Cell wall degrading enzymes in fusarium wilt of chickpea: correlation between pectinase and xylanase activities and disease development in plants infected with two pathogenic races of *Fusarium oxysporum* f. sp. *ciceris*

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Abstract: Production of cell wall degrading enzymes (CWDEs) polygalacturonase (PG), pectate lyase (PL), and xylanase was studied in chickpeas (*Cicer arietinum* L. 'P-2245') inoculated with *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato races 0 (mildly virulent, causing a yellowing syndrome) and 5 (highly virulent, causing a wilting syndrome) by the water-culture method. These CWDEs were similarly produced in both syndromes. PG and PL were the only enzymes occurring in roots and stems and attained the highest specific activity, this being generally higher for race 5 than for race 0. Gel filtration chromatography revealed a similar complement of in planta expressed pectinase isoforms, dominated by an endo-PG and two endo-PLs, the endo-PLs being differentially expressed by the two races. CWDE activities in roots and stems were positively correlated with development of yellowing and wilting. Exceptions to this were PG in stems, which was negatively correlated with the development of yellowing, and PG in roots, which showed a negative trend with development of either syndrome. The levels of CWDEs that significantly correlated with disease development were adequately described by exponential functions of disease progress. Results have implications for the role played by CWDEs in the early and later stages of pathogenesis in chickpea fusarium wilt.

Key words: Cicer arietinum, pathogenesis and virulence factors, pectic enzymes, yellowing and wilting syndromes.

Résumé : En utilisant la méthode de culture à l'eau, les auteurs ont étudié la production les enzymes qui dégradent la cellulose (CWDEs), comprenant la polygalacturonase (PG), la pectate lyase (PL), et la xylanase, chez le pois chiche (*Cicer arietinum* L. 'P-2245'), inoculé avec le *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato race 0 (modérément virulent, causant un syndrome de jaunissement) et race 5 (fortement virulent, causant un syndrome de flétrissure). Ces CWDEs sont produites avec les deux syndromes. Les PGs et Pls sont les seules enzymes présentes dans les racines et les tiges et atteignent la plus forte concentration spécifique, celle-ci étant généralement plus élevée avec la race 5 que la race 0. La chromatographie par filtration sur gel révèle un complément similaire des isoformes de la pectinase exprimées in planta, dominé par une endo-PG et deux endo-PLs, les endo-PLs s'exprimant différentiellement chez les deux races. Les activités CWDE dans les racines et dans les tiges sont positivement corrélées avec le développement du jaunissement et de la flétrissure. Les PGs caulinaires font exception, étant négativement corrélées avec le développement du jaunissement, et les PGs racinaires montrant une tendance négative avec le développement de l'un ou l'autre de ces syndromes. Les teneurs en CWDEs montrant une corrélation significative avec le développement de la maladie sont adéquatement décrites comme fonctions exponentielles des progrès de la maladie.

Mots clés : Cicer arietinum, facteurs de pathogenèse et de virulence, enzymes pectiques, syndromes de jaunissement et de flétrissure.

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Introduction

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato, severely limits chickpea (*Cicer arietinum* L.) production worldwide (Trapero-Casas and Jiménez-Díaz 1985; Jalali and Chand 1992) but can destroy the crop completely under specific conditions (Haware and Nene 1980; Halila and Strange 1996). *Fusarium oxysporum* f. sp. *ciceris* (Foc) is monophyletic but exhibits considerable pathogenic variation (Jiménez-Gasco et al. 2002). Two pathotypes have been distinguished based on the distinct yellowing or wilting symptoms they cause in the plant (Trapero-Casas and Jiménez-Díaz 1985). In addition, eight pathogenic races (races 0, 1A, 1B/C, 2, 3, 4, 5, and 6) can be identified by disease reactions in a set of differential chickpea cultivars (Haware and Nene 1982; Jiménez-Díaz et al. 1994) as well as by specific PCR markers (Jiménez-Gasco and Jiménez-Díaz 2003). Race 0 induces foliar yellowing and is the least virulent of all races, while race 5 is the most virulent race and induces severe wilt (Jiménez-Díaz et al. 1994; Navas-Cortés et al. 2000; Jiménez-Gasco et al. 2001).

There have been a number of studies on pathological, physiological, and biochemical aspects of fusarium wilt diseases. However, the precise mechanisms by which resistance and pathogenicity operate in these diseases still remain largely unknown. Cell wall degradation must play an important role in fusarium wilt pathogenesis, because the pathogen grows through the root cortex and colonizes the xylem. As a consequence, production of cell wall degrading enzymes (CWDEs), especially pectinases (polygalacturonases (PG) and pectate lyases (PL)), but also xylanases (XYL), by fusarium wilt pathogens have received much attention.

Several studies have addressed the production of CWDEs by fungal plant pathogens (e.g., Cooper 1983; Alghisi and Favaron 1995; Lang and Dörnenburg 2000; Di Pietro et al. 2003; Roncero et al. 2003), including recent work on targeted disruption of discrete CWDE genes to disclose significant reduction in pathogen virulence (Annis and Goodwin 1997). However, the precise role of those enzymes as pathogenesis and (or) virulence factors has seldom been clearly demonstrated. Genetics studies conducted with *F. oxysporum* (Di Pietro et al. 2003) as well as other plant pathogenic fungi (e.g., Annis and Goodwin 1997; Shih et al. 2000; Wu et al. 2006; and literature cited therein) have provided evidence that the production of CWDEs is frequently encoded by multigene families rather than individual genes. As a consequence of that functional redundancy, multiple isoenzymes of a given activity may be expressed, making it possible that targeted CWDE mutants still produce sufficient residual degrading activity to confer pathogenicity. The ample redundancy of CWDEs expressed by plant pathogenic fungi makes it difficult to demonstrate clear molecular evidence concerning the involvement of these enzymes in plant pathogenesis. Nevertheless, such a redundancy also suggests a major importance of CWDEs in the physiology of plant diseases and hence of their possible relevance in pathogenesis. It is in this scenario that classical, biochemical studies of correlation between in planta expression of CWDEs and disease development may still be valuable for ascertaining a possible role of those enzymes in fungal plant disease pathogenesis.

