Glycoproteins of sugarcane plants facilitate the infectivity of *Ustilago scitaminea* and *Xanthomonas albilineans*, two sugarcane pathogens

M. Blanch, M.E. Legaz, A.M. Millanes and C. Vicente

Department of Plant Physiology, Faculty of Biology, Complutense University, 28040 Madrid, Spain.

Sugarcane plants produce heterofructans composed of homofructan domains consisting of β-1,2-fructofuranoside chains which intercalate variable-length segments of polygalactitol. Sometimes, these heterofructans appear as the glycosidic moiety of glycoproteins. They are produced as a response to mechanical injuries and pathogen infections. These glycoproteins act as a defense mechanism against smut (*Ustilago scitaminea*) by inducing homotypic adhesion and by inhibiting teliospore germination. When smut teliospores are cultured on glycoproteins produced by resistant cultivars of sugarcane plants, the actin capping, which occurs before teliospore germination, is efficiently inhibited. Then, cell polarization is not achieved and the growth of germinative tube is completely inhibited. However, inoculation of sensitive cultivars with smut teliospores induce glycoprotein fractions that promote teliospore polarity after binding to their cell wall ligand, and are different from those obtained from resistant plants.

On the other hand, leaf scald, a bacterial-vascular disease of sugar cane, has *Xanthomonas albilineans* as casual organism. The pathogen is confined mainly to the leaf and stalk vascular bundles, which are often partly or completely occluded with a gum-like substance, identified as a xanthan-like polysaccharide. This xanthan-like polysaccharide produced consists of a basal tetramer that is repeated to form the macromolecule. This basal tetrasaccharide is composed by two molecules of glucose, one mannose rest and a final glucuronic acid. Since xanthans contain glucuronic acid, the ability of these bacteria to produce an active UDP glucose dehydrogenase (the enzyme that produces UDP glucuronic acid from UDPG) is often seen as a virulence factor. *Xanthomonas albilineans* produces a UDP-glucose dehydrogenase growing on sucrose but the enzyme activity rapidly decays after hydrolysis of the enzyme by bacterial proteases. Thus, *X. albilineans* axenically cultured did not secrete xanthans to liquid media but the use of inoculated sugarcane tissues for producing and characterizing xanthans is absolutely required. Glycoproteins from sugarcane, the natural host of the bacterium, also assures the production of the active enzyme by inhibiting bacterial proteases.

Keywords *Ustilago scitaminea*, *Xathomonas albilineans*, cell polarity, glycoproteins, infectivity, sugarcane, xanthans

1. Introduction

Resistance of plants to disease seems to be a multifactorial process. The response phase includes accumulation of different compounds such as: phytoalexins (i.e. low molecular mass antimicrobial compounds that accumulate at sites of infection); systemic enzymes that degrade pathogens (e.g. chitinases, β-1,3-glucanases and proteases); systemic enzymes that generate antimicrobial compounds and protective biopolymers (e.g. peroxidases and phenoloxidases); biopolymers that restrict the spread of pathogens (e.g. hydroxyproline-rich glycoproteins, lignin, callose); and regulators of the induction and/or activity of defensive compounds (e.g. elicitors of plant and microbial origin, immune signals from primed plants and compounds, which release immune signals) [1].Other glycoproteins are involved in resistance responses. Three varieties of sugarcane, defined by their relative resistance to smut, have been previously used to study the production of glycoproteins after smut infection.

2. Production of glycoproteins as a plant response to diseases caused by microorganisms.

Sugarcane produces, after infection by several pathogens, two different pools of glycoproteins containing a heterofructan as glycidic moiety and tentatively described as high molecular mass (HMMG) and mid-molecular mass (MMMG) glycoproteins [2]. The hydrolysis of the polysaccharide moiety of both HMMG and MMMG, HMMC and MMMC (high- and mid-molecular mass carbohydrates) respectively, results in free fructose

