Phytopathol. Mediterr. (2005) 44, 195–202

Pectolytic enzymes produced by *Fusarium sambucinum in vitro* and during colonisation of potato tubers

Salvatore Vitale[1], Luciana Corazza[1], Paolo Magro[2] and Gabriele Chilosi[2]

[1]Consiglio per la Ricerca e Sperimentazione in Agricoltura (C.R.A.) - Istituto Sperimentale per la Patologia Vegetale, Via C.G. Bertero 22, 00156 Roma, Italy
[2]Dipartimento di Protezione delle Piante, Università degli Studi della Tuscia, Via S. Camillo de Lellis, I-01100 Viterbo, Italy

Summary. The ability of *Fusarium sambucinum* to colonise potato tubers when artificially inoculated or in liquid culture, and which pectolytic enzymes were produced and in what amounts during such colonisation were studied. Pectin lyase (PNL) activity, represented by a set of isoenzymes focalising from isoeletric point (Ip) 6.4 to Ip 9.9, was detected in the culture filtrates. The PNL time course in infected tissues was characterised by an increase in enzyme production and a differential induction of the isoenzymes. Polygalacturonase (PG) activity, represented by a single alkaline band, was detected only in the culture filtrates. Two constitutive basic pectin methylesterase (PME) isoenzymes (Ip>10.0) were also found in both the culture filtrates and the inoculated potato tissues. In rotted tissues and culture filtrates, an increase in pH to values to over 7.0 was recorded, which are values optimal for PNL activity. In the potato-*F. sambucinum* interaction, PNL activity was the principal pectolytic component and appeared to act synergistically with the increase in ambient pH during pathogenesis. To our knowledge, this is the first report on the involvement of pectolytic enzymes during infection with *F. sambucinum*.

Key words: isoenzymes, pectin lyase, polygalacturonase, potato dry rot.

Introduction

The pectolytic enzymes pectin lyase (PNL), pectin methylesterase (PME) and polygalacturonase (PG) have important roles in infection processes (Alghisi and Favaron, 1995) and are often produced in culture and during colonisation of plant tissue sequentially as multiple isoenzymes (Chilosi and Magro, 1997). Tissue pH is an important factor during fungal colonisation since it affects the activity of individual enzymes, and thus may determine virulence efficiency (Prusky and Yakoby, 2003). It has been shown that fungal pathogens can modulate the ambient pH during host colonisation; this permits the fungus to express the genes encoding pectolytic enzymes so that these genes will function under optimal pH conditions (Yakoby *et al*., 2000b; Prusky *et al*., 2001; Drori *et al*., 2003). Some fungi, the alkaline fungi, produce an alkaline environment achieved by the active secretion of ammonia; others, the acidic fungi, produce an acidic environment, by increasing the level of organic acids and/or excreting H^+ (Prusky and Yakoby, 2003).

Fusarium sambucinum Fuckel (teleomorph *Gibberella pulicaris* [Fr.] Sacc.) is a serious pathogen of potato tubers in storage, in which, along with

Corresponding author: S. Vitale
Fax: +39 06 82070370
E-mail: salvatore.vitale@entecra.it

Fusarium solani var. *coeruleum* (Sacc.) Snyd. & Hans, it causes *Fusarium* dry rot (Boyd, 1972; Stevenson *et al.*, 2001). Early symptoms occur at wound sites, where shallow lesions become visible as small brown areas after 1 to 3 months of storage. The area of infection slowly enlarges in all directions, and the periderm over the lesion sinks and wrinkles, sometimes forming concentric rings as the underlying dead tissue desiccates. Tubers may completely rot and shrivel, eventually becoming mummified with continued storage (Stevenson *et al.*, 2001). The nature of the symptoms suggests that pectolytic enzymes are involved in host colonisation by this pathogen. The aim of the present work was to study the ability of *F. sambucinum* to produce pectolytic enzymes *in vitro* and during infection of potato tubers and to evaluate the ability of the fungus to modulate ambient pH.