In previous studies, we showed that Foc races 0 and 5 differ in the level and time course (early or late) of in vitro pectic enzyme excretion as well as in the degree of multiplicity and endo or exo mode of action of PG and PL secreted forms. This suggested that PL rather than PG may play a role as Foc pathogenicity or virulence factor in fusarium wilt of chickpea (Pérez-Artés and Tena 1989, 1990; Pérez-Artés et al. 2004). Conversely, both Foc races 0 and 5 showed a similar behaviour with respect to in vitro excretion of endoxylanases (Jorge et al. 2005). However, no information was available concerning in planta activities of those CWDEs, whose regulation may differ from that in vitro (Collmer and Keen 1986). Therefore, demonstrating production and activity of CWDEs by the pathogen during the plant–pathogen interaction and disease development is a relevant feature for concluding a possible role of those enzymes in pathogenesis.

The main objective of this study was to comparatively determine the in planta production of PG and PL along with XYL during the development of yellowing and wilting syndromes of fusarium wilt in chickpea, aimed to better understand a role of those CWDEs as possible pathogenesis or virulence factors.

Materials and methods

Fungal isolates and inoculum production

Monoconidial cultures of *F. oxysporum* f. sp. *ciceris* isolates 7802 (race 0, Foc-0) and 8012 (race 5, Foc-5) were used. These isolates were obtained from infected chickpeas (*Cicer arietinum*) in southern Spain and used in previous studies (Pérez-Artés and Tena 1989, 1990; Jiménez-Gasco et al. 2001; Cachinero et al. 2002; García-Limones et al. 2002). Isolates were stored in sterile soil tubes at 4 °C. Active cultures and inocula were produced on potato dextrose agar and potato dextrose broth (PDB), respectively, as previously described (Cachinero et al. 2002; García-Limones et al. 2002). Inocula consisted of conidia (mostly microconidia) suspensions in deionized water adjusted to 10^6 conidia·mL^{-1}. Conidia were free from PDB residues before they were used as inoculum.

Chickpea plants and inoculation

Cicer arietinum 'P-2245' was used. 'P-2245' is a 'kabuli' chickpea highly susceptible to both Foc-0 and Foc-5 (Jiménez-Díaz et al. 1989; Navas-Cortés et al. 2000). Plants were inoculated by the water-culture inoculation method (Nene and Haware 1980; Trapero-Casas and Jiménez-Díaz 1985). Briefly, seeds were surface-disinfested in 2.5% NaOCl for 3 min, sown in sterile sand in disinfested plastic trays, and the plants were grown for 7 d in a growth chamber adjusted to 25 °C, 60%–90% relative humidity, and a 14 h photoperiod of fluorescent light at 360 μE·m^{-2}·s^{-1}. Plants were selected for uniformity, carefully washed free of sand, and transferred to 6 cm diameter cylindrical plastic bottles (four plants per bottle) containing 80 mL of the inoculum suspension. Plants in bottles filled with sterile deionized water served as controls. Plants were held in position by strips of adhesive paper. The bottles were placed on an orbital shaker run at 110 r·min^{-1} (Nene and Haware 1980; Trapero-Casas and Jiménez-Díaz 1985) and incubated at

Fig. 1. Production of polygalacturonase activity by races 5 (white bars) and 0 (grey bars) of *Fusarium oxysporum* f. sp. *ciceris*. Inoculum media (*a*), and roots (*b*), and stems (*c*) of infected chickpea plants (*Cicer arietinum* 'P-2245') were sampled at times that reached the indicated disease severity scores (categorized from 0 to 4). * and ** indicate that differences in enzyme activity between syndromes at a given disease rate are significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. Bars show means ± SD ($n = 3$).

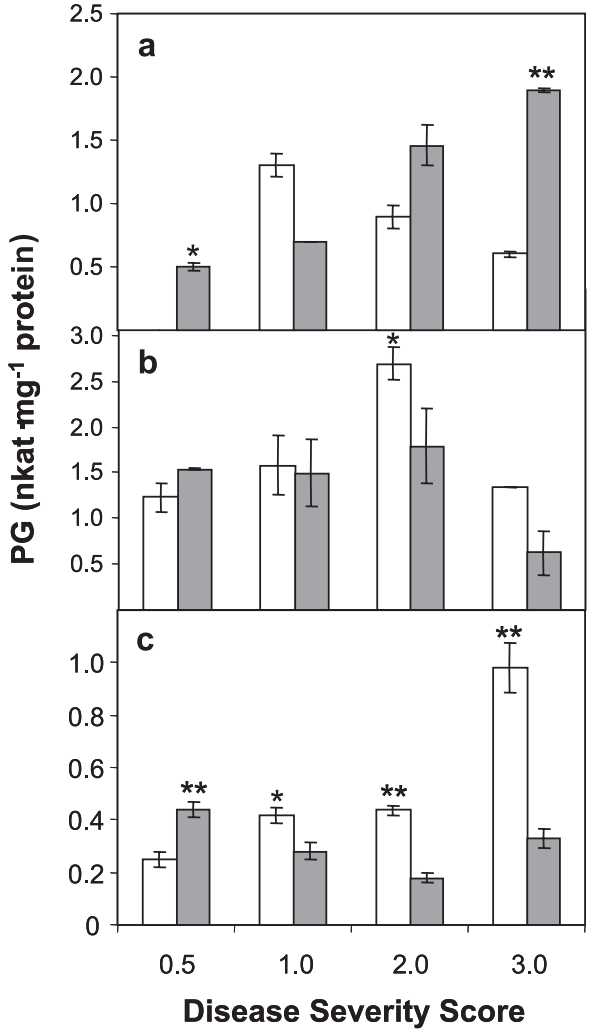
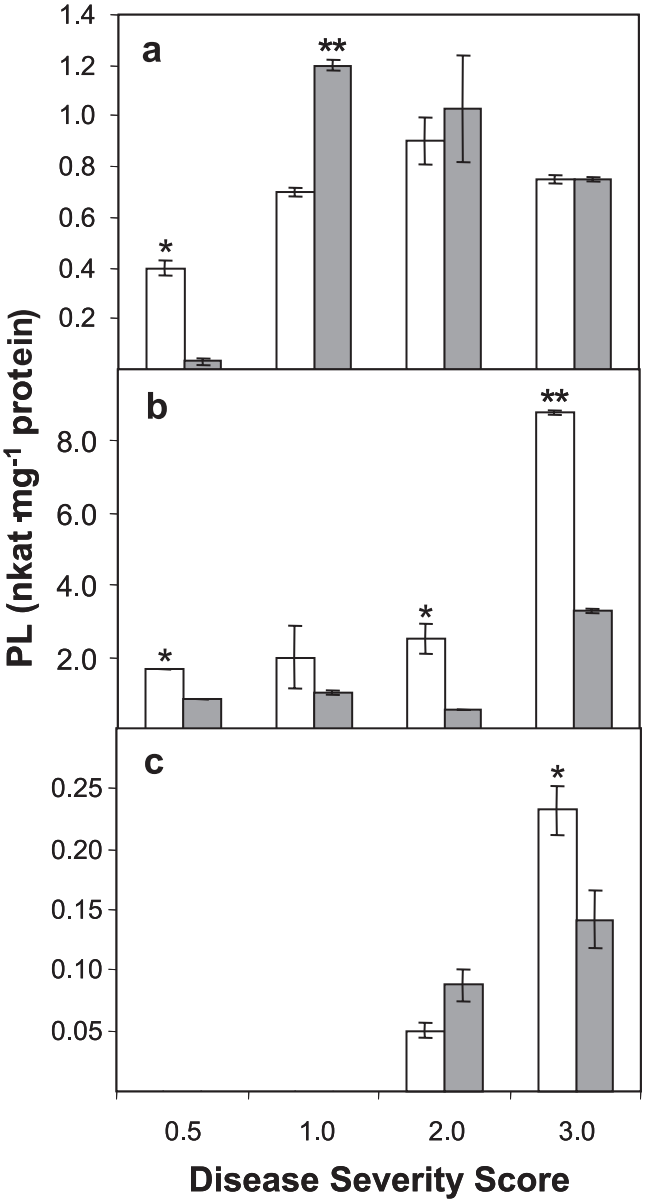


