Tropical Plant Pathology, vol. 38(6):534-538, 2013
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SHORT COMMUNICATION / *COMUNICAÇÃO*

PR-protein activities in table beet against *Cercospora beticola* after spraying chitosan or acibenzolar-S-methyl

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ABSTRACT

Cercospora leaf spot (*Cercospora beticola*) is the most important disease of table beet in the world. In this study, the preventive application of chitosan or acibenzolar-S-methyl (ASM) to control the disease was evaluated, and the involvement of pathogenesis-related proteins in the plant-pathogen interaction was verified. Beet plants cultivar Early Wonder were sprayed with distilled water, HCl 0.05 N, chitosan at 2 mg/mL or 4 mg/mL and ASM at 25 mg/L, and 24 h or three days later, inoculated with the pathogen. Leaf samples were collected at different times after spraying and enzymatic activities were quantified spectrophotometrically. It was observed that chitosan and ASM reduced disease severity in table beet leaves by 76% and 68%, respectively, on the average of two experiments, and ASM promoted the accumulation of peroxidases and β-1,3 glucanases in beet leaves. Thus, both inducers reduce cercospora leaf spot in table beet, and the effect of ASM is associated with the activation of PR-proteins, whereas chitosan act by a still unknown mechanism.
Key words: *Cercospora beticola*, glucanases, peroxidases, phenylalanine ammonia lyase, resistance induction.

Cercospora leaf spot, caused by the fungus *Cercospora beticola*, is the main beet (*Beta vulgaris*) disease worldwide. In table beet, the disease besides resulting in decreased productivity, can depreciate the quality of the product due to spots on the leaves, when the plant is commercialized with the aerial part. The pathogen survives in plant debris, and environmental conditions such as high relative humidity and temperatures between 25ºC and 35ºC favor infection and disease development (Weiland & Koch, 2004). During colonization, the fungus occupies the intercellular spaces and uses reactive oxygen species as a weapon of attack in order to cause the collapse of the plant cells (Daub & Ehrenshaft, 2000). Among the plant defense mechanisms during the infectious process is the synthesis of pathogenesis-related proteins (PR-proteins), which participate in routes of the defense metabolism or act directly against the pathogen.

Peroxidases, for example, are enzymes involved in the oxidative elimination of reactive oxygen species produced both in the process of cellular respiration and in a pathogen attack. The increase of this enzymatic activity is associated with decreased severity of diseases such as Fusarium wilt in muskmelon caused by *Fusarium oxyxporum* f. sp. *melonis* (Madadkhah et al., 2012) and brown rot of peach by *Monilinia fructicola* (Ma et al., 2013). Phenylalanine ammonia lyase, in turn, is the key-enzyme in the phenylpropanoid metabolism (Hammerschimidt & Kagan, 2001). Through this metabolic pathway, plants synthesize polyphenols used in the process of lignification, and compounds of low molecular weight called phytoalexins that act against microbial infection, such as resveratrol on the grapevine downy mildew caused by *Plasmopara viticola* (Alonso-Villaverde et al., 2011). Another form of involvement of PR-proteins in plant defense is the direct effect, as it occurs with the β-1,3 glucanases, which hydrolyze β-glucans present in the cell wall of fungi.

The synthesis and accumulation of these PR-proteins can be altered through the use of either abiotic or biotic elicitors. Chitosan, a polysaccharide obtained from deacetylation of chitin, extracted mainly from the shell of crustaceans, have the ability to activate biochemical defense responses by interacting with receptors on the surfaces of plant cells (Benhamou, 1992). Another mode of action of chitosan is the antimicrobial activity. Its positive charges may interact with negatively charged microbial surfaces, thereby restricting the movement of molecules for the microbial agent (Assis & Leoni, 2003). The ability of chitosan in reducing the severity of plant diseases has been demonstrated in peanut against *Puccinia arachidis* (Sathiyabama & Balasubramanian, 1998), common bean against *Colletotrichum lindemuthianum* (Di Piero & Garda, 2008) and apple against *C. acutatum* (Felipini & Di Piero, 2009).

