ANTIBIOTIC ACTIVITY AGAINST XANTHOMONAS GARDNERI 
AND PROTECTION OF TOMATO PLANTS BY CHITOSAN

D.S.O. Coqueiro and R.M. Di Piero
Universidade Federal de Santa Catarina (UFSC), Centro de Ciências Agrárias,
CEP 88034-001 Florianópolis, SC, Brasil

SUMMARY

*Xanthomonas gardneri* is an important species associated with bacterial leaf spot of tomato in Brazil. The losses caused by the disease can reach 100% if preventive measures are not adopted. The polysaccharide chitosan has antibiotic activity and the ability to activate defense mechanisms and may constitute a tool for managing plant diseases. This study aimed at evaluating the antibacterial effect of chitosans with different molecular weights on *X. gardneri* development and tomato plants protection. Three types of chitosan, i.e. commercial (CcOm), low molecular weight (Clmw), and medium molecular weight (Cmmw), were incorporated in nutrient agar medium to assess bacterial growth. In greenhouse tests, tomato plants were treated with chitosans and inoculated with *X. gardneri*, and the disease severity was evaluated 15 days after inoculation. Leaf samples were collected at 0, 3, 4, and 6 days after the treatment to evaluate peroxidase and β-1,3-glucanase activities. Chitosans with different molecular weights showed a similar level of antimicrobial effect on *X. gardneri*. The polysaccharides at concentrations of 1 and 3 mg ml⁻¹ reduced the leaf spot severity by 70%, when applied up to 3 days before inoculation of tomato plants. Enzymatic analysis showed an increase of peroxidase activity in Clmw-treated plants. Thus, the antibiotic activity of chitosan associated with induction of resistance may explain the control level of bacterial spot observed in this study.

Key words: bacterial spot, chitosan, induced resistance, peroxidase.

INTRODUCTION

Bacterial spot disease of tomato is caused by four species of *Xanthomonas* (*X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*). Among them, *X. gardneri* can be an important component of the disease complex (Duval, 2003). It prevails in Ontario (Canada) (Warner, 2003; Leboeuf *et al.*, 2005) and has been isolated with high frequency from symptomatic tomato plants in the midwest of Brazil. The disease can affect different parts of the plant, induces leaf shedding and reduces the quantity and quality of the yield (Silva-Lobo *et al.*, 2005). Preventive measures, such as the use of pathogen-free seeds and seedlings, disinfection of pruning tools, and the use of resistant varieties, must be implemented to minimize the deleterious effects of the infection. The use of resistant varieties would be of paramount importance, but the *Xanthomonas* species studied show wide genetic diversity, preventing the introgression of durable resistance. The use of copper fungicides has not promoted satisfactory control and has adverse effects on plant development and the environment, depending on the amount applied (Kurozawa and Pavan, 2005).

Current approaches are focused on alternative compounds that can integrate plant disease management. Chitosan, a polysaccharide present in the outer shell of crustaceans and in cell walls of fungi, has been widely investigated (Devlieghere *et al.*, 2004). It is a non toxic, biodegradable polymer, consisting of glucosamine, 2-amino-2-deoxy-β-D-glucose, that possesses antimicrobial activity and elicits defense mechanisms in plant tissues (Hadhiger and Beckman, 1980; Badaway and Rabea, 2008).

The antimicrobial effect of chitosan has been documented for several fungi, such as Botrytis cinerea (Ben-Shalom *et al.*, 2003), Fusarium solani f. sp. phaseoli (Hadhiger and Beckman, 1980), Rhizopus stolonifer (El Ghaouth *et al.*, 1992), and Penicillium expansum (Liu *et al.*, 2007). However, little is known on its effect on phytopathogenic bacteria, with only two studies showing the inhibitory action on a *Xanthomonas* species that causes leaf spot of poinsettia (Euphorbia pulcherrima) (Li *et al.*, 2008) and on Agrobacterium tumefaciens and Pectobacterium carotovorum subsp. carotovorum (Rabea *et al.*, 2009).