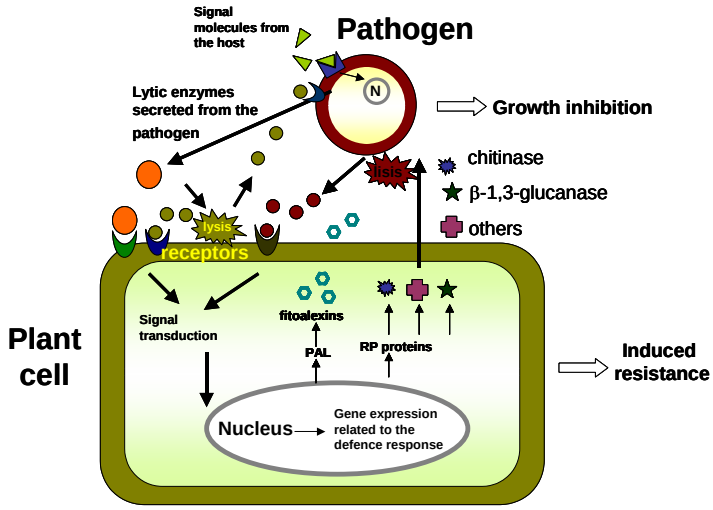

Figure 1. Chemical communication between the plant cell and its pathogen. The pathogen secretes lytic enzymes to degrade the plant cell wall and some of these lysis products can act as signal molecules to bind to cell wall receptors of the pathogen. Metabolites from the microorganism elicit plant responses of induced resistance consisting of the production of phytoalexins or resistance proteins (RP) which impede the growth of the pathogen.

and galactitol. Quantitation of products of the acidic hydrolysis reveals differences of the polymerization degree of these polysaccharides. MMMC is thought to be a $[Fructose_2{:}Galactitol_3]_n$ polymer while HMMC results to be a $[Fructose_4{:}Galactitol_5]_n$ polysaccharide [3]. HMMC and MMMC consist of a minor chain of $\beta(1\rightarrow2)$ fructofuranoside, hydrolyzed by invertase, on which a heteropolymer composed of fructose-galactitol is attached. Hexitol-hexose bond showed to be sensitive to acidic hydrolysis but unaccesible to invertase action. This ether linkage can be hydrolyzed by a sugarcane glycosidase that produced three times more fructose than galactitol. Enzyme activity was clearly dependent on the presence of Mn^{2+} in the reaction mixtures. Purified native glycosidase has an apparent molecular mass of 13.2 kDa.

The occurrence of soluble polysaccharides in sugarcane juices causes a reduction in the amount of sucrose recovered during the industrial process of crystallization [4] by forming molasses which affect the purity of the syrup, changing its physico-chemical properties.

Analysis of both HMMG and MMMG by capillary electrophoresis reveals that MMMG fraction contains two cationic and four anionic components whereas only one cationic and four anionic proteins are separated from the HMMG fraction [5]. These glycoproteins can be considered as factors of biological resistance to smut [6] since their amount increases in resistant cultivars but decrease in susceptible varieties after infection with smut teliospores. In addition, Fontaniella *et al.* [7] found that these glycoproteins selectively bind to smut teliospores, preferently those defined as anionic peptides. After binding, cell aggregation occurs in parallel to a loss of the germination ability of recruited teliospores and the emergence of the germinating tube is completely impeded.

3. Sugarcane smut

Sugarcane smut is caused by the dimorphic basidiomycete *Ustilago scitaminea*. The characteristic symptom of culmicolous smut is the formation of a long whip-like sorus in which diploid, airborne teliospores are produced [8]. Spore germination originates a pro-mycelium which give rise to four haploid sporidia after meiosis. When two compatible sporidia form germ tubes that fuse, a dikaryotic, infecting mycelium is produced [9]. Hyphal growth occurs throughout the infected plant but mostly in the parenchyma cells of the cane stalk internode. The formation of the dikarion is readily recognized *in vitro* when culture changes from a yeast-like appearance to white, fuzzy mycelial growth [10] .

