacturonases, in pathogenesis is important because they are involved in the first step in the interaction between fungus and host. Similarly, knowledge of PGIPs is valuable because of the role that they play in combating the action of the endoPGs produced by fungal pathogens. Knowledge of the role of polygalacturonases could lead to the design of novel PGIPs that have a broad specificity to inhibit endoPGs from different fungal pathogens. The aim of this study was, therefore, to detect and compare the amounts of polygalacturonases produced by the fungal pathogens. Our subsequent aim was to assess the inhibition of these enzymes by extracts from two *Eucalyptus grandis* clones that have opposite disease tolerance levels against *Cryphonectria cubensis, Botryosphaeria dothidea, Phytophthora cinnamomi* and *Coniothyrium zuluense*

MATERIALS AND METHODS

Fungal Species

Isolates used in this study were specifically chosen from other studies on the pathogens of interest and where they had been selected for their high levels of virulence. Thus, isolates CMW2113, CMW7218, CMW7217 and CMW2100 were chosen for the pathogens *C. cubensis, B. dothidea, P. cinnamomi* and *C. zuluense*, respectively (Van Heerden *et al.*, 2001; Bernard Slippers (personal communication); Linde *et al.*, 1999; Van Zyl, 1999). The cultures are all maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Prior to testing, the pathogens were grown on a 2 % malt extract agar (2g malt extract, 2g agar, 100 ml water) in the dark.

Plant Material

Tissue culture microplants of *E. grandis* clones, TAG5 and ZG14 were used in this study. Clone TAG5 is known to be reasonably tolerant to stem and root diseases and clone ZG14 is known from prior pathogenicity tests to be highly susceptible to infection by *B. dothidea, C. zuluense, C. cubensis* and *P. cinnamomi.* They are also respectively tolerant and susceptible to damage by insects such as termites (Van Heerden *et al.*, 2001; Bernard Slippers (personal communication); Linde *et al.*, 1999, Van Zyl, 1995).

Eucalyptus cuttings were grown on modified solid MS medium at pH 5.8 (Murashige and Skoog, 1962) at 24 ÚC under fluorescent lights with 16-hour light and 8-hour dark cycles. Modifications to the MS medium included the

addition of vitamins (10 ml^{-1} of 1000X stock), sucrose (4 gl^{-1}), Benzylaminopurine (0.2 mgl^{-1}), Naphthalene acetic acid (0.01 mgl^{-1}). The 1000X vitamin stock consisted of the following: myo-inositol (10 gl^{-1}), thiamine-HCl (67.3 mgl^{-1}), nicotinic acid (246.2 mgl^{-1}), pyridoxine-HCl (61.69 mgl^{-1}), calcium panthothenic acid (47.65 mgl^{-1}), biotin (4.9 mgl^{-1}), choline (13.96 mgl^{-1}), riboflavin (37.64 mgl^{-1}), ascorbic acid (17.61 mgl^{-1}) and glycine (2 mgl^{-1}) (Sigma Chemical Company).

Production of polygalacturonases

Polygalacturonases were induced *in vitro* by taking five mycelial plugs (4 mm^2) from the actively growing margins of each of the fungi and inoculating these into minimum salts liquid medium (100 ml) in Erlenmeyer flasks. The medium contained: 0.5 g yeast extract (Merck); 1.0 g NaOH; 3.0 g $_{DL}$-Malic acid; 2.0 g NH_4NO_3; 1.0 g KH_2PO4; 0.1 g $MgSO_4$ and supplemented with polygalacturonic acid (PGA) (5g/l) (Sigma Chemical Company) as a carbon source in a litre of sterile distilled water (Errampalli and Kohn, 1995). The cultures were incubated in the dark with shaking (100 rpm) at 25 °C for 10 days. Samples from the culture vessels were collected on each of these days. Mycelium was separated by suction filtration with a Buchner funnel. The filtrates were then filter-sterilised through 0.22-micron disposable syringe filters (Millipore, USA) and stored at 4 °C. All samples were assayed for polygalacturonase activity in three independent repeats. Protein concentrations were determined by measurement of absorbance values at 280 nm using bovine serum albumin as a standard. Equal amounts of protein were used in the assays. Production of polygalacturonases was recorded as absorbance values at wavelength 410 nm. A higher absorbance value corresponded to more polygalacturonase enzyme produced. The maximum absorbance was reached after 60 min for all four fungal pathogens (data not shown).