Plant defense responses induced by chitosan involve callose deposition, lignification, phytoalexins, and pathogenesis-related proteins (PRs) synthesis (Cavalcanti *et al.*, 2007; Faoro *et al.*, 2008; Iriti *et al.*, 2008). In addition, chitosan activates MAP kinases in several plant species (Lizama *et al.*, 2007). Cucumber, barley and bean plants
treated with chitosan showed lower severity of diseases caused by B. cinerea, Blumeria graminis f. sp. hordei, and Colletotrichum lindemuthianum, respectively (Ben-Shalom et al., 2003; Faoro et al., 2008; Di Piero and Garda, 2008). In tomato plants, Abd-El-Kareem et al. (2006) reported an antibiotic effect of chitosan on Rhizoctonia solani, F. solani and Sclerotium rolfsii, apart from over 80% reduction of root rot incidence when the plants were grown in soil enriched with the polysaccharide.

The mentioned scarcity of reports on bacterial disease control by chitosan prompted this work for determining its ability to induce resistance of tomato plants to X. gardneri.

MATERIALS AND METHODS

Plant material and growth conditions. Tomato seeds of cv. Santa Cruz Kada were planted in styrofoam trays containing Germina Plant substrate (Terra Fértil, Florestal, Brazil). After fifteen days, the seedlings were transferred to pots with a 2 litre capacity, containing organic compounds and soil (1:5, v/v). Experiments were carried out in a greenhouse with plants at the five true leaf stage.

Chitosans and Bion. Three types of chitosans were used: (i) low molecular weight chitosan (Clmw, 50-190 Kda, Sigma Aldrich Chemicals, Brazil) with 75-85% deacetylation degree; (ii) medium molecular weight chitosan (Cmmw; 190-310 Kda; Sigma Aldrich Chemicals, Brazil) with 75-85% deacetylation degree, and (iii) a commercial chitosan (Ccom) with 85% deacetylation degree, supplied by the Departamento de Ciência e Tecnologia de Alimentos, Universidade Federal de Santa Catarina, Santa Catarina, Brazil. Chitosan suspensions were prepared according to Bhaskara Reddy et al. (1999), using HCl 0.05 N as a solvent and adjusting the pH of the solutions to 5.6. Bion (acibenzolar-S-methyl; ASM) was provided by Syngenta (Brazil) in the form of a commercial product registered for tomato crops against bacterial spot, was the positive control. After 3 days of treatment, X. gardneri (0.3 OD) was inoculated by spraying the bacterial suspension on the abaxial and adaxial surfaces of the leaves and keeping the plants in a moist chamber for 48 h.

Two experiments were conducted to evaluate the effect of the different intervals between treatment and inoculation. The plants were treated with chitosans at 1 mg ml⁻¹ and inoculated after 24, 48, and 72 h. In another experiment, tomato plants were treated with Clmw (3 mg ml⁻¹) or ASM (50 mg l⁻¹) and inoculated after an interval of 3 and 6 days. The procedures were identical to that used for the test previously described.

The experiments were set to be completely randomized, with four or five replicates per treatment, depending on the experiment, where one plot was represented by a pot with two plants. An estimate of the percentage of infected leaf tissue was made by visual analysis with the aid of a diagrammatic scale (Mello et al., 1997) 15 days post inoculation. Three leaves from the intermediate portion of each plant were evaluated, for a total of six leaves per plot.

Antimicrobial activity of chitosan against X. gardneri. Chitosans (Ccom, Clmw, and Cmmw), prepared as previously described, and the nutrient agar medium (NA, 20 g l⁻¹) were autoclaved separately at 120°C for 20 min. Each chitosan was added to NA at final concentrations of 0, 0.25, 1, and 4 mg ml⁻¹, and the mixture was distributed onto Petri plates. HCl 0.05 N (pH 5.6) incorporated in the medium was used as control. Aliquots of 200 µl of a bacterial suspension (adjusted to 0.3 OD) were spread on the plate surface using a Drigalsky stick, and the plates were incubated for 48 h at 25°C. After verifying the typi-
calness of the colonies of X. gardneri, bacterial growth was evaluated by preparing a cell suspension in 80 ml distilled water and the absorbance was monitored at 600 nm. The experiments, which were performed twice, were arranged in a completely randomized design and analyzed through a factorial ANOVA 3x4, with three types of chitosan and four concentrations. Each treatment had at least four replicates, where a replicate was represented by one plate.

