Article

Amaranth cystatin prevents and controls early blight in tomato

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Abstract

Early blight is a disease caused by *Alternaria alternata* in tomato and other vegetables. This fungus affects the leaves, stem bases and fruits, causing economic losses. Different fungicides are currently used to control fungal diseases; however, these increase production costs and pose a risk to health and the environment. Therefore, the use of biological products, including phytocystatins, represent an attractive alternative for the control of plant diseases. Phytocystatins are widely distributed proteins in plants, which inhibit the activity of cysteine-like proteases and affect the growth and development of some phytopathogenic fungi. Preliminary work showed *in vitro* tests that amaranth cystatin produced recombinantly in *Escherichia coli*, inhibited the growth and development of some phytopathogenic fungi, including *Alternaria alternata*. In the present work, the effect of foliar application of amaranth cystatin in the prevention and control of early blight in tomato plants was determined. Greenhouse tests carried out in the municipalities of Irapuato and Celaya, in the state of Guanajuato (Mexico), during 2018, show that the foliar application of amaranth cystatin (168 µg and 335 µg of cystatin/plant) prevents and controls the development of blight early in different tomato varieties in crops under commercial greenhouse production. These results show the potential of cystatin in the control of fungal diseases.

Keywords: *Alternaria* sp., *Solanum lycopersicum* L., phytocystatins.

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Introduction

Tomato (*Solanum lycopersicum* L.) is the edible fruit of an herbaceous plant in the Solanaceae family, which includes 3 000 species and 90 different genera. The tomato originated in the Andean region that currently corresponds to part of Chile, Bolivia, Ecuador, Colombia and Perú. Although the tomato was domesticated in America, it has been suggested that Mexico was the most likely domestication region, while Peru is considered the center of diversity of wild relatives (Bai and Lindhout, 2007). The tomato has a great diversity of culinary uses and is consumed worldwide. Tomato production worldwide is estimated at 177 million tons and grown on 5 million hectares.

Among the main tomato producing countries are China, India, the United States of America, Turkey and Egypt (FAOSTAT, 2016). Mexico has positioned itself as the tenth largest tomato producer in the world, contributing 2.3% to world vegetable production. Tomato is the main agricultural product that is exported in Mexico, and its main commercial destination is the United States, which acquires 90.1% of the total exported volume (SIAP, 2018). Among the exported tomato varieties, those known as heirloom or heirloom tomatoes stand out.

An important characteristic of these tomatoes is that they have not been crossed, nor hybridized, so they retain their flavor and texture, compared to hybrid tomatoes, which is why they are in demand in the export market (Jordan, 2007). Some of the most commercialized heirloom tomato varieties are: Brandywine (BW) large tomato, with dark pink skin and soft red pulp, open-pollinated, undetermined, valued for its excellent flavor and large size (Barret *et al*., 2012).

Cherokee purple (CP) Tennessee heirloom, indeterminate tomato, fruit from dark pink to purple, medium to large in size, its multilocular interior varies from purple to brown and green, rich, complex and sweet flavor (Ozores *et al*., 2012); striped german (SG) indeterminate tomato, medium to large red and yellow bicolor fruit, with variable ribs, fruity flavor and smooth texture (Ozores and McAvoy, 2014). As for the local market, the Rio Grande variety is one of the most widely used in greenhouse and open field production in important producing states nationwide, such as Sinaloa. This variety is characterized by being of indeterminate habit, high yield and saladette-type fruit (Santiago *et al*., 1998; García-Hernández *et al*., 2001).

Tomato cultivation in the state of Guanajuato plays an important role in the country's economy as it directly and indirectly generates thousands of jobs a year. However, in recent cycles, the profitability of the crop has been seriously threatened by various factors, including phytosanitary problems, which reduce yields and affect the economy of farmers. Among the most important phytosanitary problems are diseases caused by phytopathogenic fungi, such as *Alternaria*, the causative agent of early blight (CESAVEG, 2011).

The early blight in tomato causes great losses in the crop, due to the fact that it affects the leaf area of the plant and causes the death of the leaves and that no fruits are produced in the areas affected by the fungus (Wyenandt *et al*., 2006). *Alternaria* sp. has recently been identified as part of the damping-off complex or tomato seedling dryer, which generates losses of 30% to 50% of already established seedlings (Reyes, 2017).

For the control of early blight, the use of chemical pesticides is used, which not only increases the production costs of the crop, but also generates negative impacts on human health and the environment (Nesler *et al*., 2015). Given this panorama, the use of biological products represents an alternative for disease control. In this sense, naturally occurring phytocystatins (cysteine protease inhibitors) hold promise for the biocontrol of phytopathogenic fungi, as they are bioactive compounds, friendly to the environment and not posing a health risk.