Materials and methods

Fungus and fungal culture

Fusarium sambucinum isolate MCF ISPaVe 1228 obtained from rotted potato tubers was cultured on potato dextrose agar (PDA) (Oxoid, Unipath Ltd, Basingstoke, England) at 24°C. For enzyme preparation the isolate was surface-cultured in Czapek's liquid medium (pH 5.0) containing $NaNO_3$ (2 g l^{-1}), KH_2PO_4 (1 g l^{-1}), $MgSO_4 \times 7H_2O$ (0.5 g l^{-1}), KCl (0.5 g l^{-1}), $FeSO_4 \times 7H_2O$ (0.01 g l^{-1}), $ZnSO_4 \times 7H_2O$ (0.01 g l^{-1}) and 10 g l^{-1} of citrus pectin (Sigma Chemical Co., St. Louis, MO, USA) as the sole carbon source. The inoculum was one agar disc (7 mm diameter) cut from the edge of 7-day-old mycelium cultured on PDA. The mycelium was grown at 24°C in 250 ml Erlenmeyer flasks containing 50 ml of medium.

Plant material and inoculation

Potato tubers (*Solanum tuberosum*, cv. Spunta) were washed under running water for 3 min, surface sterilized by immersion in 3% (w:v) sodium hypochlorite for 1.5 min and rinsed with sterile water.

Sterile tubers were inoculated with disks of PDA (7 mm diameter, 2 mm thick) bearing mycelium from 7-day-old cultures. One disk was inserted into a 2-mm-deep hole in each tuber made with a sterile 7 mm diameter cork borer. Control tubers were inoculated with sterile PDA disks. After inoculation, the potato tubers were covered with polyethylene bags and stored at 15°C on wet filter paper. Infected and control tubers were collected at the site of infection 1, 3, 5, 7, 10, 14, 18, and 21 days after inoculation. At each of these inspection dates 5 potato tubers were examined.

Enzyme extraction from fungal cultures, spores and inoculated potato tubers

At each inspection date the liquid from three Erlenmeyer flasks was collected and the mycelium removed by filtration with sterile paper in a Buchner funnel. The culture filtrates were tested for pH, centrifuged at 15,000 *g* for 15 min at 4°C, and the supernatants dialysed with distilled water at 4°C.

Enzymes from inoculated tubers were prepared by grinding infected tissues in an ice-cooled mortar in 0.05 M Tris-HCl buffer, pH 8.0 (1 g tissue ml^{-1} buffer) containing 0.1 M KCl, 5 g l^{-1} cysteine and 10 g l^{-1} insoluble polyvinylpolypyrolidone (Sigma Chemical Co.). The slurry was strained through two layers of cheesecloth, centrifuged at 15,000 *g* for 20 min at 4°C and dialysed with three changes of distilled water at 4°C. Uninoculated tubers were prepared in the same way.

Pectolytic enzyme assays

PNL activity was assayed spectrophotometrically by measuring the increase of absorbance at 235 nm. An increase of 1.73 indicated the formation of 1 μmol of unsaturated uronide (Zucker and Hankin, 1970). One unit of enzyme activity (U) catalysed the formation of 1 μmol of unsaturated uronide min^{-1} from 2.5 g l^{-1} (w:v) citrus pectin in Tris-HCl buffer (0.1 M, pH 8.5) at 35°C.

PG activity was determined by measuring the increase of reducing-end groups over time by the method of Nelson (1944), using galacturonic acid (Sigma Chemical Co.) as a standard. One unit of PG activity (RU) produced 1 mmol of reducing groups min^{-1} from 2.5 g l^{-1} polygalacturonic acid (Sigma Chemical Co.) in Na-acetate buffer (0.1 M, pH 5.0) at 35°C.