On the other hand, acibenzolar-S-methyl (ASM) is a resistance inducer registered and commercially available for the prevention of fungal and bacterial diseases in crops such as bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicon*) (MAPA, 2013). The molecule is absorbed and translocated by the plant and acts as a second messenger, resulting in the expression of genes related to systemic acquired resistance (Benhamou & Belanger, 1998). Its mode of action is mainly related to increased activity of

phenylalanine ammonia lyase and the synthesis of phenolic compounds (Stadnik & Buchenauer, 1999).

The aim of this study was to evaluate the effect of chitosan and ASM for the control of cercospora leaf spot and analyze the PR-protein accumulation in plants after treatment with inducers and inoculation with the pathogen.

Experiments were conducted under greenhouse conditions using table beet cultivar Early Wonder. Seeds were sown in 128-cell polystyrene trays filled with commercial substrates. When plants reached the four-leaf stage, each plant was individually transferred to 2 L pots containing mineral soil and organic matter at a 6:1 ratio. Plants were treated two weeks after transplanting, at the six-leaf stage.

The suspensions of chitosan with 85% deacetylation were prepared by dissolving the product in HCl 0.05 N, with constant stirring and pH adjustment to 5.6 using 2 N NaOH. The ASM solutions were prepared from dissolving the commercial product Bion 500 WG in sterile distilled water. The fungal inoculum was obtained by adding 10 mL sterile distilled water to Petri dishes containing 10-day old cultures of *C. beticola* on V8 agar medium, which was subsequently scraped with a Drigalski spatula. In all experiments, inoculations were carried out by spraying a suspension containing $1x10^4$ conidia/mL on the beet seedlings to run off and the plants were kept in a moist chamber for 36 h.

In the first experiment, plants were treated with distilled water, 0.05N HCl pH 5.6, chitosan at 2 mg/mL and 4 mg/mL or ASM at 25 mg/L and after 24 h were inoculated with the pathogen. In the second bioassay, plants were sprayed with 0.05 N HCl pH 5.6, chitosan at 2 mg/mL or ASM at 25 mg/L and 72 h later were challenged with the pathogen as described above. In this experiment, leaf samples were collected at 2, 4, 6, 8 and 10 days after spraying in order to determine the activity of PR-proteins. Disease severity was evaluated at 20 days after the inoculation through the computational program Quant v.1.0.2. (Vale et al., 2001).

For protein extraction, each sample composed of approximately 200 mg of leaf tissue was macerated in liquid nitrogen, and then homogenized in 1.5 mL of 100 mM phosphate buffer pH 7.0 containing 1 mM phenylmethanesulfonyl fluoride, 0.5% polyvinylpyrrolidone and 1 mM ethylenediaminetetraacetic acid. The homogenates were placed in 2 mL microtubes, and centrifuged at 20000 g for 30 min at 4ºC. The supernatant, called protein extract, was collected and used to determine enzyme activities. The protein content of the sample was evaluated by the method of Bradford (1976). β-1,3 glucanase (EC 3.2.1.39) activity was determined from 40 µL of protein extract added to 460 µL of 100 mM acetate buffer pH 5 containing laminarin at 1 mg/mL at 44ºC following 1 h. Each sample was compared to a blank control, which contained all reagents in the same amounts, with the exception of laminarin (Di Piero & Pascholati, 2004). After 1 h, the reducing sugars were quantified (Lever, 1972). The reaction product absorbance was measured at 595 nm. Reducing sugar content was determined through a standard glucose curve, and results were expressed as µkatal glucose formed from laminarin hydrolysis per mg protein (µkatal/mg protein). Peroxidase activity (EC 1.11.1.7) was measured from a reaction that included 45 µL of protein extract, and 2.55 mL of 50 mM phosphate buffer pH 6 containing 0.25% guaiacol and 0.1 M hydrogen peroxide. The reaction was carried out for 4 min at 40ºC, and the absorbance values recorded in a spectrophotometer Femto 700 Plus at 470 nm, in 30 s intervals, from the first reaction minute. The results were expressed as optical density units at 470 nm per min per mg protein (OD470nm/min/mg protein) (Hammerschmidt et al., 1982). The determination of phenylalanine ammonia lyase (EC 4.3.1.5) activity was carried out according Falcón et al. (2008). Therefore, 40 µL of protein extract were added to 460 µL of 100 mM sodium borate buffer pH 8.8 containing 50 mM phenylalanine. After 1 h incubation at 40ºC, the samples were immersed for 5 min in ice bath and the reaction stopped by adding 200 µL of 5 N HCl. Finally, 300 µL of distilled water were added to the mixture and the absorbance was measured at 290 nm. The experiments were conducted using a randomised complete design with five replicates per treatment; each plot was composed of a pot containing one plant. Analysis of variance (ANOVA), F-tests ($P < 0.05$), and Student-Newman-Keuls (SNK) ($P < 0.05$) were conducted in experiments to test for significant differences among means.