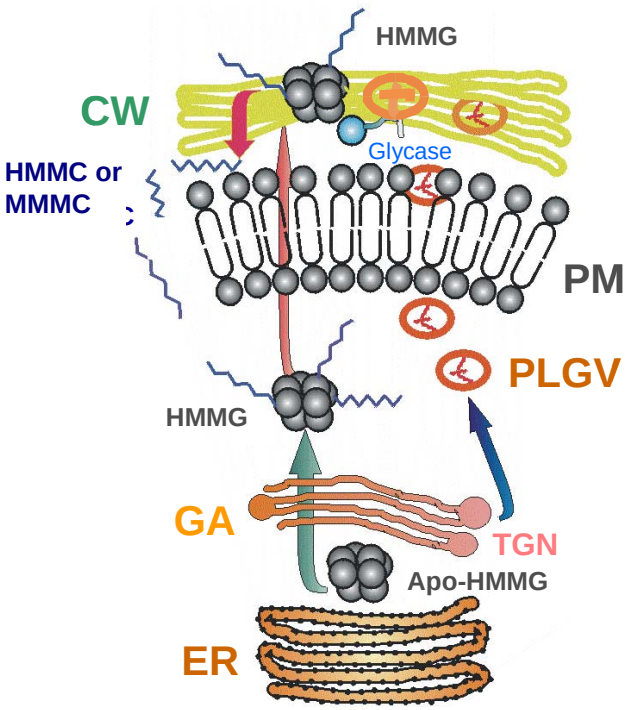

Figure 2. Parenchymatous cells of sugarcane stalks are able to produce proteins (Apo-HMMG) which are glycosylated (HMMG) in Golgi cisternae (TGN) to be moved through the cytoplasm (PLGV) and secreted across the plasma membrane (PM) into the cell wall (CW). A glycosidase (Glycase) system catalyzes the total or partial hydrolysis of these glycoproteins and the resulting polysaccharides (HMMC and MMMC) are removed from the cell wall to appear as soluble macromolecules in the cytoplasm.

One of the major changes previous to, or during spore germination concerns cytoplasm polarization, a process mediated by actin [11-13], that is essential for fungal tip growth. *Ustilago maydis*, the causal pathogen of corn smut disease, divides by budding at its unicellular yeast-like stage. During budding, the actin cytoskeleton polarizes to sites of active growth [14]. Because the bud tip is the site of the incorporation of new cell wall material during bud growth, the concentration of actin at this site suggests that actin plays an active role in the secretion of cell wall polymers.

4. The role of sugarcane glycoproteins in the inhibition/promotion of the germination of smut teliospores.

HMMG and MMMG fractions recovered from Barbados 42-231 cv. contain chitinase activity, the level of which was minor in HMMG than in the second fraction, disappearing from the first one and getting down in the second one when they were obtained from inoculated plants. Put both fractions, HMMG or MMMG, in contact with smut teliospores, chitinase of healthy plants fastly and completely binds to those, whereas the low amount of MMMG chitinase obtained from inoculated plants binds to theliospores only in a 36 %.

The differences among these glycoproteins and their activities obtained from resistant or susceptible cultivars to smut can be summarized in a following way:

1 º The proteomic endowment of Mayarí 55-14 cv., resistant to the disease, shows a high degree of diversity and microheterogeneity, estimated by means of capillary electrophoresis [15], whereas a low level of complexity is revealed for glycoproteins obtained from. Barbados cv, sensitive to smut.

2 º The cv Mayarí shows high chitinase activity in both HMMG and MMMG and high capacity of interaction with teliospores, whereas it is minor in Barbados and very low if the plant has been before infected [16].