Isoenzyme identification

Isoenzyme separation by isoeletric focusing (IEF) was performed horizontally on a Multiphor II apparatus (Pharmacia Biotech, Uppsala, Sweden) using 0.4-mm-thick polyacrilamide gels containing 5% (v:v) ampholytes (Pharmacia Biotech)

covering the pH range 3.5–10.0. The run was carried out at a constant power of 5 W for 1.5 h. Ultrathin agarose overlay gels (0.4 mm) for PNL and PG detection were prepared as described by Ried and Collmer (1985) with modifications: for PNL detection the 10 g l^{-1} agarose (Sigma Chemical Co.) gel contained 1 g l^{-1} pectin in 50 mM Tris-HCl buffer, pH 8.5; and for PG detection the 10 g l^{-1} agarose gel contained 1 g l^{-1} polygalacturonic acid in 50 mM Na-acetate buffer at pH 5.0. IEF polyacrylamide gels overlaid with ultrathin agarose gels were incubated at 100% humidity and 35°C for 60–90 min. Activity bands were visualised by staining the agarose gel for 15 min in 0.5 g l^{-1} ruthenium red (Sigma Chemical Co.) solution, followed by rinsing in distilled water. PNLs or PGs appeared as a white band, pectin methyl esterase (PME) isoenzymes as a dark red band. The isoeletric point (Ip) values of the pectolytic isoenzymes were estimated from a regression equation of standard proteins (Pharmacia Biotech) vs. distance migrated.

Disease assessment

Visual ratings of disease development were made at 1, 3, 5, 7, 10, 14, 18, and 21 days after inoculation on tubers cut through the middle point of the inoculation site. The rotted area around each inoculation site was calculated by multiplying the depth of each site by its width.

pH measurements

The pH of liquid cultures was measured with a pH electrode (Model HI 223 Hanna Instruments, Woonsocket, RI, USA), sampled at different time intervals. The pH of tissue was taken by placing the electrode directly against the colonised potato tissue.

Results

Production of pectolytic enzymes *in vitro* culture

Fusarium sambucinum isolate MCF ISPaVe 1228 produced both PNL and PG enzymatic activity when grown on liquid medium containing pectin as the sole carbon source (Fig. 1 and 2). The