Fig. 2. Production of pectate lyase activity by races 5 (white bars) and 0 (grey bars) of *Fusarium oxysporum* f. sp. *ciceris*. Inoculum media (*a*), and roots (*b*), and stems (*c*) of infected chickpea plants (*Cicer arietinum* 'P-2245') were sampled at times that reached the indicated disease severity scores (categorized from 0 to 4). * and ** indicate that differences in enzyme activity between syndromes at a given disease rate are significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. Bars show means ± SD ($n = 3$).

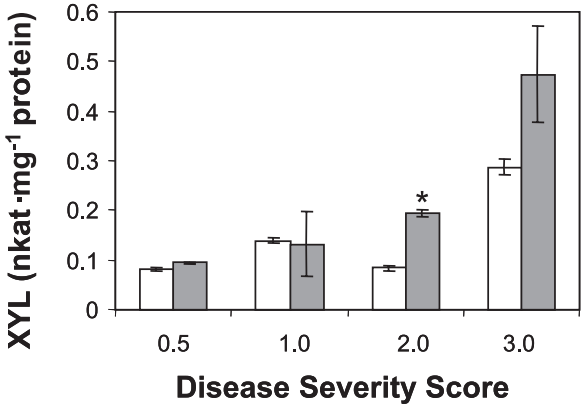


the same conditions as above. Sterile Hoagland's nutrient solution (Hoagland and Arnon 1950) was added daily, or at weekly intervals after the first sampling, to provide nutrients to the plants and to replace water lost. Three independent experiments were done, each consisting of four inoculation treatments: Foc-0, Foc-5, and their corresponding noninoculated controls. There were 16 replicated bottles per treatment arranged in a randomized complete block design.

Disease assessment and tissue sampling

Severity of symptoms on individual plants was assessed at 2 d intervals using a 0–4 rating scale according to percentage of foliage with yellowing or necrosis in acropetal progression (0, 0%; 1, 1%–33%; 2, 34%–66%; 3, 67%–100%; and 4, dead plant) (Navas-Cortés et al. 2000; Cachinero et al. 2002). A total of 16 plants in four bottles (four plants per bottle) were arbitrarily sampled at each of the times when average disease severity score reached 0.5, 1.0, 2.0, and 3.0 values, respectively. Because Foc-0 and Foc-5 differ in virulence to 'P-2245' (Navas-Cortés et al. 2000), those average severity values occurred at about 10, 14, 17, and 20 d after inoculation for the former and 8, 10, 12, and 14 d after inoculation for the latter. At each of the sampling dates, both the inoculum media and plants were collected. The inoculum media were centrifuged (15 000 *g*, 30 min, 4 °C), filtered through a 0.2 µm Millipore filter (Millipore Ibérica, Madrid, Spain), and stored at –20 °C until they

Fig. 3. Production of xylanase activity by races 5 (white bars) and 0 (grey bars) of *Fusarium oxysporum* f. sp. *ciceris*. Roots of infected chickpea plants (*Cicer arietinum* 'P-2245') were sampled at times that reached the indicated disease severity scores (categorized from 0 to 4). * indicates that differences in enzyme activity between syndromes at a given disease rate are significant at $P \leq 0.05$. Bars show means ± SD ($n = 3$).



were used for enzyme assays (usually 1–2 weeks). In these conditions, the inoculum media maintained their levels of CWDE activities without noticeable loss for at least 3 months. Plants were thoroughly washed in distilled water, their root and shoot portions were separated, and the stems free from leaves were divided into lower and upper halves. Both roots and the lowermost half of a stem for each plant were frozen in liquid nitrogen and stored at –20 °C for enzyme extraction. Isolations were made from stem segments of one plant arbitrarily chosen from each of the four sampled bottles to determine the occurrence of vascular infection by the pathogen (Cachinero et al. 2002).