In plants, cystatins are natural and specific inhibitors of the cysteine-like proteases of the papain C1A family, which generally interfere with the activity of these proteases through close and reversible interaction (Chu *et al*., 2011). To date, several functions have been proposed for cystatins in plants, such as the regulation of endogenous protein turnover during growth and development processes, as well as senescence and programmed cell death (Díaz-Mendoza *et al*., 2014). Cystatins have also been documented to participate in the accumulation and mobilization of proteins stored in seeds (Szewińska *et al*., 2016).

Another key function is protection against pests and plant diseases, since they inhibit the activity of cysteine proteases that insects and microorganisms need for their growth and proliferation (Van Wik *et al*., 2014). In the laboratory, the amaranth cystatin gene was isolated and cloned into an expression vector to produce recombinant cystatin in *Escherichia coli* (Valdes-Rodríguez *et al*., 2007). Subsequent studies have shown that amaranth cystatin (AhCPI) inhibits the growth of phytopathogenic fungi, such as *Fusarium oxysporum*, *Sclerotium cepivorum*, and *Rhizoctonia solani* (Valdes-Rodríguez *et al*., 2010). As well as mycotoxin-producing fungi such as *Aspergilllus parasiticus* (Guzmán-de-Peña *et al*., 2015).

Recently, in the laboratory of Biochemistry and Molecular Biology of Proteins of Cinvestav-Irapuato, it was demonstrated in *in vitro* tests that amaranth cystatin inhibits the growth of *Alternaria* sp., causal agent of early blight in tomato (Valdes-Rodríguez *et al*., 2018). In the present work it was proposed to evaluate the effect of amaranth cystatin in the prevention and control of early blight in greenhouse trials with different tomato varieties.

Materials and methods

Preparation of *Alternaria alternata* spore suspension

In previous works, *Alternaria* sp., causal agent of early blight in tomato plants, was isolated and identified (Valdes-Rodriguez *et al*., 2018); however, it was recently identified as *A. alternata*. The purified isolate was grown for 10 days on plates with PDA medium (potato dextrose agar 3.9% pH 5.6) at 28 °C. From these cultures the spores were collected by shaking with 10 mL of 0.01% triton and counted by observations under the light microscope (Leica Microsystems, Germany) with the 10x objective in a Neubauer camera. Dilutions with distilled water were prepared to obtain a concentration of 1×10^5 spores mL^{-1} to evaluate the curative effect and 6×10^5 spores mL^{-1} for the preventive effect.

Recombinant cystatin preparation

Recombinant cystatin was produced with some modifications according to the previously described method (Valdes-Rodríguez *et al*., 2010). The cystatin-producing strain of *E. coli* was grown in continuous agitation at 37 °C in Super Broth medium in the presence of 100 µg mL^{-1} of carbenicillin and 25 µg mL^{-1} of kanamycin, until reaching an optical density of 0.5 to 600 nm. Cystatin expression was induced for 4 h with 0.1 mM IPTG and the bacteria recovered by centrifugation (12 000 rpm for 20 min) were resuspended in sterile deionized water.

The bacterial cells were lysed with a sonicator (Branson Sonifier 450, USA) programmed with a wave amplitude of 40%, applying 10 pulses of 30 s, with a time interval of 30 s between each pulse to avoid heating the suspension. Cell debris was removed by centrifugation at 18 000 rpm for 25 min and the obtained supernatant (cystatin lysate), the protein content was determined according to the Bradford micro-method (Bio-Rad Laboratories, USA), using bovine albumin serum as standard. As a control, an *E. coli* lysate was prepared, in which the production of cystatin was not induced, uninduced lysate (LNI). The lysates obtained were analyzed by electrophoresis on SDS polyacrylamide gels, according to the method of Laemmli (1970).

Growth conditions of tomato plants

In the present work, tomato plants of the Rio Grande variety were used, as well as the heirloom varieties: Brandywine, Cherokee Purple and Striped German, which were donated by Agro Invernaderos Gasca SPR of RL. The seed germinated in seedlings with general mix (flat earth, leaf earth, Sunshine Mix 3, vermiculite and perlite) were transplanted after 45 days into 3.5 L pots containing the same substrate. The plants were grown in the greenhouse in the autumn-winter season with an average temperature of 27 °C. Irrigation was supplied with distilled water according to the needs of the plants and fertilization was carried out every week with Ferviafol 20-30-10 (Agroquimicos Rivas SA of CV, Celaya, Guanajuato, Mexico).