In the first experiment, there were reductions of 72%, 72% and 55% in cercospora leaf spot severity when chitosan was applied at 2 mg/mL or 4 mg/mL, and ASM at 25 mg/L, respectively, 24 h before inoculation. Spraying with HCl pH 5.6 did not differ from the control with distilled water (data not shown). The ability of the products to control the disease was confirmed in the second experiment, when applied 72 h before inoculation, with chitosan at 2 mg/mL reducing the disease severity in 80% and ASM at 25 mg/L in 91% (Figure 1A).

The reduction in disease by chitosan and ASM was reported in the literature for several foliar and fruit diseases. Sathiyabama & Balasubramanian (1998) obtained a reduction of peanut rust (*Puccinia arachidis*) by applying 1 mg/mL chitosan preventively. In addition, there was a decrease of the production of uredinospores *in vivo*. Ma et al. (2013) found that immersion of peach fruits in a solution with 0.5 mg/mL of chitosan reduced the incidence of *Monilinia fructicola*. In the case of ASM, the product is commercially recommended for different crops, such as cotton, potato, citrus, bean, melon and tomato (MAPA, 2013), However, there is no information on the use of chitosan and ASM for the control of cercospora leaf spot in table beet.

Beet leaves treated with ASM showed an increase in β-1,3 glucanases after inoculation, indicating that the plants were pre-sensitized by the product (Figure 1B).