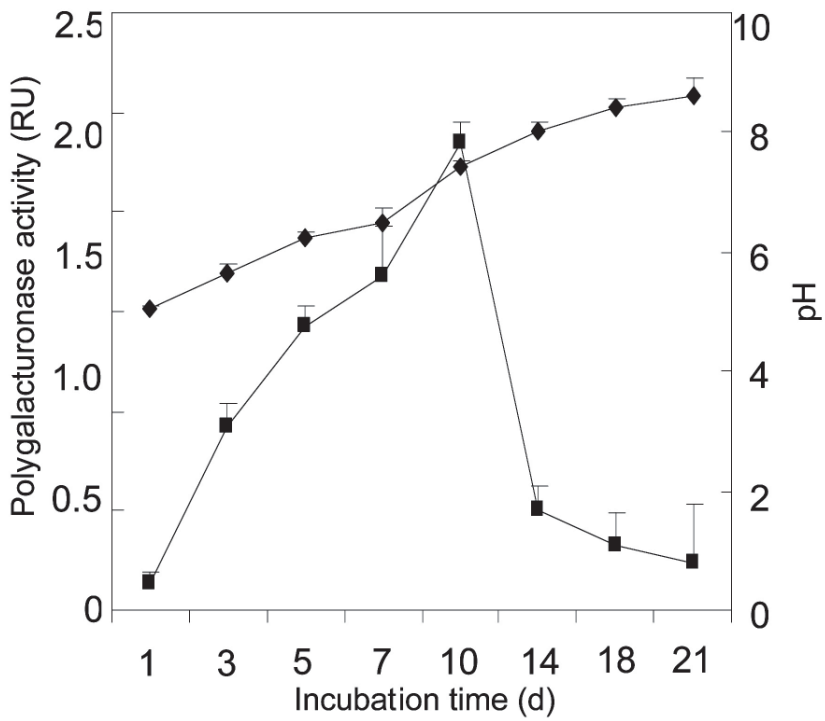

Fig. 1. Time course of polygalacturonase (■) produced in liquid medium supplemented with pectin as sole carbon source by *Fusarium sambucinum* isolate MCF ISPaVe 1228 and pH variation (♦). Polygalacturonase activity is expressed as Reducing Units. Bars represent the standard error of means of at least three experiments.

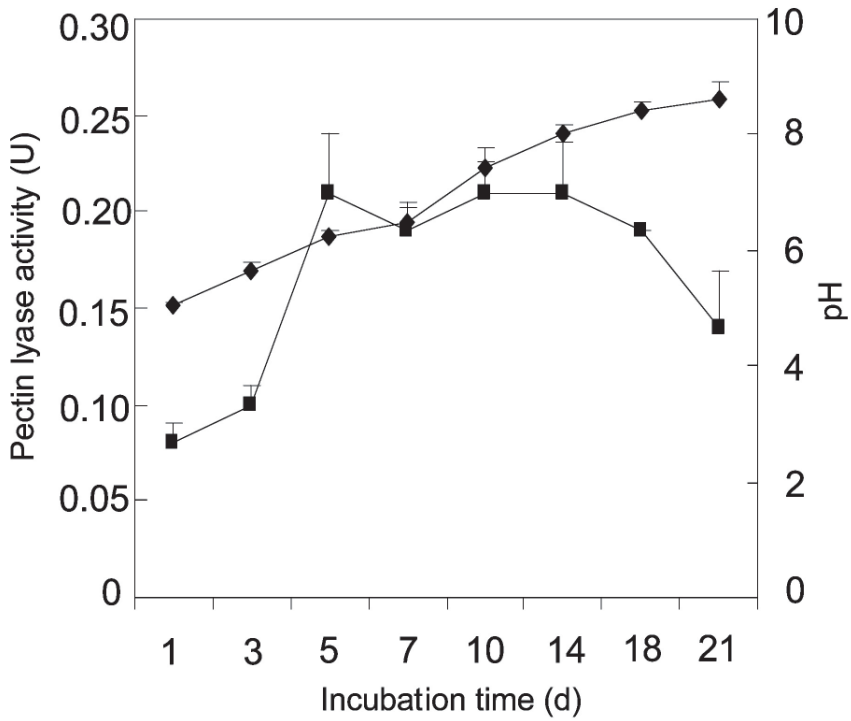

Fig. 2. Time course of pectin lyase (■) produced in liquid medium supplemented with pectin as sole carbon source by *Fusarium sambucinum* isolate MCF ISPaVe 1228 and pH variation (♦). Enzyme activity is expressed as Units. Bars represent the standard error of means of at least three experiments.

time course of PG was characterised by an increase in activity, attaining maximum value at 10 d, followed by a marked decrease. PNL activity rapidly rose, reaching peak activity at 5 d, then levelled off, followed by a slow decline from 18 d onwards.

The dialysed extracts from the culture filtrates were analysed by thin layer polyacrylamide gel IEF and evaluated for their PNL and PG isoenzyme patterns. Seven PNL isoforms were observed in the liquid cultures of both isolates focusing at Ip 6.4, 7.0, 7.5, 8.1, 8.9, 9.4, 9.9. (Fig. 3). The PNL isoenzyme pattern at 10 d was strongly expressed, reflecting the higher PNL activity assayed spectrophotometrically. From 18 d onwards the 9.9 band was not detected.

One PG band at Ip 8.8 was observed at 10 d after growth in culture filtrate (data not shown) in accordance with the results of the quantitative PG assay. From 3 d to 14 d two dark alkaline bands (Ip>10.0) associated with PME activity of fungal origin were also detected in the culture filtrates (Fig. 3).