Curative effect of amaranth cystatin on the development of early blight in tomato

The trial was established under a completely randomized factorial experimental design, with 25 plants (5 for each treatment) of tomato of the Río Grande variety, 114 days after sowing (dds). For infection, four incisions of the plants were made with small incisions with a scalpel and sprayed with 1 mL of a spore suspension of *Alternaria alternata* (1×10^5 spores $plant^{-1}$). The plants were covered with a polythene bag to increase the relative humidity.

After seven days of inoculation and once the symptoms of the disease appeared, batches of 5 plants were sprayed with different doses (84, 168 and 335 µg protein $plant^{-1}$) of the cystatin lysate. 21 days after the first cystatin spray, a second application was made under the same conditions. Plants sprayed with water and *E. coli* cell lysate in which cystatin production was not induced (LNI) were used as controls. Ten days after the second application, the severity of the damage caused by *Alternaria* was visually evaluated according to the scale described by Chaerani *et al*. (2007).

The severity of the damage caused by *A. alternata* was evaluated on all the leaves of all tomato plants, on a scale of 0 to 5, where 0 represented 0% infection, 1: 1-10%, 2: 11- 25%, 3: 26-50%, 4: 51-75%, and 5: 76-100% infection. Finally, the average percentage value of the damage observed in all the leaves by plants analyzed was considered using the following modified formula by Chaerani *et al*. (2007).

$$\text{Infection percentage} = \frac{\text{sum of infection values per plant}}{\text{total number of leaves sampled}} \times 100$$

Preventive effect of amaranth cystatin on *Alternaria alternata* infection

The trial was established under a completely randomized factorial experimental design as in the previous trial. In this test, tomato seedlings of 57 dds of the varieties: Brandywine, Cherokee Purple and Striped German were used, which were sprayed three times with the cystatin lysate (335 µg of cystatin plant^{-1}) with a periodicity of three and 25 days. Three days after the last application, the plants were inoculated with 1 mL of a suspension of *A. alternata* spores (6 x 10^5 spores mL^{-1}).

The plants were covered with a polyethylene bag to increase the relative humidity as in the curative test. Twelve days later, the severity of the damage was evaluated as described in the previous section. Five plants per variety were included in the trial for each of the treatments. Plants sprayed with water and with the *E. coli* lysate in which cystatin production was not induced (335 µg protein plant^{-1}) were included as controls.

Evaluation of the curative and preventive effect of cystatin in tomato growers in greenhouses

The healing effect of amaranth cystatin was also evaluated at Agro Invernaderos Gasca SPR of RL, located in Celaya, Guanajuato, which produce and export the varieties: Brandywine, Cherokee Purple and Striped German. These tomato plants had characteristic symptoms of early blight, such as leaf yellowing of annular concentric rings, in addition to necrotic areas on the edge of the leaves.

In the greenhouse, rows of plants in the reproductive stage of each of the afore mentioned varieties were selected, distributed randomly. For the Brandywine variety 9 rows with 10 to 15 plants each were selected, for the Cherokee Purple variety 3 rows with 15 plants each and for the Striped German variety 2 rows with 15 and 18 plants each, which were sprayed with different dose of cystatin lysate, every month for three months. In the case of the Brandywine variety, the plants (122) were sprayed with 168 µg of cystatin plant^{-1}, while the Cherokee Purple (45 plants) and Striped German (33 plants) were applied 84 and 335 µg of cystatin plant^{-1}, respectively.

The effect of cystatin was visually evaluated 10 days after each application of cystatin and the general appearance of cystatin-treated plants and plants treated with conventional chemical control based on the use of Cupravit Hidro was compared (Bayer of México, SA of CV) at a dose of 2 kg ha^{-1}. The preventive effect of cystatin in tomato growers in greenhouses was evaluated under the same conditions as the curative effect. In this case, 60 tomato seedlings of the

Brandywine variety of 60 dds were sprayed with 335 µg of cystatin plant^{-1}, 30 days later a second application of cystatin was made (168 µg of cystatin plant^{-1}). Twenty days later, the effect of cystatin on tomato plants was visually evaluated.

Statistical analysis

The results were analyzed by means of an Anova and the statistical significance of the means was determined with the Tukey test at a significance level of $p \leq 0.05$. To carry out this analysis, the statistical system SPSS Statistics version 17.0 (IBM) was used.

Results and discussion

Preliminary work has shown *in vitro* tests that amaranth cystatin is capable of inhibiting the growth and development of *Alternaria* sp., The causative agent of early blight in the tomato producing zone in the state of Guanajuato (Valdes-Rodríguez *et al*., 2018). Based on these results, in the present work the effect of amaranth cystatin in the control and prevention of this disease was evaluated in greenhouse trials.