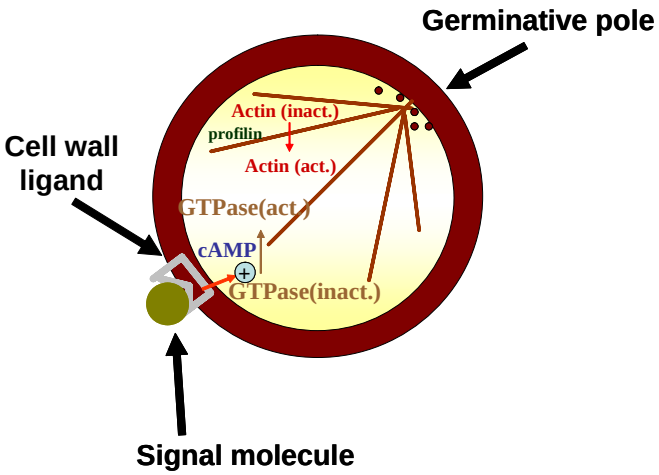

Figure 3. Signal molecules from the plant cells (HMMG or MMMG) bind to specific receptors in the cell wall of smut teliospores to activate a metabolic cascade involving GTPase activated by cyclic AMP and profiling that promotes actin polarization. This rearrangement of the cytoskeleton indicates the position of the germinative pole through which the protrusion and growth of the fungal hypha is achieved.

3 º Glycoproteins from the cv. Mayarí show a high capacity of secretion of proteins from teliospores of the pathogenic fungus, which makes them detectable for the plant, whereas the above mentioned capacity is very low in the cv. Barbados, which significantly reduces its ability to recognize the pathogen.

4 º The ability of cane glycoproteins to join specific receptors has been studied by labelling with fluorescein isothiocyanate and by the

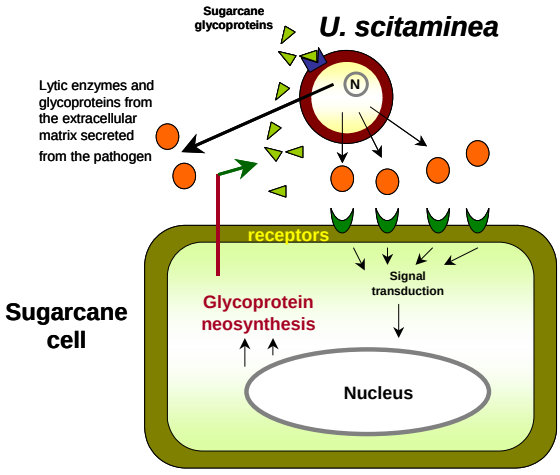

Figure 4. Smut teliospores secrete signal molecules (lytic enzymes and other glycoproteins) which act as ligands for several plant cell wall receptors. The binding promotes the biosynthesis of both HMMG and MMMG which activates teliospore germination when they are produced by sensitive cultivars, or inhibition of the germination when they are produced by resistant cultivars.

observation of teliospores treated with labelled glycoproteins by fluorescence microscopy. The binding of the glycoproteins of the resistant cultivar to the wall of smut teliospores disable cell polarization and the reorganization of its cytoskeleton in order to mark the germinative pole. This effect was observed by labelling the fungal actin by reaction with phaloidin to which the suitable fluorophore was previously

bound [15]. On the contrary, glycoproteins from Barbados are unable to disable this reorganization but they promote cellular polarization and spore germination.

5 º Consistent with this capability to bind to the walls of smut teliospores, HMMG and MMMG from the resistant Mayari cv. show high affinity to bind to N-acetyl-D-glucosamine residues in glycoproteins of the teliospore wall whereas the efficiency of Barbados HMMG and MMMG for the same receptor is very low. This interaction has been studied after a selective extraction of glycoproteins from teliospore walls and their retention in a bead of activated agarose to which HMMG or MMMG from both cultivars had joined. The major recovery of fungal protein from the agarose bead was achieved by elution with a 50 mM N-acetyl-D-glucosamine solution.