**Determination of peroxidase and β-1,3-glucanase activities.** Tomato plants were sprayed with Clmw at 3 mg ml⁻¹, ASM 50 mg l⁻¹, or HCl 0.05 N (pH 5.6) and inoculated with X. gardneri (0.3 OD) 3 days after treatment. Sampling was made at 0, 3, 4, and 6 days after treatment, and collected leaf tissue (5 leaflets) was immediately frozen at -20°C.

For peroxidase and β-1,3-glucanase activity, leaf samples were macerated in liquid nitrogen and homogenized for 2 min in a mortar in 2 ml of ice-cold 0.1 M sodium acetate buffer (pH 5.2) containing 1 mM ethylenediamine tetraacetic acid (EDTA), 1% polyvinylpyrrolidone (PVP), and 1 mM phenylmethanesulfonylfluoride (PMSF). The homogenate was centrifuged at 20,000 g for 30 min, and the supernatant (protein extract) was used as the source of enzymes. All steps were performed at 0-4°C. Total soluble proteins in the extracts were quantified according to Bradford (1976), with bovine serum albumin (BSA) as a standard.

Peroxidase activity was determined by direct spectrophotometry measuring the conversion of guaiacol in tetraguaiacol as described by Hammerschmidt et al. (1982) with a few modifications. The reaction consisted of adding 20 µl of protein extract to a 2.9 ml solution containing 50 mM sodium acetate buffer (pH 6.0), 20.2 mM guaiacol, and 90 mM hydrogen peroxide. The reaction was conducted at 30°C for 4 min, and the absorbance was read at 470 nm every 30 sec. Peroxidase activity was expressed as the variation of one unit of absorbance at 470 nm per milligram of soluble protein per minute (OD 470 nm mg protein⁻¹ min⁻¹).

The β-1,3-glucanase activity was determined by colorimetric quantification of reducing sugars released from laminarin (Laminaria digitata; Sigma Aldrich Chemicals, Brazil) by adding 25 µl of protein extract to 475 µl laminarin (750 µg ml⁻¹) dissolved in 0.1 M sodium acetate buffer (pH 5.2). The reaction was conducted for 1 h at 44°C. After incubation, an aliquot of 30 µl was diluted in 470 µl distilled water and subjected to the method of Lever (1972) to determine reducing sugars using p-hydroxybenzoic acid. The β-1,3-glucanase activity was expressed in µKatal per milligram of protein (µKatal mg protein⁻¹), and 1 Katal was defined as the enzymatic activity catalyzing the formation of 1 mole of equivalent-glucose/second.

For biochemical determination, experiments were arranged in completely randomized design with three replicates per treatment. One replicate was represented by one plant, and five leaflets (ca. 500 mg fresh weight) were collected from each plant at random for the analyses.

**Statistical analysis.** Analysis of variance (one-way or factorial ANOVA) and Tukey’s test (multiple comparisons) were used to evaluate the difference between the means of variables analyzed. Data without homogeneity of variances were analyzed using the Kruskal-Wallis non-parametric test, and all means were compared in an independent way through Mann-Whitney test (P<0.05).

Analyses were conducted using the statistical software Statistica 8.0 (Statsoft, 2007) and Sisvar (Ferreira, 2003).

**RESULTS AND DISCUSSION**

Experimental evidence has shown the ability of chitosan to modify the plant defense metabolism in many crop species and inhibit the growth of pathogens. This dual effect may lead the polysaccharide to control plant diseases when applied preventively. In this study, chitosans of low molecular weight, medium molecular weight, and commercial controlled tomato bacterial spot at a similar level, reducing disease severity by 70% (Fig. 1). Chitosans (1 mg ml⁻¹) were sprayed onto plants 72 h before inoculation with X. gardneri, and their effects were comparable to that afforded by the commercial resistance inducer, ASM, used as a control.

Plant response to elicitors depends on the pathogen, the elicitor concentration, and the interval between inoculations. The interval between inoculations and treatments was at least 0.05. Error bars indicate standard deviation.