Production of PGIP extracts

To induce PGIPs, the plantlets were

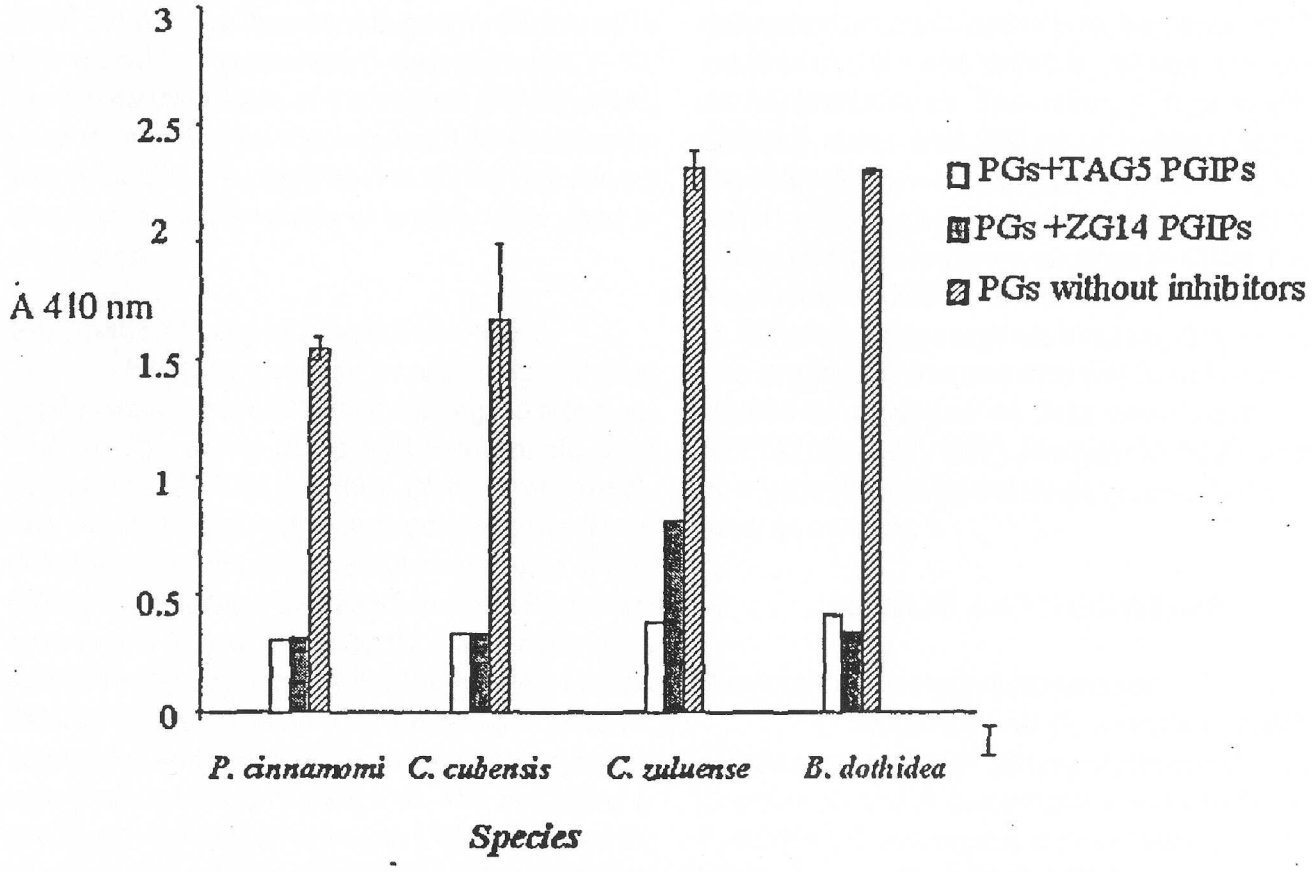

Fig. - 1: Comparing the levels of polygalacturonase production and inhibition of fungal polygalacturonases by PGIPs from by TAG5 and ZG14 at t=60 min. The absorbance values of the untreated samples were subtracted from those of the salicylic acid-treated samples. The bars indicate the standard error of the means of three replicates.