Enzyme extraction from plants

Frozen root and stem samples were ground to a fine powder under liquid nitrogen. For extraction of soluble proteins, this power was suspended in four volumes of ice-cold 100 mmol·L^{-1} sodium citrate buffer (pH 4.6) supplemented with 50 mmol·L^{-1} EDTA, 10 mmol·L^{-1} sodium ascorbate, 10 mmol·L^{-1} β-mercaptoethanol, 1 mmol·L^{-1} phenylmethanesulfonyl fluoride (PMSF) and 5% (*m*/*v*) polyvinylpolypyrrolidone, and the homogenate was centrifuged at 17 000 *g* for 15 min. The supernatant was filtrated through a PD-10 column (Sephadex G-25; Sigma, St. Louis, Missouri, USA) using 10 mmol·L^{-1} Tris–HCl buffer (pH 7.5) as eluting solvent, and the collected protein peak solution was frozen in liquid nitrogen and stored at –20 °C for further analysis. All manipulations were carried out at 0–4 °C.

Enzymatic activity assays

Both the inoculum media and plant extracts were assayed for polygalacturonase (PG), pectate lyase (PL), and xylanase (XYL) activities. For PG and XYL activities, the release of reducing groups from polygalacturonic acid and birchwood xylan (both from Sigma) was determined at 520 nm using the Somogyi–Nelson method (Pérez-Artés and Tena 1989). Reaction mixtures consisted of 400 µL of 0.1% (*m*/*v*) polygalacturonic acid or xylan in 50 mmol·L^{-1} sodium acetate buffer (pH 4.5) and 100 µL enzyme extract, and were incubated at 37 °C for 30 min. Enzyme activity was expressed in nkat, defined as the amount of enzyme that releases an amount of reducing groups equivalent to 1 nmol·s^{-1} of either galacturonic acid (ϵ_m = 5498 mol·L^{-1}·cm^{-1}, for PG), or xylose (ϵ_m = 5518 mol·L^{-1}·cm^{-1}, for XYL). PL activity was assayed by the increase in absorbance at 232 nm (ε_m = 4600 mol·L^{-1}·cm^{-1}) produced by the release of unsaturated uronides (Hislop et al. 1979). The reaction mixture consisted of 700 µL of 0.1% (*m*/*v*) polygalacturonic acid and 2 mmol·L^{-1} $CaCl_2$ in 100 mmol·L^{-1} glycine–NaOH buffer (pH 10), and 100 µL enzyme extract, and it was incubated at 37 °C for 30 min. Enzyme activity was expressed in nkat, defined as the amount of enzyme that releases an amount of unsaturated uronides equivalent to 1 nmol·s^{-1}.

Separation of polygalacturonase and pectate lyase isoenzymes by gel filtration

Inoculum media and enzymatic extracts from roots and stems of diseased plants (rated with a 3.0 severity score) were appropriately concentrated by freeze-drying and subjected to gel filtration chromatography. That was done on a Sephacryl S-200 HiPrep 16/60 column coupled to a FPLC System (Pharmacia Biotech, Uppsala, Sweden), using 50 mmol·L^{-1} Tris–HCl buffer (pH 7.5) with 100 mmol·L^{-1} KCl as eluting solvent, at a flow rate of 0.8 mL·min^{-1}. Twenty 2 mL fractions were collected and assayed for PG and PL activities. The exclusion volume of the column was determined with blue dextran, and the following proteins were used as molecular mass markers (Sigma): cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa).

Data analyses

Enzyme activity levels (PG, PL, or XYL) for combinations of enzyme source (inoculum media, root or stem tissues), infection by Foc-0 or Foc-5, and sampling dates were compared by ANOVA using the statistical package Statistix (NH, Analytical Software, Roseville, Minnesota, USA). Variance homogeneity of data was tested by the Barlett's test of equal variance before ANOVA. A disease intensity index (DII) was calculated using symptom severity data by the following equation (Acimovic 1979): $DII = (\Sigma\ S_i\ N_i)\ (4N_t)^{-1}$; where S_i is the symptom severity, N_i represents the number of plants with S_i symptom severity, and N_t is the total number of inoculated plants. DII (on a scale 0 and 1) expresses the mean amount of disease at any given moment as a proportion of the maximum possible disease level. The accumulated DII over time in days from the date of inoculation was used to obtain curves of disease progress. The area under the DII progress curves (AUDPC) was calculated by trapezoidal integration method (Campbell and Madden 1990) as $AUDPC = \Sigma_i^{n-1}\ (DII_i + DII_{i+1}) \times 0.5\ (t_{i+1} - t_i)$, in which n is the number of assessment times, and DII_i and DII_{i+1} are DII at time t_i and t_{i+1}, respectively. The AUDPC was further standardized by the duration of disease development in days ($t_n - t_1$) to obtaining the standardized area under the DII progress curve (SAUDPC). Pearson simple correlation analyses were done between either DII or SAUDPC and levels of the CWDEs in the study. To further determine any possible relationship between induction of CWDEs and disease devel-

Table 1. Correlation coefficients between levels of cell wall degrading enzymes and development of chickpea (*Cicer arietinum*) wilting and yellowing disease syndromes induced by *Fusarium oxysporum* f. sp. *ciceris* races 5 (Foc-5) and 0 (Foc-0).

	Foc-5 (wilting syndrome)		Foc-0 (yellowing syndrome)	
Enzyme type and source	DII	SAUDPC	DII	SAUDPC
PG activity				
Inoculum media	0.61**	0.44	0.11	0.13
Roots	–0.43	–0.47	–0.39	–0.50
Stems	0.79**	0.89***	–0.78**	–0.79**
PL activity				
Inoculum media	0.58**	0.51*	0.04	0.05
Roots	0.68*	0.85***	0.61*	0.79**
Stems	0.78**	0.89***	0.87***	0.86***
XYL activity				
Roots	0.68*	0.80**	0.76**	0.89***

Note: PG (polygalacturonase), PL (pectate lyase), and XYL (xylanase) activity levels in the inoculum media and in roots and stems of inoculated plants were correlated with a disease intensity index (DII) and the standardized area under the DII progress curve over time after inoculation (SAUDPC). *, **, and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

opment, activity data significantly correlating with disease progress were fitted to SAUDPC data according to an expanded exponential model, as follows: Enzyme activity = B1·exp(B2·SAUDPC) + B3; where B1 is the constant of integration, B2 is the relative rate of enzyme activity increase, and B3 is the asymptotic value of enzymatic activity. Regression analyses were conducted using the least-squares programme for nonlinear models (NLIN) procedure of the statistical analysis system (SAS Institute Inc., Cary, North Carolina, USA). The coefficient of determination (R^2), the mean square error, the asymptotic standard error associated with the estimated parameter, and the pattern of the standardized residuals plotted against either predicted values or the independent variable were used to evaluate the appropriateness of the model to describe the data (Campbell and Madden 1990).