During growth, the pH of the culture medium steadily increased from pH 5.0 to a maximum of 8.6 at 21 d (Fig. 1 and 2).

Disease development, pectolytic activity and pH in potato tuber extracts

After infection with *F. sambucinum,* brown discolorations around the infection sites were observed within 3 days, then the infected tissues became sunken and finally they rotted. Typical dry rot symptoms were observed 2 weeks after infection. The pathogen was successfully re-isolated from most of the inoculated tubers, confirming that symptoms were due to the fungus. The pH of healthy potato tissue was 5.8. During tissue colonisation, the pathogen caused an increase in pH, bringing it to values from 7.1 to 7.3.

The time course of PNL activity was characterised by a peak at d 18, followed by a pronounced decline thereafter (Fig. 4). The PNL pattern of potato tissues inoculated with *F. sambucinum* was characterised by the expression of the bands al-

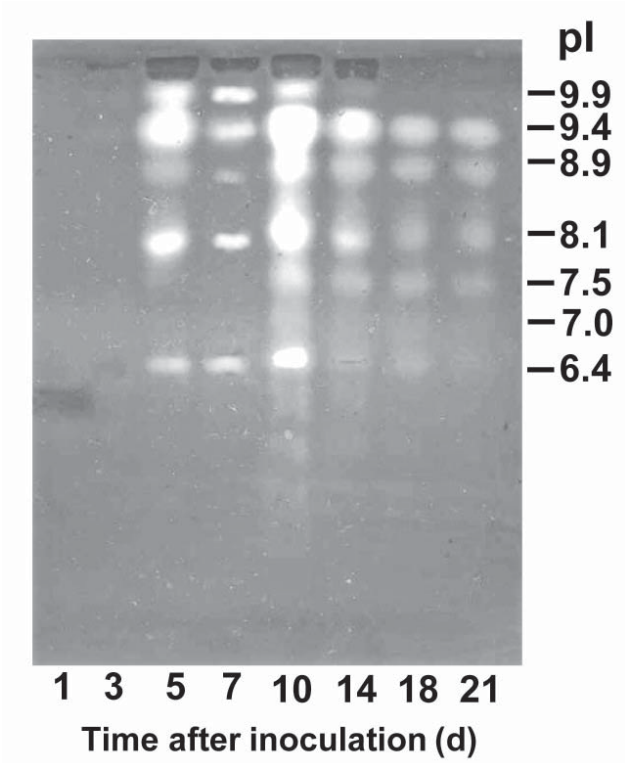

Fig. 3. Pectin lyase isoenzyme patterns from culture filtrate of *Fusarium sambucinum* isolate MCF ISPaVe 1228 grown in liquid medium supplemented with pectin as carbon source at 1, 3, 5, 7, 10, 14, 18, 21 days. Samples were separated on an isolelectric focusing (IEF) ultrathin gel (pH 3.5–10.0), followed by agarose overlay activity staining. Positions and pI values of pectin lyase bands are indicated on the right.