5. Leaf scald disease

Xanthomonas albilineans is a member of Pseudomonaceae, a yellow-pigmented, Gram-negative plant pathogenic bacterium [17]. This microorganism produces a xanthan-like polysaccharide which occludes both xylem and phloem producing desiccation of leaves [18]. This xanthan-like polysaccharide consisted of a basal tetramer composed by two molecules of glucose, one mannose rest and a final glucuronic acid that is highly repeated to form the macromolecule [19]. The occurrence of glucuronate rest in the polysaccharide requires the action of an UDP-glucose dehydrogenase which catalyses the NAD^+-dependent oxidation of UDP-glucose to UDP-glucuronic acid. It belongs to a small group of dehydrogenases that are able to carry out the two-fold oxidation of an alcohol to an acid without the release of an aldehyde as intermediate [20]. This enzyme has a wide range of functions. In plants, UDP-glucose dehydrogenase is the main enzyme in the pathway of synthesis of hemicelluloses and pectins, which are the components of newly formed cell walls [21].

6. Regulation of UDPglucose dehydrogenase by sugarcane glycoproteins

X. albilineans in liquid culture is able to produce an enzyme which catalyses a redox reaction using UDPG as substrate and NADPH as a cofactor. However, the absence of redox reaction by using NAD+ or NADP+ instead of NADPH implies that the enzyme does no catalyse the conventional reaction of UDPG dehydrogenase (EC 1.1.1.22):

$$\text{UDP-glucose} + NAD^+ + H_2O \rightarrow \text{UDP-glucuronic acid} + NADH$$

as described for the protein identified from animals, plants and bacteria [22-25], but the alternative reaction

$$\text{UDP-glucose} + NADPH + O_2 \rightarrow \text{UDP-glucuronic acid} + NADP^+ + H_2O$$

as deduced from the oxygen dependence found for the enzymatic oxidation of NADPH.

The time-course of UDPG dehydrogenase activity shows that it strongly decreased in bacterial cells growing in Wilbrink media for 15 h culture to remain unchanged to 24h at very low level. The initial loss of UDPG dehydrogenase activity was repeated by bacterial cells growing in Wilbrink media supplied with HMMG but strongly increased later, from 15h to 24h culture. When bacteria were grown on media supplemented with MMMG, the initial decrease of UDPG dehydrogenase activity was abolished and the enzyme activity strongly increased at the end of the time of culture.

By including 0.1 mM 8-azaguanine in culture media, the activity of the enzyme was maintained unchanged along the time of culture, even when HMMG or MMMG were included in the culture media, although values of activity obtained when sugarcane glycoproteins were added to the media were always