Results

In planta production of CWDEs

Neither symptoms nor enzyme activity occurred in the control plants. Plants inoculated with Foc-0 showed yellowing, and those inoculated with Foc-5 showed wilting. Isolations from inoculated plants indicated that vascular infection of the entire stem occurred by the time plants were first sampled, 8 and 10 d after inoculation with Foc-5 and Foc-0, respectively.

Pectinase and xylanase activities were detected in infected plants, but both the level and pattern of accumulation varied either with the enzyme type, enzyme source, and (or) disease syndrome affecting the plant. PG activity occurred in the infected plant tissues as soon as symptoms started to develop (severity score of 0.5) (Fig. 1). Level of PG ranged from about 1 to 3 nkat·mg^{-1} protein in roots and was lower than 1 nkat·mg^{-1} protein in stems. PG activity in infected roots increased slightly and similarly both for Foc-0 (yellowing) and Fo-5 (wilting), as severity of symptoms increased from 0.5 to 2.0 (within a scale of 0–4), and then it decreased (Fig. 1*b*). Conversely, in stems, PG activity clearly increased as severity of wilting increased in Foc-5 infected plants, and decreased as severity of yellowing increased in Foc-0 infected ones (Fig. 1*c*). PG activity in Foc-5 infected stems was significantly higher ($P < 0.05$) than that in Foc-0 infected ones for severity scores of 1.0–3.0. In the inoculum medium, PG activity increased as yellowing developed, but for Foc-5 inoculated plants, it was detectable only when plants were clearly symptomatic and then decreased as severity of symptoms increased (Fig. 1*a*).

PL activity was detected in all infected roots sampled, but it was measurable in stems of severely affected plants only (severity scores 2 and 3) (Fig. 2). The level of PL activity was much higher in infected roots (up to about 9 nkat·mg^{-1} protein) than in infected stems (lower than 0.25 nkat·mg^{-1} protein) (Figs. 2*b* and 2*c*). In general, PL activity in infected roots and stems increased as disease developed, and it was higher for the wilting (Foc-5 infected) than for the yellowing (Foc-0 infected) plants, although differences were not always statistically significant ($P < 0.05$). PL activity in the inoculum medium was lower than 1.2 nkat·mg^{-1} protein. This activity increased as disease started to develop in the plants until symptoms severity reached a score of 1 for Foc-0 and 2 for Foc-5; and it was higher for Foc-5 compared with Foc-0 at initiation of symptoms, but the reverse occurred at the severity score of 1 (Fig. 2*a*).

Finally, XYL activity was detected only in root tissue of infected plants, where it increased as disease developed (Fig. 3). In general, XYL activity was higher in yellowing, Foc-0 infected plants compared with the wilting ones infected by Foc-5, although differences were statistically significant ($P < 0.05$) when disease severity reached a score of 2, only (Fig. 3).

Relationhip between in planta production of CWDEs and the development of fusarium wilt

Results of linear correlation analyses between each of PG, PL, and XYL activities in infected root and stem tissues, as well as in Foc-0 and Foc-5 inoculum media, and both the disease intensity index (DII) and the standardized area under the DII progress curve (SAUDPC) are shown in Table 1.

Table 2. Nonlinear regression analysis of levels of cell wall degrading enzymes over the development of chickpea (*Cicer arietinum*) wilting and yellowing disease syndromes induced by *Fusarium oxysporum* f. sp. *ciceris* races 5 (Foc-5) and 0 (Foc-0), respectively.

	Parameter estimates[a]			Statistics[b]	
Source, enzyme	B1 ± SE	B2 ± SE	B3 ± SE	MSE	R^2
Wilting syndrome (Foc-5 inoculated plants)					
Roots					
PL	0.021±0.001	14.430±0.000	1.690±0.201	0.304	0.70
XYL	0.018±0.003	6.746±0.861	0.035±0.000	0.003	0.75
Stems					
PG	0.068±0.011	6.584±0.001	0.196±0.054	0.008	0.93
PL	0.008±0.001	8.191±0.001	0.000±0.000	0.001	0.84
Yellowing syndrome (Foc-0 inoculated plants)					
Roots					
PL	0.069±0.010	11.604±0.001	0.626±0.192	0.217	0.85
XYL	0.057±0.006	6.511±0.081	0.027±0.005	0.003	0.88
Stems					
PG	0.366±0.003	–9.024±1.193	0.162±0.002	0.003	0.79
PL	0.012±0.002	8.367±0.000	0.000±0.000	0.004	0.74

Note: PG (polygalacturonase), PL (pectate lyase), and XYL (xylanase) activity levels previously found to be significantly correlated with disease development were fitted to data of the standardized area under the disease intensity index progress curve (SAUDPC) according to the following expanded exponential model: enzyme activity = B1 × exp(B2 × SAUDPC) + B3.

[a]B1, constant of integration; B2, relative rate of enzymatic activity increase; and B3, asymptotic value of enzymatic activity; SE, asymptotic standard error for the parameter estimates.

[b]R^2, coefficient of determination; MSE, final mean square error.

PG activity in roots and inoculum media showed negative and positive trends, respectively, with disease development, but correlations were not statistically significant ($P > 0.05$) (Table 1). Conversely, PG activity in infected stems was positively correlated ($P < 0.01$) with the increase in severity of wilting (Foc-5) and negatively correlated ($P < 0.01$) with that of yellowing (Foc-0) (Table 1). Contrary to that, PL activity in roots and stem was positively and significantly correlated with the increase in the amount of disease both for yellowing (Foc-0) and wilting (Foc-5) (Table1). PL activity in the inoculum medium was significantly correlated with development of wilting only. Finally, XYL activity in infected root tissues was positive and significantly correlated with development of either of the disease syndromes (Table 1).