**Fig. 1.** Effect of commercial (Ccom), low molecular weight (Clmw) and medium molecular weight (Cmmw) chitosan at 1 mg ml⁻¹ on the severity of bacterial spot (Xanthomonas gardneri). HCl 0.05 N (pH 5.6) represents the negative control and acibenzolar-S-methyl (ASM) at 50 mg l⁻¹ the positive control. Treatments were applied 3 days before inoculation and the evaluation made 15 days after inoculation. Means with the same letters are not significantly different by Tukey’s test (P ≥ 0.05). Error bars indicate standard deviation.
treatment and inoculation. Resistance expression can occur several hours to weeks after the application of an inducer (Hammerschmidt and Kuc, 1982). In our experiments, treatment of tomato plants with chitosans 72, 48, or 24 h before inoculation reduced disease severity by 58%, 72%, and 67%, respectively (Fig. 2), showing that the polysaccharide exhibited the same level of protection against X. gardneri at short intervals. On the other hand, with interval of 6 days, there was no reduction in the disease severity by Clmw when compared to the control. The control resistance inducer ASM was effective in reducing the bacterial disease when sprayed 3 and 6 days before inoculation (Fig. 3). A larger interval (6 days) between treatments and inoculation with X. gardneri resulted in a reduction of the polysaccharide residual effect on the bacteria, increasing the chances of plant infection. Moreover, some defense mechanisms may have been activated within the first hours or days after plant treatment with chitosan, but this state of activation (or induced resistance) may not have lasted until the time of bacterial infection.

Reportedly, chitosan was effective in protecting cucumber plant against B. cinerea when applied until 24 h before inoculation, reducing the disease by 87% (Ben-Shalom et al., 2003). In bean, a disease protection against anthracnose of up to 50% provided by commercial chitosan at 2 mg ml⁻¹ was observed when intervals of 1 and 4 days between treatment and inoculation with Colletotrichum lindemuthianum were compared, and the best results came from the longer interval (Di Piero and Garda, 2008). In tobacco, foliar application of chitosan at 0.5 mg ml⁻¹, 5 days before inoculation, conferred 55% protection against Phytophthora parasitica nicotianae compared with the control (Falcón et al., 2008). In this case, the authors concluded that the protective effect was due to induced systemic resistance because the polysaccharide was sprayed on the aerial parts of the plants, and the challenge with the pathogen was via root inoculation.

Chitosan also exhibited a direct effect against the pathogen in vitro. The three chitosans at 0.25 mg ml⁻¹ did not affect the growth of X. gardneri, but a significant effect was observed from 1 mg ml⁻¹, Ccom and Clmw having the highest antibacterial activity (Fig. 4). Moreover, bacterial growth was normally observed on NA with HCl 0.05 N (OD = 1.01 ± 0.027) compared with pure NA (OD = 0.79 ± 0.241).

**Fig. 2.** Effect of commercial (Ccom), of low molecular weight (Clmw) and medium molecular weight (Cmmw) chitosan at 1 mg ml⁻¹, on the severity of bacterial spot. Chitosans were applied at different time intervals before inoculation. HCl 0.05 N (pH 5.6) represents the negative control and was applied 72 h before inoculation. Evaluation was made 15 days after inoculation. Means with the same letters are not significantly different by Tukey’s test (P ≥ 0.05). Error bars indicate standard deviation.

**Fig. 3.** Effect of different intervals between treatments [low molecular weight chitosan (Clmw) at 3 mg ml⁻¹, Acibenzolar-S-methyl (ASM) at 50 mg l⁻¹ and HCl 0.05 N (pH 5.6)] and inoculation, on the severity of bacterial spot. HCl 0.05 N, used as control, was applied 6 days before inoculation. Evaluation was made 15 days after inoculation. Means with the same letters are not significantly different by Tukey’s test (P ≥ 0.05). Error bars indicate standard deviation.