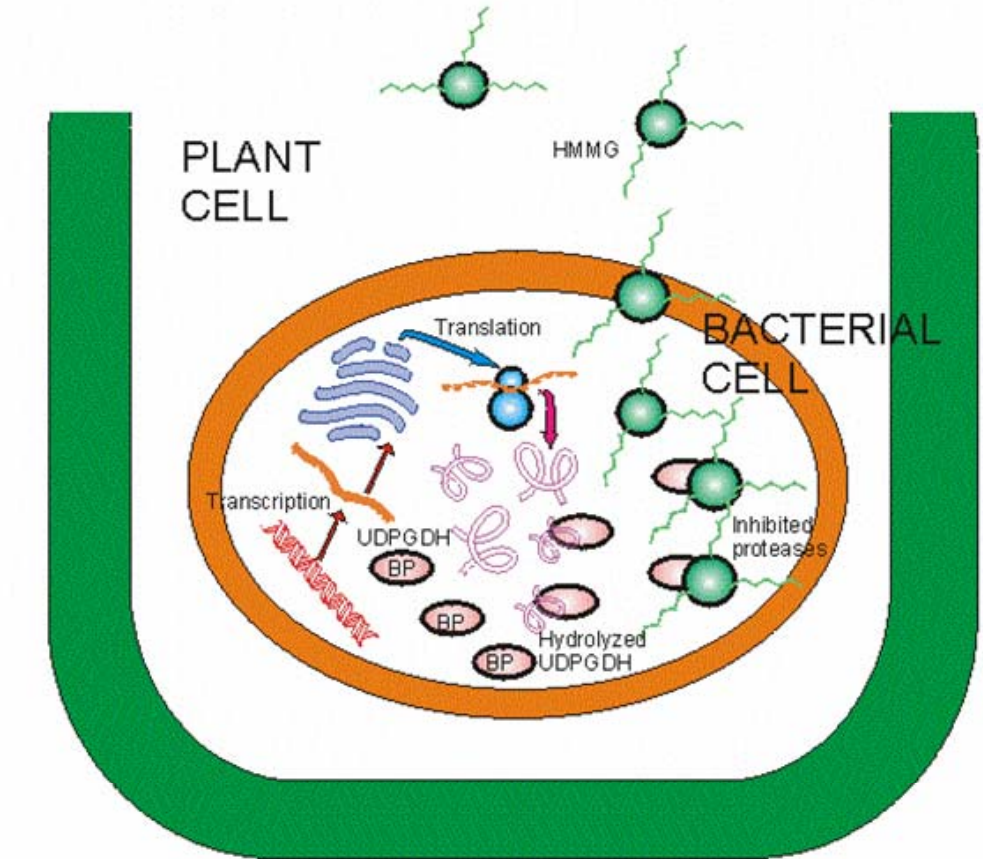

Figure 5. Elicitors produce by bacteria invading plant tissues promote the synthesis of sugarcane glycoproteins (HMMG) that are internalized into the bacterial cells. When bacteria synthesized UDPG dehydrogenase (UDPGDH), the enzyme can be inactivated by bacterial proteases (BP), impeding the production of UDPglucuronic acid and, consequently, the synthesis and secretion of xanthans. However, cane glycoproteins synthesized by sensitive cultivars act as powerful inhibitors of these bacterial proteases, facilitating then the synthesis of xanthans.

slightly higher than those obtained when the inhibitor of transcription was used alone. Then, it could be concluded that sugarcane glycoproteins do not compete with 8-azaguanine to enhance transcription of the corresponding mRNA. When 0.1 mM chloramphenicol was included in the Willbrink media, only MMMG seemed to compete with the inhibitor by enhancing the enzyme production at 24h culture. Both inhibitors, 8-azaguanine and chloramphenicol nullified the reported loss of UDPG dehydrogenase activity in the absence of sugarcane glycoproteins. UDPG dehydrogenase activity was completely nullified by increasing the concentration of inhibitors from 100 μM to 300 μM. These results could be explained by considering that 100 μM 8-azaguanine or chloramphenicol mainly inhibit protease synthesis whereas the same inhibitors at a concentration of 300 μM could inhibit the synthesis of both proteases and UDPG dehydrogenase.

Dow *et al.* [26,27] reported that proteases could be used to degrade protein in the plant cell wall, allowing bacterial spread or overcome host defences. The role of proteases in the pathogenicity depends on the host–pathogen system [28], in which proteases act in the cleavage of peptide bonds and play a role in many physiological functions [29]. However, in absence of the plant host, bacterial proteases could be used to hydrolyze bacterial proteins, such as UDPG dehydrogenase. Since sugarcane glycoproteins inhibit bacterial proteases in vitro and permit then the production of UDPG dehydrogenase, this can be

interpreted as an interdependence between host and pathogen, probably derived from a coevolution process. This could explain why *X. albilineans* did not produce xanthans in culture whereas the gum was secreted from bacteria invading sugarcane tissues [30].

Acknowledgements This work was supported by a grant, BFU2006-14263, from the Ministerio de Educación (Spain). We are grateful to Miss Raquel Alonso for the excellent technical support.