We further explored a functional relationship among production of CWDEs and chickpea yellowing and wilting caused by Foc-0 and Foc-5, respectively, by means of nonlinear regression analyses using enzyme activities that had previously shown significantly correlated with SAUDPC. Results of analyses are summarized in Table 2.

Both for Foc-0 and Foc-5 infections, the levels of PL and XYL activities in root tissues increased with increasing SAUDPC according to an expanded positive exponential model (Table 2; Fig. 4), suggesting that enzymes are induced as disease developed. For PL activity, the B2 rate parameter of enzyme activity increase in Foc-5 infected roots was higher than that in roots infected by Foc-0, whereas that rate parameter for XYL activity was similar in Foc-0 and Foc-5 infected roots (Table 2). Conversely, the asymptotic value of PL and XYL activities (B3) was higher in Foc-5 infected roots than in Foc-0 infected ones (Table 2).

PL activity increased both in Foc-0 and Foc-5 infected stems with increasing SAUDPC according to an exponential model. Conversely, PG activity decreased with increasing SAUDPC according to an expanded negative exponential model in Foc-0 infected plants, and increased according to an expanded positive exponential model in Foc-5 infected ones (Fig. 5; Table 2). In addition, the rate of PG activity increase (B2) in Foc-5 infected stems was lower compared with the rate of PG activity decrease in Foc-0 infected stems. However, the asymptotic value for PG activity (B3) was similar in stems infected either by Foc-0 or Foc-5 (Table 2). For PL fits, the rate parameter was higher for the yellowing, Foc-0 infected plants compared with that in the wilting, Foc-5 infected ones, even though both values were relatively close and the asymptotic value was null in both cases (Table 2).

Polygalacturonase and pectate lyase isoenzymes expressed in fusarium wilt affected plants

Gel filtration was demonstrated as a simple and reliable method for differentiating extracellular pectic enzymes produced by Foc-0 and Foc-5 (Pérez-Artés and Tena 1990; Pérez-Artés et al. 2004); therefore we used this method to determine profiles of PG and PL isoforms present in infected roots and stems, as well as in the corresponding inoculum media, using samples collected when severity of symptoms reached a score of 3 (Fig. 6). By comparing profiles in the inoculum media and in extracts from infected roots and stem in this study, we aimed to determine if plants contributed to the assayed pectic activities in infected tissues. Secondly, by comparing profiles in Foc-5 and Foc-0 infected tissues, we intended to elucidate if there were dif-

Fig. 4. Expanded exponential relationship between root levels of PL (pectate lyase) and XYL (xylanase) activities and disease progress (SAUDPC). Enzyme activities were assayed in extracts from root tissues of infected 'P-2245' chickpea (*Cicer arietinum*) plants during development of chickpea wilting and yellowing disease syndromes induced by *Fusarium oxysporum* f. sp. *ciceris* races 5 (Foc-5) (solid line) and 0 (Foc-0) (broken line), respectively.

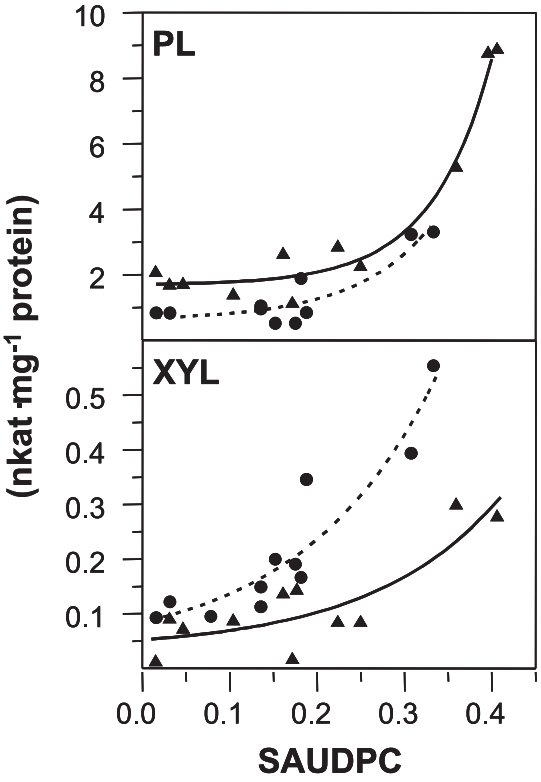
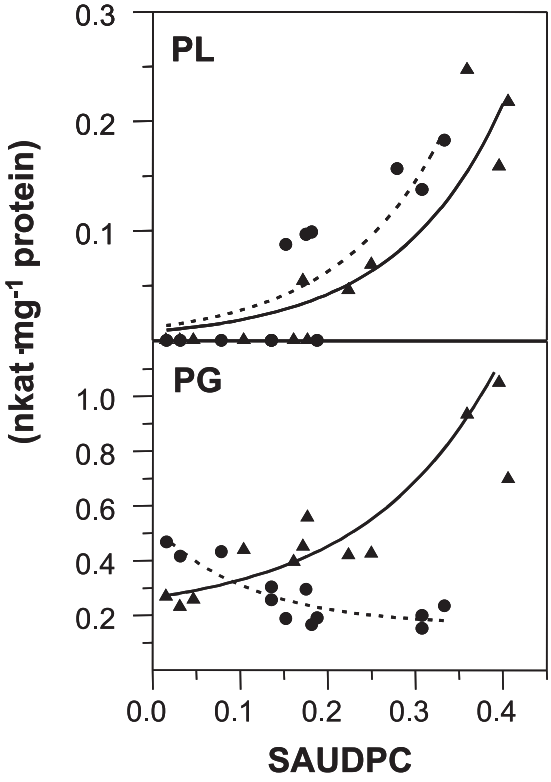


Fig. 5. Expanded exponential relationship between stem levels of PL (pectate lyase) and PG (polygalacturonase) activities and disease progress (SAUDPC). Enzyme activities were assayed in extracts from stem tissues of infected 'P-2245' chickpea (*Cicer arietinum*) plants during development of chickpea wilting and yellowing disease syndromes induced by *Fusarium oxysporum* f. sp. *ciceris* races 5 (Foc-5) (solid line) and 0 (Foc-0) (broken line), respectively.