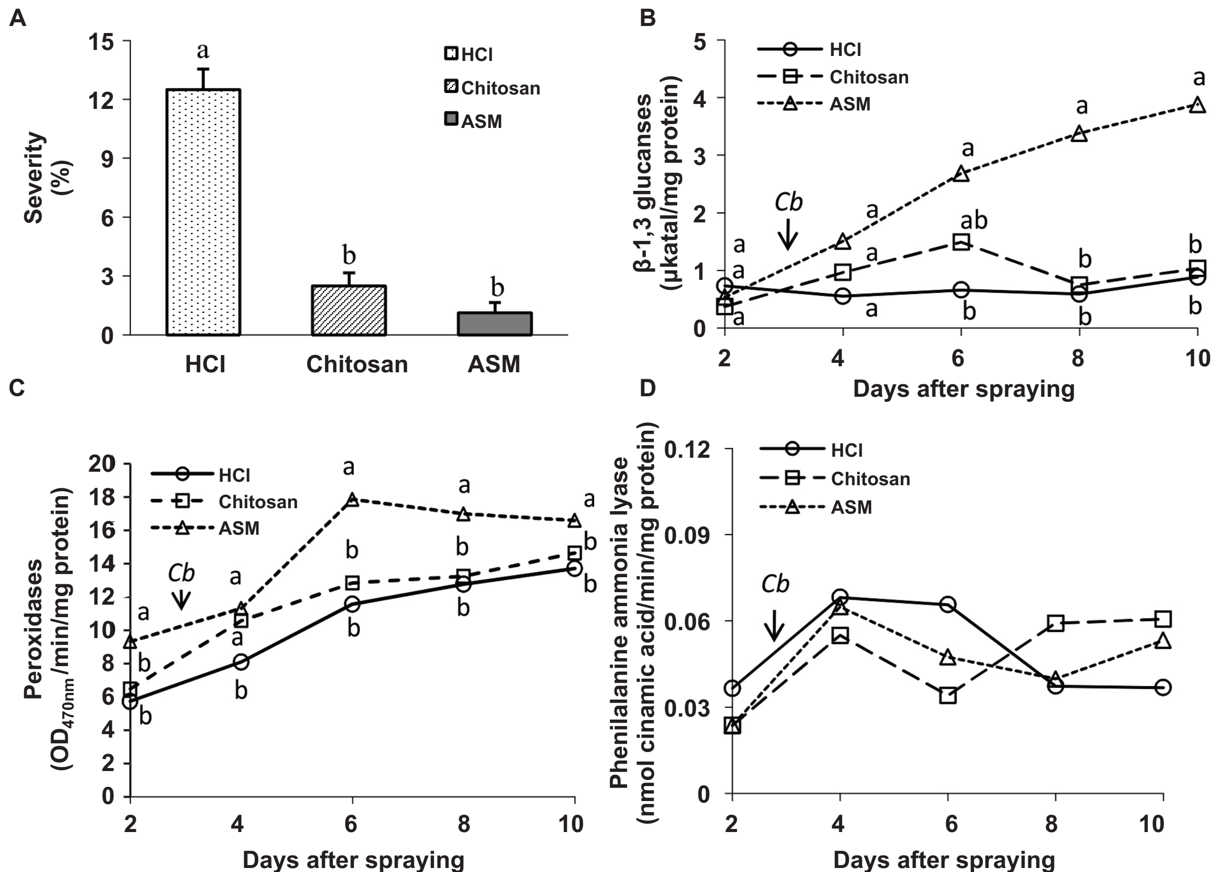

FIGURE 1 - Effects of chitosan and ASM on cercospora leaf spot severity (**A**) and activities of β-1,3 glucanases (**B**), peroxidases (**C**) and phenylalanine ammonia lyase (**D**). Beet leaves were sprayed with hydrochloric acid (HCl), chitosan (2 mg/mL) or acibenzolar-S-methyl (ASM, 25 mg/L). Plants were challenged with *Cercospora beticola* (Cb) three days after spraying. Enzyme activities were determined spectrophotometrically. Bars followed by the same letter in "A" and markers followed by the same letter in "B" and "C" within each time period do not significantly differ based on SNK tests ($P < 0.05$).

These enzymes are able to hydrolyze β-1,3 glucan of the fungal cell wall. The basic forms are within the vacuoles and acid forms are found in cell walls (Pascholati & Leite, 1995). The latter defend the plant during the colonization of intercellular spaces by the pathogen, a place that *C. beticola* occupies during the initial process of colonization (Pal & Mukhopadhyay, 1984). This increase in β-1,3 glucanase activity may have contributed to lower disease levels in plants treated preventively with ASM. Increased levels of this enzyme after application of ASM has been shown to be associated with the reduction of diseases such as bacterial wilt in tomato (*Xanthomonas campestris* pv. *campestris*) (Cavalcanti et al., 2006), fusariosis in cowpea (*Fusarium oxysporum* f. sp. *tracheiphilum*) (Rodrigues et al., 2006), and leaf spot of cotton (*Xanthomonas axonopodis* pv. *malvacearum*) (Ishida et al., 2008).

Plants sprayed with ASM showed higher peroxidase activity before inoculation (Figure 1C). Peroxidases are related to greater stress tolerance and to the elimination of reactive oxygen species resulting from the metabolism (Resende et al., 2003). However, *C. beticola* uses reactive oxygen species as chemical weapons to destroy the cell membranes of plants (Daub & Ehrenshaft, 2000). Thus, the higher peroxidase activity may protect the plant by eliminating these highly cytotoxic molecules generated by the pathogen. The highest activity of this enzyme is related to the control of plant diseases in other crops. Viecelli et al. (2009) showed a reduction of the severity of angular leaf spot of bean caused by *Pseudocercospora griseola* when the plants were treated with 75 mg/L of ASM and observed that the control was associated with increased peroxidase activity.