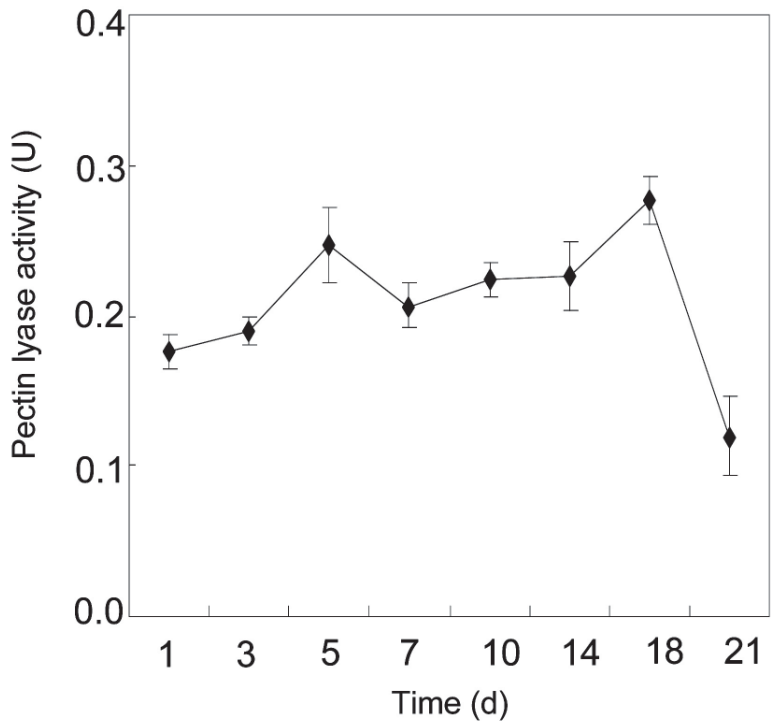

Fig. 4. Pectin lyase activity from potato tissues at 1, 3, 5, 7, 10, 14, 18, 21 days after infection with *Fusarium sambucinum* isolate MCF ISPaVe 1228. Bars represent the standard error of means of at least three experiments.

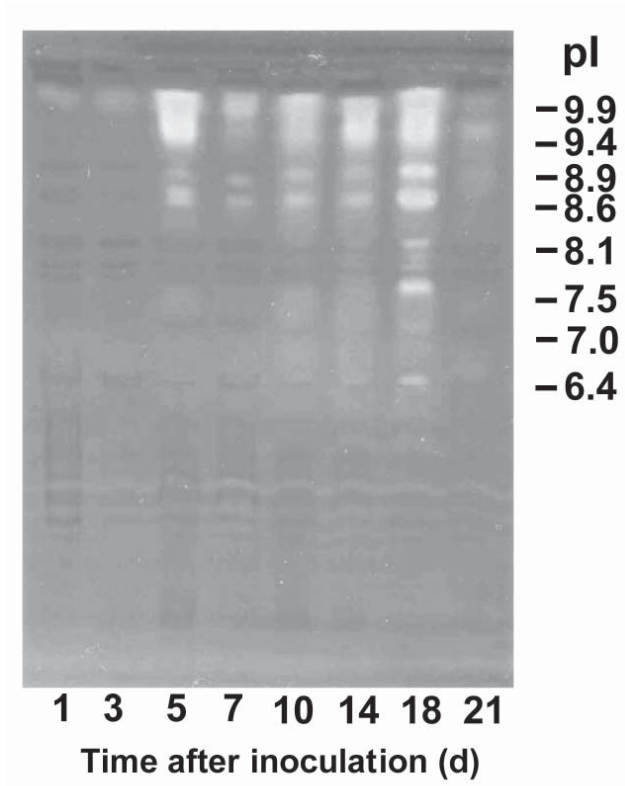

Fig. 5. Pectin lyase isoenzyme patterns from potato tissues at 1, 3, 5, 7, 10, 14, 18, 21 days after infection with *Fusarium sambucinum* isolate MCF ISPaVe 1228. Samples were separated on an isolectric focusing (IEF) ultrathin gel (pH 3.5–10.0), followed by agarose overlay activity staining. Positions and pI values of the pectin lyase bands are indicated on the right.

ready recorded from the liquid culture plus the induction of an extra band focalising at Ip 8.6 (Fig. 5). As with PNL activity recorded during the spectrophotometric PNL assay, the isoenzyme pattern at 18 d was the most complex and yielded the most intense activity staining. No PG activity was detected in the inoculated extracts, nor was any PG or PNL activity detected in extracts of control tubers. A complex pattern of PME dark red bands, presumably of plant origin, was detected in both inoculated and non-inoculated extracts.