removed from the culture bottles and salicylic acid (50 mM) was sprayed onto the stems. The plantlets were then returned to solid MS medium, aseptically. The culture vessels containing the treated plantlets were covered with aluminium foil. Control plants were not subjected to the treatment with salicylic acid. After 24 hr, PGIPs were extracted with salt washes using a protocol modified from Salvi and co-workers (1990). The plants were finely ground using a mortar and pestle for 4 min in cold 4 mM sodium acetate buffer (pH 5.2) containing 500 mM sodium chloride (2 ml buffer/g plant tissue) at 4 °C. The suspension was vacuum filtered through a Whatman No. 113 filter paper and the filtrates were centrifuged at 10 000 g for 30 min. The supernatant was dialysed (membrane: molecular weight cut off 8000) against 2 litres of 20 mM sodium acetate (pH 5.2) at 4 °C overnight. The sample was centrifuged at 10 000 g for 30 min and the supernatant was concentrated by placing it in a dialysis bag and covering it with several changes of polyethylene glycol powder (Mwt 6000) until the volume was reduced four times. This PGIP extract was dialysed for 24 h with 2 litres of 20 mM sodium acetate. The PGIP extract was then centrifuged for 30 min at 10 000 g and the supernatant was stored at 4 °C. Protein concentration of the various PGIP extracts were determined by measurement of absorbance values at 280 nm using bovine serum albumin as standard. Equal amounts of protein were used in the assays.

Enzymatic activity of fungal endoPGs

Polygalacturonase activity in the different filtrates was determined by measuring the reducing-end groups using the p-hydroxybenzoic acid hydrazide (PAHBAH) method (York *et al.*, 1985). The activity of each polygalacturonase extract was determined by incubating 50 µl of extract in a 1 ml solution containing PGA (25g/l) and 40 mM sodium acetate (pH 5.0) for 1 h at 30 °C. This reaction was terminated by addition of 1.5 ml of freshly prepared PAHBAH (50g/l). The sample tubes were boiled for 10 minutes and cooled to room temperature before taking absorbance readings at 410 nm using a spectrophotometer (Pharmacia LKB. Ultrospec III, Sweden). The assays were repeated three times. Statistical analyses of data were done using SYSTAT software (SYSTAT, 1997). Assays without enzyme served as controls. The absorbance readings were compared for all the species and for each repeat. Differences in the ability of species to produce polygalacturonases were analysed using analysis of variance and Tukey 's multiple comparison method.

Inhibition of fungal polygalacturonases by PGIP extracts

To determine the inhibition capacity of extracts from ZG14 and TAG5, a modified PAHBAH assay was used (York *et al.*, 1985). In this assay, equal concentrations of PGIP extracts and equal concentrations of polygalacturonase extract were used to enable comparisons of inhibition levels. The activity of each PGIP extract, including an untreated control to rule out artefact inhibition, was determined by incubating 50 µl of PGIP extract and 50-µl polygalacturonase extract. The mixture was incubated at 25 °C for 20 min. From this mixture, 80 µl was added to a 1 ml solution containing PGA (25g/l) and 40 mM sodium acetate (pH 5.0) and then incubated for 1 h at 30 °C. One hour was chosen because it was the time taken for optimum polygalacturonase activity to be reached. The reaction mixture was boiled for 10 min and placed on ice immediately. Thereafter, 250 µl of sterile distilled water and 750 µl of freshly prepared PAHBAH (50g/l) were added. The tubes were boiled for 10 minutes and cooled to room temperature before taking absorbance readings at 410 nm using a spectrophotometer (Pharmacia LKB. Ultrospec III, Sweden). The assays were repeated three times. The readings at time (t=0 min) were used as blanks. Statistical analyses of data were done using SYSTAT (SYSTAT, 1997). Assays with PGIP extracts from untreated plants and those without PGIPs were used as controls.

RESULTS AND DISCUSSION

Production of polygalacturonases

C. zuluense and *B. dothidea* produced greater amounts of polygalacturonases than *C. cubensis* and *P. cinnamomi* ($F=6400.972$, $df=3$, $P=0.0001$). *C. zuluense* and *B. dothidea* had similar levels of production of polygalacturonases and the same was observed for *C. cubensis* and *P. cinnamomi* (Fig. -1).

Inhibition of fungal polygalacturonases by PGIP extracts

Polygalacturonases from *C. zuluense* and *B. dothidea* were inhibited to the same extent by PGIPs extracts from TAG5 (F=18.797, df=11, P=0.001). Polygalacturonases from *C. cubensis* and *P. cinnamomi* were inhibited more strongly than those from the other two pathogens (Fig. 1). The polygalacturonases from *C. cubensis* and *B. dothidea* were inhibited similarly while those from *P. cinnamomi* were most inhibited by the PGIPs from ZG14.

TAG5 and ZG14 PGIPs inhibited *P. cinnamomi* polygalacturonases at approximately the same level. The same was observed for *C. cubensis*. Interestingly, TAG5 PGIPs inhibited *C. zuluense* polygalacturonases more than those from ZG14 (Fig. 1). An opposite result was observed for the polygalacturonases extracted from *B. dothidea*.