The activity of phenylalanine ammonia lyase, in turn, increased after challenge with the pathogen, but did not differ between plants that received different treatments. One type of plant defense response against infection by pathogens is the production of defense molecules. This includes the phytoalexins, through the route of phenylpropanoids, catalyzed initially by phenylalanine ammonia lyase (Siegrist et al., 1998). The results reported here indicate that this was a normal reaction mechanism of the plant in contact with the pathogen, not influenced by

the inducers tested and insufficient to prevent infection and disease development (Figure 1D).

Although several studies relate the ability of chitosan to induce the synthesis of defense enzymes, in the conditions of the present study it was observed that this polysaccharide did not promote an increase in the activity of the evaluated PR-proteins. Thus, the reduction of the cercospora leaf spot in beet by the application of chitosan may be due to the antimicrobial effect of the product on *C. beticola* or to the activation of other types of defense mechanisms unrelated to PR-proteins. The fungicidal effect of chitosan has been documented in the literature on *Pythium aphanidermatum*, *C. acutatum*, *B. cinerea* and *P. expansum*, among others (El Ghaouth et al., 1994; Liu et al., 2007; Felipini & Di Piero, 2009). Further studies need to be carried out to verify this effect on *C. beticola*.

Therefore, both chitosan and ASM reduce cercospora leaf spot in table beet. In the case of ASM, the effect occurs concomitantly to the increment of the peroxidase and β-1,3 glucanase activities, whereas chitosan act by a mechanism not revealed in our studies.

REFERENCES

Alonso-Villaverde V, Voinesco F, Viret O, Spring J, Gindro K (2011) The effectiveness of stilbenes in resistant Vitaceae: ultrastructural and biochemical events during *Plasmopara viticola* infection process. Plant Physiology and Biochemistry 49:265-274.

Assis OBG, Leoni AM (2003) Filmes comestíveis de quitosana. Revista Biotecnologia, Ciência e Desenvolvimento 30:33-38.

Bradford MA (1976) A rapid and sensitive method for the quantitation of microgram quanties of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Benhamou N (1992) Ultrastructural and cytochemical aspects of chitosan on *Fusarium oxysporum* f. sp. *radicis-lycopersici*, agent of tomato crown and root rot. Phytopathology 82:1185-1193.

Benhamou N, Belanger RR (1998) Induction of systemic resistance to *Pythium* damping-off in cucumber plants by benzothiadiazole: Ultrastructure and cytochemistry of the host response. Plant Journal 14:13-21.

Cavalcanti FR, Resende MLV, Pereira RB, Costa JCB, Carvalho CPS (2006) Atividades de quitinase e beta-1,3-glucanase após eliciação das defesas do tomateiro contra a mancha-bacteriana. Pesquisa Agropecuária Brasileira 41:1721-1730.

Daub ME, Ehrenshaft M (2000) The photoactivated *Cercospora* toxin cercosporin: contributions to plant disease and fundamental biology. Annual Review of Phytopathology 38:461-490.

Di Piero RM, Garda MV (2008) Quitosana reduz a severidade da antracnose e aumenta a atividade de glucanase em feijoeiro-comum. Pesquisa Agropecuária Brasileira 43:1121-1128.

Di Piero RM, Pascholati SF (2004) Effect of *Lentinula edodes* and *Agaricus blazei* on the interaction between tomato plants and *Xanthomonas vesicatoria.* Summa Phytopathologica 30:58-63.

El Ghaouth A, Arul J, Grenier J, Benhamou N, Asselin A (1994) Effect of chitosan on cucumber plants: suppression of *Pythium aphanidermatum* and induction of defence reactions. Phytopathology 84:313-320.

Falcón AB, Cabrera JC, Costales D, Ramirez MA, Cabrera G, Toledo V, Martinez-Telles MA (2008) The effect of size and acetylation degree of chitosan derivatives on tobacco plant protection against *Phytophthora parasitica nicotianae*. World Journal of Microbiology & Biotechnology 24:103-112.