**Fig. 4.** Antimicrobial activity of commercial (Ccom), a low molecular weight (Clmw) and medium molecular weight (Cmmw) at different concentrations on the growth of Xanthomonas gardneri estimated by optical density of cell suspensions at 600 nm. Chitosan and control HCl 0.05 N were added to nutrient agar (NA) medium. Data were processed according to the Kruskal-Wallis nonparametric test and all means compared independently through the Mann-Whitney test for P < 0.05.
As mentioned, there are many reports on the antimicrobial effect of chitosan on fungal pathogens (Hadj-wiger and Beckman, 1980; Ben-Shalom et al., 2003; Chien et al., 2007; Guo et al., 2006; Hernández-Lauraz-do et al., 2008) but the effect of the polysaccharide against phytopathogenic bacteria is little known. Li et al. (2008) proved the activity of chitosan on X. arboricola pv. poinsetticola and X. axonopodis pv. poinsetticola, causal agents of leaf spot of poinsettia. For these species, maximum inhibition was observed at 0.1 mg ml\(^{-1}\). Contrary to these results, a significant bacterial inhibition was observed in the present study only by chitosan at 1 mg ml\(^{-1}\). More recently, however, Rabea et al. (2009) showed that a low molecular weight chitosan at 0.5 and 1.05 mg ml\(^{-1}\) markedly inhibited the growth of Agrobacterium tumefaciens and P. carotovorum subsp. carotovorum, respectively, which is similar to what found for X. gardneri.

Different characteristics can interfere in the biological activity of chitosan, such as its cationic nature. It has been suggested that there is an interaction between the positive charges of the polysaccharide with the negatively charged residues from macromolecules exposed on the cell surface of pathogens, resulting in alteration of membrane permeability and release of cellular content in the environment (Benhamou, 1996). The chitosans used in our study have a similar deacetylation degree, approximately 85%, which seems to have been sufficient to allow interaction between the amino groups of the polysaccharides (concentration above 1 mg ml\(^{-1}\)) with the bacterial membrane.

The molecular weight also interferes with the antimicrobial activity of chitosan. Studies on fungi have shown that chitosans with low molecular weight exhibit the highest antifungal activity (Chien et al., 2007; Liu et al., 2007; Badaway and Rabea, 2008; Hernández-Laurazo et al., 2008). In agreement with this, we have also observed that the low molecular weight chitosan had a higher effect on X. gardneri than medium molecular weight chitosan, especially when used at 1 mg ml\(^{-1}\). The most accepted hypothesis is that smaller polymers can cross the cell membrane of the microorganisms more easily and cause cell death. In the study of human pathogenic bacteria, Zheng and Zhu (2003) reported that chitosan can form a polymer membrane on the cell surface, preventing the entry of nutrients. Furthermore, low molecular weight chitosan enters the cell and, through its cationic nature, can absorb and flocculate electronegative substances, disturbing the physiological activity of the cells and causing bacterial death. Yakovlev et al. (2007) also showed that a low molecular weight chitosan is effective in inhibiting bacterial RNase.

The highest antifungal effect of low molecular weight chitosan could explain the greater level of decay control when this polysaccharide is used in postharvest. Chitosans with different molecular weights showed a potential to control gray mold (B. cinerea) in tomato. Chitosan with a lower molecular weight reduced the disease incidence by 100%, at concentrations of 0.5, 1, 2, and 4 mg ml\(^{-1}\), when fruits were inoculated and stored for 21 days at 2°C, whereas chitosan with higher molecular weight was effective when used at 2 mg ml\(^{-1}\) only (Badaway and Rabea, 2008). Similarly, coating of mandarin fruits with a low molecular weight chitosan at 2 mg ml\(^{-1}\) was more effective in controlling Penicillium digitatum and P. italicum than a high molecular weight chitosan (Chien et al., 2007).

Regarding the activation of plant defense mechanisms, different pathogenesis-related proteins can be induced by treatment with elicitors and subsequent inoculation with the pathogen. Four days after treatment (1 day post inoculation with X. gardneri), an increase of peroxidase activity in tomato plants treated with Clmw was observed as compared with plants treated with HCl (P = 0.09). At 6 days after treatment (3 days post inoculation), plants treated with chitosan exhibited enzymatic activity statistically superior to that of the control (P = 0.03). ASM had an effect similar to that of chitosan but not significant when compared with the negative control at 6 days after treatment (Fig. 5). For glucanase, there was an increase in enzymatic activity in tomato leaves 3 days after treatment, with a stabilization from the fourth day. In any case, throughout the study period, significant differences between treatments were not observed (Fig. 6).