In this study, we have shown that the generally resistant *Eucalyptus grandis* clone TAG5, produces PGIPs that have a greater capacity to inhibit polygalacturonases of the serious pathogen *C. zuluense* than the generally susceptible clone ZG14. These results suggest that PGIP is involved in the ability of TAG5 to protect itself against endoPG–mediated damage from *C. zuluense*. Similar observations have been made in studies of leek (*Allium porrum*) plants where PGIPs where shown to protect leek plants from endoPG-mediated damage (Favaron *et al.*, 1997). In addition, our results suggest that TAG5 produces PGIPs that are specifically able to inhibit the polygalacturonases produced by *C. zuluense*. Similarly, the results of this study also suggest that *C. zuluense* requires polygalacturonases as part of its suite of characteristics linked to pathogenicity on *Eucalyptus*. Similar findings have been reported for *Aspergillus flavus* infections on cotton (Cleveland and Cotty, 1991) and *Botrytis cinerea* infection of tomatoes and apples (Ten Have *et al.*, 1998).

The inhibition of polygalacturonases of *C. zuluense* by TAG5 and ZG14 PGIPs is particularly intriguing. We have previously observed that in *Eucalyptus* spp., there is high amino acid sequence similarity amongst PGIPs (Chimwamurombe *et al.*, 2001) and that the PGIP peptide sequences of TAG5 and ZG14 are identical. However, we observed what seems to potentially be a differential PGIP specificity to inhibit *C. zuluense* PGs. Differential glycosylation of PGIPs of identical amino acids has been reported previously (Leckie *et al.*, 1999). We therefore suggest that differential glycosylation patterns in the â-strand/ â-turn motif of the PGIPs of the TAG5 and ZG14 *Eucalyptus* clones could explain the differential inhibition specificity that is observed. Differential glycosylation is believed to interfere with the ligand binding capacity of PGIP and hence specificity to polygalacturonases (Leckie *et al.*, 1999).

C. cubensis and *P. cinnamomi* endoPGs were equally inhibited by TAG5 and ZG14 PGIP extracts. Although there are many factors that control pathogenicity of fungi, our results suggest that PGs are probably not a major factor in the pathogenicity of either fungus. Additionally, we have previously observed that isolates of *C. cubensis* with varying virulence produce similar amounts of endoPGs (Chimwamurombe *et al.*, 2001). Therefore, it is not surprising that *C. cubensis* endoPGs were inhibited equally by extracts of both the disease tolerant and the disease susceptible clones. If endoPGs were a major component of the pathogenicity determinants of *C. cubensis* on *Eucalyptus*, then differences in inhibition by the two *Eucalyptus* PGIP extracts should be detected.

PGIP extracts from ZG14 inhibited the polygalacturonases from *B. dothidea* to a greater extent than those from TAG5. This observation was intriguing as an opposite result was expected. There are two possible explanations for this observation. Firstly, it is known that *B. dothidea* is an endophyte and latent pathogen of *Eucalyptus* (Smith *et al.*, 1994, 1996), which only leads to disease after trees have been stressed. The tissue culture plants that were used in this study were already under tissue culture stress and thus the TAG5 plants, which are naturally tolerant to infection by *B. dothidea*, could have been stressed by the study conditions, even though the ZG14 plants were also under the same stress. Another possible explanation for the contradictory results could be that the PGIPs from ZG14 have greater specificity for polygalacturonase of *B. dothidea* than those from TAG5. Thus ZG14

PGIPs would inhibit *B. dothidea* polygalacturonases more than those from TAG5. This would then imply that polygalacturonases may not be a limiting factor in the pathogenicity of *B. dothidea*. This would be consistent with reports from *Cochliobolus carbonum* on a maize (Scott-Craig *et al.*, 1990) and *Cryphonectria parasitica* on chestnuts (Gao *et al.*, 1996) where endoPG disruption studies showed that the pathogenicity of these fungi is not dependent on endoPGs.

The overall results of this study have shown that polygalacturonases are not limiting factors in the pathogenicity of *C. cubensis*, *P. cinnamomi* and *B. dothidea* while they are apparently important for the pathogenicity of *C. zuluense* on *Eucalyptus* plants. TAG5 and ZG14 PGIPs have different capacities to inhibit polygalacturonases from *C. zuluense* and this, at least in part, explains the protection of TAG5 against polygalacturonase-mediated damage by *C. zuluense* in field experiments (Van Zyl, 1999). It is clear from these results that expression of TAG5 PGIPs in ZG14 may improve the tolerance of ZG14 plants, and potentially could provide an option to reduce endoPG-mediated damage of ZG14 plants by *C. zuluense*.

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