Felipini RB, Di Piero RM (2009) Redução da severidade da podridão-amarga de maçã em pós-colheita pela imersão de frutos em quitosana. Pesquisa Agropecuária Brasileira 44:1591-1597.

Hammerschimidt R, Kagan IA (2001) Phytoalexins into the 21st century. Physiological and Molecular Plant Pathology 59:59-61.

Hammerschmidt R, Nuckles EM, Kuc J (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiological Plant Pathology 20:73-82.

Ishida AKN, Souza RM, Resende MLV, Cavalcanti FR, Oliveira DL, Pozza EA (2008) *Rhizobacterium* and acibenzolar-S-methyl (ASM) in resistance induction against bacterial blight and expression of defense responses in cotton. Tropical Plant Pathology 33:027-034.

Lever MA (1972) A new reaction for colorimetric determination of carbohydrates. Analitical Biochemistry 47:273-279.

Liu L, Tian S, Meng X, Xu Y (2007) Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruits. Postharvest Biology and Technology 44:300-306.

Ma Z, Yang L, Yan H, Kennedy JF, Meng X (2013) Chitosan and oligochitosan enhance the resistance of peach fruit to brown rot. Carbohydrate Polymers 94:272-277.

Madadkhah E, Lotfi M, Nabipour A, Rahmanpour S, Banihashemi Z, Shoorooei M (2012) Enzymatic activities in roots of melon genotypes infected with *Fusarium oxysporum* f. sp. *melonis* race 1. Scientia Horticulturae 135:171-176.

MAPA. Sistema de agrotóxicos fitossanitários. Available at: http://extranet.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. Accessed on March 10, 2013.

Pal V, Mukhopadhyay AN (1984) Study of the cellulolytic and pectolytic enzymes in nine biological forms of *Cercospora beticola* Sacc. Acta Phytopathologica Academiae Scientiarum Hungaricae 19:263-269.

Pascholati SF, Leite B (1995) Hospedeiro: Mecanismos de resistência. In: Bergamin Filho A, Kimati H, Amorim L (Eds.) Manual de Fitopatologia: Princípios e Conceitos. São Paulo SP, Brazil. Ceres. pp. 417-453.

Resende MLV, Salgado SML, Chaves ZM (2003) Espécies ativas de oxigênio na resposta de defesa de plantas a patógenos. Fitopatologia Brasileira 28:123-130.

Rodrigues AAC, Neto EB, Coelho RSB (2006) Indução de resistência a *Fusarium oxysporum* f. sp. *tracheiphilum* em caupi: eficiência de indutores abióticos e atividade enzimática elicitada. Fitopatologia Brasileira 31:492-499.

Sathiyabama M, Balasubramanian FL (1998) Chitosan induces resistance components in *Arachis hypogaea* against leaf rust caused by *Puccinia arachidis* Speg. Crop Protection 17:307-313.

Siegrist J, Mühlenbeck S, Buchenauer H (1998) Cultured

parsley cells, a model system for the rapid testing of abiotic and natural substances as inducers of systemic acquired resistance. Physiological and Molecular Plant Pathology 53:223-238.

Stadnik MJ, Buchenauer H (1999) Accumulation of autofluorogenic compounds at the penetration site of *Blumeria graminis* f. sp. *tritici* is associated with both benzothiadiazole-induced and quantitative resistance in wheat. Journal of Phytopathology 147:615-622.

Vale FXR, Fernandes Filho EI, Liberato JR (2001) Quantificação de doenças. Quant v.1.0.1 [software]. Viçosa MG, Brazil. UFV.

Viecelli CA, Stangarlin JR, Kuhn OJ, Schwan-Estrada KRF (2009) Indução de resistência em feijoeiro por filtrado de cultura de *Pycnoporus sanguineus* contra *Pseudocercospora griseola*. Tropical Plant Pathology 34:087-096.

Weiland JJ, Koch G (2004) Sugarbeet leaf spot disease (*Cercospora beticola* Sacc.). Molecular Plant Pathology 5:157-166.

TPP 2013-0116 - Received 4 July 2013 - Accepted 17 September 2013
Section Editor: Jorge Teodoro de Souza