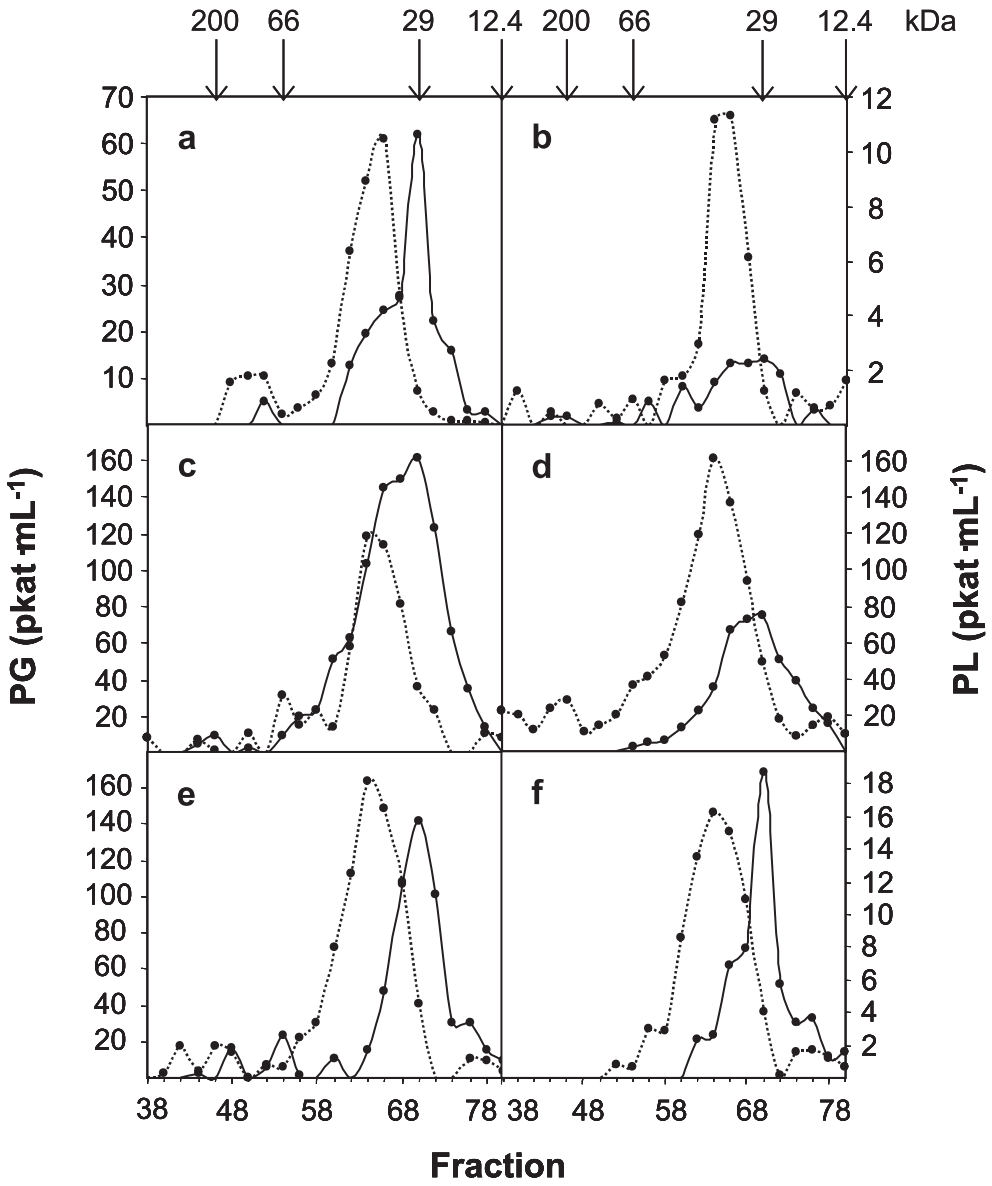
ferences in pectic isoforms expressed by either races in planta. Results support that plant tissue extracts contained enzyme forms from fungal rather than plant origin, since all main PG and PL peaks in those extracts occurred also in the inoculum media. Regarding pectic enzyme forms, there was one main PG peak of 42.7 kDa present both in the inoculum media and in the assayed root and stem tissues infected by either of races. Similarly, there were at least two PL peaks of 30.3 and 38.6 kDa, respectively, but their expression varied depending upon either the enzyme source or the pathogen race. The 30.3 kDa PL isoform was predominant in the Foc-5 inoculum medium, whereas both the 30.3 and 38.6 kDa PL isoforms were similarly present in that of Foc-0. The two PL isoforms were present in root tissues infected by either Foc-0 or Foc-5, but the amount in Foc-0 infected tissues was much lower compared with that in Foc-5 infected ones. Finally, the 30.3 kDa PL was the predominant isoform in Foc-0 infected stems, whereas a more balanced mixture of the two PL isoforms occurred in Foc-5 infected ones. This was indicated by the wider shape of the 30.3 kDa PL peak corresponding with this latter sample, which suggests an important contribution of the 38.6 kDa PL merged into the 30.3 kDa peak. Thin-layer chromatography analyses of assay mixtures of the above pectic enzyme peaks revealed that they contained endo-acting forms as evidenced by the formation of oligomeric degradation products (not shown).

Discussion

Results of this study concern three main aspects of a putative CWDE implication in chickpea wilt pathogenesis: (*i*) differences between in planta and in vitro expression of pectic enzymes by races of the pathogen differing in virulence to susceptible plants; (*ii*) plant organ-specific expression of the studied pathogen CWDEs in diseased plants; and (*iii*) the relationship between pathogen CWDEs and development of characteristic disease syndromes.