Discussion

Fusarium sambucinum isolate MCF ISPaVe 1228 tested in this study produced PNL both *in vitro* and during colonisation of potato tubers. This is to our knowledge the first report that pectolytic enzymes are involved in the development of dry rot caused by this fungus. *F. sambucinum* also produced PG *in vitro* as a single band. However, since PG was not detected during tissue colonisation it may not be involved in pathogenicity during this plant-pathogen interaction. There are some indications that the PNLs produced by some fungal pathogens are determinants for pathogenicity; these indications have been obtained from studies on hypovirulent isolates (Marcus *et al*., 1986), non-pathogenic mutants (Wattad *et al*., 1995), antibody-blocking enzymes (Crawford and Kolattukudy, 1987), the enhancement of virulence by gene transfer (Yakoby *et al*., 2000a), and on the correlation between the level of PNL and virulence (Chilosi and Magro, 1998). In the present study PNL produced by *F. sambucinum* was found to be the preponderant pectolytic enzyme during potato colonisation. This suggests that the PNLs may play a critical role in determining the pathogenic success of this pathogen. Moreover, the ability of this pathogen to produce multiple PNLs may give

it an advantage by enhancing its versatility and its expression of virulence during potato tuber colonisation, possibly in combination with other factors. One of these other factors may be the ability of *F. sambucinum* to modulate the ambient pH, bringing it closer to the optimum for PNL. *F. sambucinum* produced one basic PNL at the beginning of host colonisation and this was followed by the differential induction of a number of other isoenzymes. The regulation of pectolytic enzymes during attack of fungal pathogens is reported to be induced by factors that include catabolite repression, oligomer production and changes in ambient pH levels (Alghisi and Favaron, 1995; Annis and Goodwin, 1997; Prusky and Yakoby, 2003). Factors affecting the pH level such as the secretion of ammonia by the fungus (Wijesundera *et al*., 1989) may act synergistically with PNLs to create pH conditions better suited for enzyme activity, and enhance the ability of the fungus to invade host tissues. The induction of PNLs by *F. sambucinum* in parallel with the increase of pH described in this study is reminiscent of the behaviour of other pathogenic fungi. The natural increase in pH during avocado ripening was shown to regulate pectate lyase secretion by the postharvest pathogen *C. gloeosporioides*. This pathogen also produced ammonia locally in host tissue, resulting in a pH increase that enhanced enzyme secretion and virulence (Prusky *et al*., 2001). *Didymella bryoniae,* a cucurbit pathogen, was also found to produce PNLs as the preponderant pectolytic component during stem colonisation, in parallel with a rapid increase of pH in the colonised tissues (Chilosi and Magro, 1998). In potato, the change in pH during potato tissue colonisation by *F. sambucinum* may promote the synthesis and activity of other hydrolytic enzymes that are potentially a part of the invasion strategy. It has been shown that a protease produced by *F. solani* f. sp. *eumartii*, another important causative agent of potato dry rot, has optimal activity at pH 8.0 and hydrolyses specific polypeptides of potato intercellular washing fluids and cell walls as well as degrading pathogenesis-related proteins (Olivieri *et al*., 2002). The development of strategies targeting the pH-regulating processes may represent a novel tool for blocking the onset and development of dry rot of potato by *F. sambucinum*.

Acknowledgements

This study was carried out as part of the Italian Project "Miglioramento genetico della patata" financially supported by the Ministry of the Agriculture and Forestry (MiPAF).

Literature cited

Alghisi P. and F. Favaron, 1995. Pectin-degrading enzymes and plant-parasite interactions. *European Journal of Plant Pathology* 101, 365–375.

Annis S.L. and P.H. Goodwin, 1997. Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *European Journal of Plant Pathology* 103, 1–14.

Boyd A.E.W., 1972. Potato storage diseases. *Review of Plant Pathology* 51, 297–321.