Peroxidase is known to participate in numerous physiological processes of great importance, among which the deposition of phenolic compounds and lignin biosynthesis. The peroxidase activity is responsible for polymerization of phenylpropanoid units and, conse-

![Fig. 5. Activity of peroxidase in tomato leaves treated with low molecular weight chitosan (Clmw - ◊) at 3 mg ml\(^{-1}\), Acibenzolar-S-methyl (ASM - □) at 50 mg l\(^{-1}\), or hydrochloric acid 0.05 N (HCl - △) and inoculated with Xanthomonas gardneri (Xg) 3 days later, as indicated by the arrow. Within each day, values with the same letters are not significantly different by Tukey’s test (P ≥ 0.05). Error bars indicate standard deviation.](image-url)
cotyledons in a cultivar inoculated with avirulent races with the aim of evaluating peroxidase activity in cotton. For the authors, these observations suggested the involvement of cotton peroxidase in various functions in the defense response to the bacterium. In the present study, chitosan stimulated the increase of peroxidase activity in the presence of X. gardneri. Our results are in agreement with those of other authors that evaluated the potential of inducers to control bacterial diseases. In the interaction between eggplant and Ralstonia solanacearum, peroxidase activity increased when plants were previously treated with extracts of the Agaricus blazei mushroom, and this increase was associated with a significant reduction in the incidence of wilted leaves (Silva et al., 2008). Schneider and Ullrich (1994) reported that the protection of tobacco plants against Pseudomonas syringae pv. tabaci, induced by filtrates of a P. fluorescens strain, was associated with induction of peroxidase. However, like in this study, Schneider and Ullrich (1994) did not find a positive relationship between increased β-1,3-glucanase activity and resistance induction in cucumber leaves, when plants were previously treated with elicitors and challenged with Sphaerotheca fuliginea.

Consequently, lignin formation, which results in reinforcement of cell walls and can lead to resistance to pathogens. In addition, peroxidase participates in hypersensitive reactions in response to pathogens. Delannoy et al. (2003) conducted a time course experiment with the aim of evaluating peroxidase activity in cotton cotyledons in a cultivar inoculated with avirulent races of X. campestris pv. malvacearum. A significant increase in activity was observed from 8 h post infection in the incompatible interaction, as compared with the water-infiltrated control, with a peak activity occurring 48 h after inoculation. Transient accumulation of peroxidase was detected earlier (3 h after inoculation) by immunocytolocalization and coincided with the oxidative burst. For the authors, these observations suggested the involvement of cotton peroxidase in various functions in the defense response to the bacterium.

**Fig. 6.** Activity of glucanase in tomato leaves treated with low molecular weight chitosan (Clmw -○) at 3 mg ml⁻¹, Acibenzolar-S-methyl (ASM -□ at 50 mg l⁻¹, or hydrochloric acid 0.05 N (HCl -△) and inoculated with Xanthomonas gardneri (Xg) 3 days later, as indicated by the arrow. Error bars indicate standard deviation. There was not statistic difference between treatments, according with the F-test ($P \geq 0.05$).

In conclusion, chitosan has a definitive antibacterial effect against X. gardneri and increases the activity of peroxidase in tomato plants. These modes of action may help to reduce plant infection and colonization, resulting in lower severity of tomato bacterial spot.

**ACKNOWLEDGEMENTS**

The authors thank the Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC, Brazil) for financial support, the Sakata Seed Sudamerica for providing the bacterial strain, and Alice M. Quezado-Duval, Ph.D., for help in the identification of Xanthomonas gardneri.

**REFERENCES**


Chien P., Sheu F., Lin H., 2007. Coating citrus (Murcott tan-
gor) fruit with low molecular weight chitosan increases postharvest quality and shelf life. *Food Chemistry* **100**: 1160-1164.


Ferreira D.F., 2003. SISVAR, 4.3 (Build 45). Universidade Federal de Lavras, Minas Gerais, Brazil.


Yafei C., Yong Z., Xiaoming Z., Peng G., Hailong A., Yuguang D., Yingrong H., Hui L., Yuhong Z., 2009. Functions of oligochitosan induced protein kinase in *Tobacco mosaic virus* resistance and pathogenesis related proteins in...


Protection of tomato plants by chitosan

Received September 6, 2010
Accepted February 11, 2011