Gel filtration fractionation of PG and PL isozymes (Fig. 6) convincingly demonstrate that, as described for other fungi (Collmer and Keen 1986), CWDEs expressed by *F. oxysporum* f. sp. *ciceris* in planta differ from those expressed in vitro on artificial substrates or plant cell wall preparations. Thus, while both Foc-0 and Foc-5 induced in planta a unique main endo-PG peak of molecular mass 42.7 kDa, on pectin or chickpea plant cell walls as sole carbon sources Foc-0 produced this endo-PG activity peak as well as an exo-PG peak of molecular mass 76 kDa, whereas Foc-5 only produced this latter exo-PG peak (Pérez-Artés and Tena 1989, 1990; Pérez-Artés et al. 2004). Because of the unique ability of endo forms to initiate efficient plant cell wall degradation, endo- rather than exo-PGs have been considered as the main putative factor in plant pathogenesis (Karr and Albersheim 1970; Albersheim and Anderson-

Fig. 6. Profiles of in planta expressed PG (polygalacturonase) (broken line) and PL (pectate lyase) (solid line) isoforms. Inoculum media (*a*, *b*), and extracts from roots (*c*, *d*), and stems (*e*, *f*) tissues of 'P-2245' chickpea (*Cicer arietinum*) plants infected by races of *Fusarium oxysporum* f. sp. *ciceris* (Foc), sampled at time of disease severity score 3 (categorized from 0 to 4), were subjected to gel filtration chromatography. (*a*, *c*, *e*) Foc race 5; and (*b*, *d*, *f*) Foc race 0.



Prouty 1975). Thus, inability of the highly virulent Foc-5 to produce endo-PG in vitro as opposed to high ability of both the mildly virulent Foc-0 and nonpathogenic but cortical invader *F. oxysporum* to produce this isoform (Pérez-Artés et al. 2004) were considered evidence against a role of PGs as pathogenesis or virulence factors in fusarium wilt of chickpea (Pérez-Artés and Tena 1990; Pérez-Artés et al. 2004). This earlier conclusion is now questioned by results of in planta CWDEs induction in this present study. Conversely, results from in planta induction would support a possible role of PL in wilting proposed from in vitro studies (Pérez-Artés and Tena 1990), since this pectic enzyme was produced by the highly virulent Foc-5 to a larger extent and more complex isoenzyme pattern compared with that of the mildly virulent Foc-0 (Figs. 2 and 6).

In general, the several CWDEs studied were similarly produced both in plants infected by Foc-0 (yellowing syndrome) and Foc-5 (wilting syndrome), but there were spatial differences in the expression of individual enzyme activities. Thus, PG and PL activities were detected in infected roots and stems, as well as the inoculum media, whereas XYL activity was detected in roots only. Moreover, levels of specific enzymatic activity in each fraction were about one order of magnitude higher for PG and PL than for XYL. These results suggest that pectic enzymes play the most important role in chickpea wilt pathogenesis, since they are the unique CWDEs being present in roots and stem at different stages of the infection process. Although some artifacts (i.e., selective enzyme bound or inactivation; Cooper and Wood 1980) might influence those differences in activities, such differences may also reflect true variation in the spatial or temporal expression of the respective CWDE forms. This

possibility was recently illustrated in fusarium wilt of tomato by molecular genetics approaches, whereby *F. oxysporum* f. sp. *lycopersici* expressed certain PG, PL, and XYL genes in a plant organ (root or stem) or infection stage specific manner, even though other genes were expressed in undiscriminate form with respect to either plant organ or stage of infection (Di Pietro et al. 2003; Roncero et al. 2003). Similarly, differences in CWDEs expression have also been pointed out for other plant pathogenic fungi when comparing their growth in culture on different carbon sources or during different stages of host infection (e.g., Shih et al. 2000).

A third conclusion from our results is that production of CWDEs was found to be correlated with development of fusarium wilt. However, correlation between CWDEs activity and disease development was a specific rather than a general characteristic, as both its trend (positive or negative) and significance were influenced by either enzyme type and (or) source (Table 1). Consequently, these correlations seem to indicate specific roles of the various enzyme activities in plant tissues during disease progress rather than a simple relationship of such activities with the presumable increase of fungal biomass within the plant. Furthermore, for all significant positive or negative correlations found between CWDEs levels and disease development, the variation of both of them was adequately explained by positive or negative exponential equations, respectively (Table 2; Figs. 4 and 5). While statistically significant quantitative differences between the yellowing and wilting syndromes occurred for most CWDEs, generally in favour of the latter; a clear qualitative difference existed in the pattern of variation of PG in stems with development of the two disease syndromes. This variation was positive for the wilting caused by infection with the most virulent Foc-5 race and negative for the yellowing induced by infection with the least virulent Foc-0 race. The negative correlation between expression of PG activity in stem and development of yellowing would suggest that such enzyme activity may play a role in early rather than late stages of pathogenesis in this disease syndrome, and hence might be important during initiation of the infection process. Such a relationship would be similar to that found in fusarium wilt of carnation caused by *F. oxysporum* f. sp. *dianthi,* whereby the level of PG activity in stem at an early phase of disease development was correlated with the final disease severity (Baayen et al. 1997). Nevertheless, the expressed PG activity relies to a large extent on endo-acting enzymes, and these PG forms may have a dual role as putative pathogenicity determinants or host defence response elicitors (Alghisi and Favaron 1995). Therefore, it might be also argued that early endo-PG induction in Foc-0 infected plants compared with that in Foc-5 infected ones could determine a lesser amount of disease (and reduced virulence) as a consequence of an earlier alert of the host defensive machinery. That the levels of some CWDEs were found significantly and positively correlated with disease development; that is, PG in stem infected by Foc-5 (wilting syndrome), as well as PL in root and stem, and XYL in roots infected by either Foc-0 (yellowing syndrome) or Foc-5, suggest that those enzyme activities might play a major role in the final stages of disease development. This conclusion would suit best PL activity in stems, because this activity was detected only when severe symptoms had developed (Fig. 2), and it was an exponential function of disease progression with null value of predicted asymptotic activity (Fig. 4). In the remaining cases for which CWDEs were positively correlated with the disease development, the enzyme activities were detected at the lowest score of symptom severity and were adequately explained by an expanded exponential model with predicted asymptotic activity values different from zero. Therefore, a contribution of such activities to the initial stages of infection and establishment of disease cannot be ruled out.

In conclusion, the highly significant correlations between CWDE levels in infected chickpea root and (or) stem and disease development, as well as the absence of correlation with enzymes excreted in the inoculum media, strongly support a role of such enzymes in pathogenesis of fusarium wilt in chickpea with early PG induction in Foc-0 infected plants and high level of PL induction in Foc-5 infected ones as main differences between the yellowing and wilting disease syndromes, respectively.

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