Chilosi G. and P. Magro, 1997. Pectin lyase and polygalacturonase isoenzyme production by *Botrytis cinerea* during the early stages of infection on different host plants. *Journal of Plant Pathology* 78, 61–69.

Chilosi G. and P. Magro, 1998. Pectolytic enzymes produced *in vitro* and during colonization of melon tissues by *Didymella bryoniae*. *Plant Pathology* 47, 700–705.

Crawford M.S. and P.E. Kolattukudy, 1987. Pectate lyase from *Fusarium solani* f. sp. *pisi*: purification, characterization, *in vitro* translation of the mRNA, and involvement in pathogenicity. *Archives of Biochemistry and Biophysics* 258, 196–205.

Drori N., H. Kramer-Haimovich, J. Rollins, A. Dinoor, Y. Okon, O. Pines and D. Prusky, 2003. External pH and nitrogen sources affect secretion of pectate lyase by *Colletotrichum gloeosporioides*. *Applied and Environmental Microbiology* 69, 3258–3262.

Marcus L., I. Barash, B. Sneh, Y. Koltin and A. Finkler, 1986. Purification and characterization of pectolytic enzymes produced by virulent and hypovirulent isolates of *Rhizoctonia solani* Kuhn. *Physiological and Molecular Plant Pathology* 29, 325–336.

Nelson N., 1944. A photometric adaptation of the Somogyi method for determination of glucose. *Journal of Biological Chemistry* 153, 375–380.

Olivieri F., M.G. Zanetti, C.R. Oliva, A.A. Covarrubias and C.A. Casalongué, 2002. Characterisation of an extracellular serine protease of *Fusarium eumartii* and its action on pathogenesis related proteins. *European Journal of Plant Pathology* 108, 63–72.

Prusky D., J.L. McEvoy, B. Leverentz and W.S. Conway, 2001. Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. *Molecular Plant-Microbe Interactions* 14, 1105–1113.

Prusky D., J.L. McEvoy, R. Saftner, W.S. Conway and R. Jones, 2003. The relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. *Phytopathology* 94, 44–51.

Prusky D. and N. Yakoby, 2003. Pathogenic fungi: leading or led by ambient pH? *Molecular Plant Pathology* 4, 509–516.

Ried J.L. and A. Collmer, 1985. Activity stain for rapid characterization of pectic enzymes in isoelectric focusing and sodiumdodecyl sulphate-polyacrylamide gels. *Applied and Environmental Microbiology* 50, 615–622.

Stevenson W.R., R. Loria, G.D. Franc and D.P. Weingartner, 2001. *Compendium of Potato Diseases*. 2th edition, APS press, St. Paul, MI, USA, 106 pp.

Wattad C., S. Freeman, A. Dinoor and D. Prusky, 1995. A nonpathogenic mutant of *Colletotrichum magna* is deficient in extracellular secretion of pectate lyase. *Molecular Plant-Microbe Interactions* 8, 621–626.

Wijesundera R.L.C., J.A. Bailey, R.J.W. Byrde and A.H. Fielding, 1989. Cell wall degrading enzymes of *Colletotrichum lindemuthianum*: their role in the development of bean anthracnose. *Physiological and Molecular Plant Pathology* 34, 403–413.

Yakoby N., S. Freeman, A. Dinoor, N.T. Keen and D. Prusky, 2000. Expression of pectate lyase from *Colletotrichum gloeosporioides* in *C. magna* promotes pathogenicity. *Molecular Plant-Microbe Interactions* 13, 887–891.

Yakoby N., I. Kobiler, A. Dinoor and D. Prusky, 2000. pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. *Applied Environmental Microbiology* 66, 1026–1030.

Zucker M. and L. Hankin, 1970. Regulation of pectate lyase synthesis in *Pseudomonas fluorescens* and *Erwinia carotovora*. *Journal of Bacteriology* 104, 13–18.

Accepted for publication: May 24, 2005