References

[1] J. Kuc, Ciba Foundation Symposia **154**, 213 (1990).
[2] M.E. Legaz, M.M. Pedrosa, R. Armas, M. Martínez and C. Vicente, Journal of Chromatography, **697**, 329 (1995).
[3] M.E. Legaz, L. Martín, M.M. Pedrosa, C. Vicente, R. de Armas, M. Martínez, I. Medina and C.W. Rodríguez, Plant Physiology **92**, 679 (1990).
[4] B. Fontaniella, A.M. Millanes, D. Piñón, C.W. Rodríguez, C. Vicente, M.E. Legaz, Food Science and Biotechnology **12**, 346 (2003).
[5] M.E Legaz, M.M. Pedrosa, R. de Armas, C.W. Rodríguez, V. de los Ríos and C. Vicente, Analytica Chimica Acta **372**, 201, (1998).
[6] M. Martínez, I. Medina, S. Naranjo, C.W. Rodriguez, R. de Armas, D. Piñon, C. Vicente and M.E. Legaz, International Sugar Journal **102**, 445 (2000).
[7] B. Fontaniella, A. Márquez, C.W. Rodríguez, D. Piñon, M.T. Solas, C. Vicente and M.E. Legaz, Plant Physiology and Biochemistry **40**, 881 (2002).
[8] G. Lee-Lovick, Review on Plant Pathology **57**, 181 (1978).
[9] K.C. Alexander and K.V. Srinivasan, Current Science **35**, 603 (1966).
[10] S. Schenk, Molecular aspects of the sugarcane smut disease pathogen, *Ustilago scitaminea*, in: Sugacane Pathology, G.P. Rao, A. Bergamin-Filho, R.C. Magarey, L.J.C. Autrey (Eds.), Science Publishers Inc, Enfield, NH, 1999, pp.131.
[11] M. Evangelista, S. Zigmond and C. Boone, Journal of Cell Science **116**, 2603 (2003).
[12] J. Magdalena, T.H. Millard and L.M. Machesky, Journal of Cell Science **116**, 743 (2003).
[13] R. Wedlich-Soldner, S. Altschuler, L. Wu and R. Li, Science **299**, 1231 (2003).
[14] F. Banuett and I. Herskowitz, Fungal Genetics and Biology **37**, 149 (2002).
[15] A.M. Millanes, B. Fontaniella, M.E. Legaz and C. Vicente, Journal of Plant Physiology **162**, 253 (2005).
[16] R. de Armas, R. Santiago, M.E. Legaz and C. Vicente**,** Australasian Plant Pathology 36, 32 (2007).
[17] C. Kai-Wei, W. Shu-Fen and T. Yi-Hsiung, Biochemical and Biophysical Research Communications **287**, 550 (2001).
[18] M.T. Solas, D. Piñon, R. Acevedo, B. Fontaniella, M.E. Legaz and C. Vicente, European Journal of Plant Pathology **109**, 351 (2003).
[19] B. Fontaniella, C.W. Rodriguez, D. Piñon, C. Vicente and M.E. Legaz, Journal of Chromatography B. **770**, 275 (2002).
[20] R.E. Campbell, R.F. Sala, I. van de Rijn and M.E. Tanner, Journal of Biological Chemistry **272**, 3416 (1997).
[21] A. Kärkönen, A. Murigneux, J.P. Martinant, E. Pepey, C. Tatout, B.J. Dudley and S.C. Fry,. Biochemical Journal **391**, 409 (2005).
[22] H. Ankel, E. Ankel and D.S. Feingold, Biochemistry **5**, 1864 (1966).
[23] R. Jaenicke and R. Rudolph, Biochemistry **25**, 7283 (1986).
[24] V. Sieberth, G.P. Rigg, I.S. Roberts and K. Jann, Journal of Bacteriology **177**, 4562 (1995).
[25] D.C. Stewart and L. Copeland, Plant Science **147**, 119 (1999).
[26] J.M. Dow, B.R. Clarke, D.E. Milligan, J.L. Tang and M.J. Daniels, Applied and Environmental Microbiology **56**, 2994 (1990).
[27] J.M. Dow, L. Crossman, K. Findlay, Y.Q. He, J.X. Feng and J.L. Tang, Proceedings of the National. Academy of Sciences **100**, 10995 (2003).
[28] S.M. Fry, J.S. Huang and R.D. Milholland, Phytopathology **84**, 357 (1994).
[29] F.J Romero, L.A. García, J.A. Salas, M. Díaz and L.M. Quirós, Process Biochem. **36**, 507 (2001).
[30] Y. Blanco, M. Blanch, D. Piñón, M.E. Legaz and C. Vicente, Journal of Bioscience and Bioengineering **99**, 366 (2005).