Recombinant cystatin

To confirm the presence of cystatin in the bacterial extracts used, an electrophoresis analysis was performed as shown in Figure 1, where the presence of a 28 kDa band corresponding to cystatin was observed, while in the cell lysate uninduced this prominent band is not perceived.

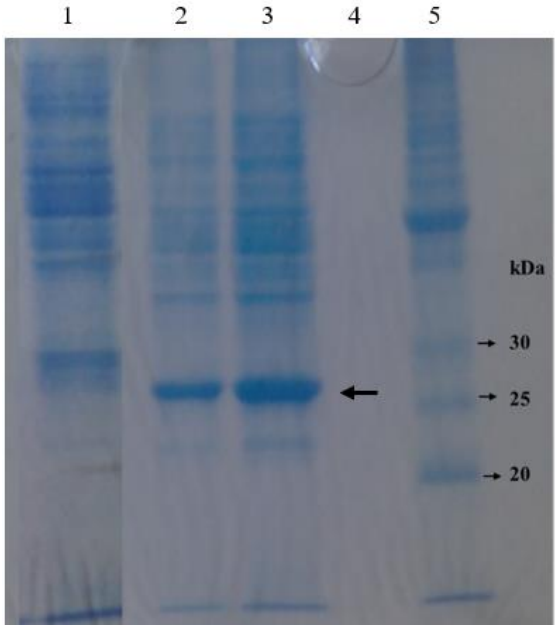

Figure 1. Electrophoretic profile of cell lysates of the *E. coli* strain, producer of cystatin. Cell lysates were prepared as described in materials and methods and analyzed on 12% SDS-polyacrylamide gels. Lane 1, cell lysate in which cystatin expression was not induced (4.5 µg protein). Cell lysate induced 3.5 µg protein (lane 2) and 8.7 µg protein (lane 3), lane 4 empty. BenchMark Protein Ladder marker (Thermo Fisher Scientific Inc., Waltham, Massachusetts) (lane 5). The arrow indicates the Cystatin band (28 kDa).

Curative effect of amaranth cystatin on the development of early blight in tomato

The results obtained indicate that cystatin controlled the development of early blight on Rio Grande variety tomato leaves. During the evaluations of the severity of the damage caused by *A. alternata*, the appearance of chlorosis and necrosis at the edge of the leaves was observed, as well as dark-colored concentric rings characteristic of the damage caused by *A. alternata*.

No significant differences in the level of damage were found between the control plants sprayed with water and those treated with the uninduced lysate, which indicates that the bacterial lysate per se does not produce any type of protection in tomato plants. On the other hand, it was found that as the cystatin concentration increased, the severity of the damage caused by *A. alternata* gradually decreased, reaching a significant reduction (95%) with the highest doses of cystatin (Figure 2).

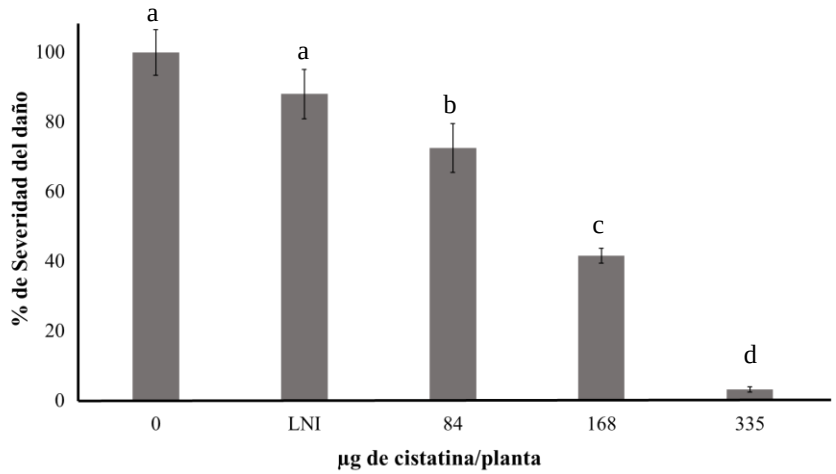

Figure 2. Severity of the damage produced by *Alternaria alternata* in tomato plants var. Río Grande treated with different concentrations of amaranth cystatin. Tomato plants infected with *A. alternata* were treated with different doses of the cystatin lysate (2 applications) and after 10 days from the last application the damage was evaluated. Plants sprayed with water (0) and *E. coli* cell lysate in which cystatin production was not induced (LNI) were used as controls. The bars above the columns indicate the standard error (n= 5). Different letters indicate significant differences between treatments. Tukey ($p \leq 0.05$).

The protective effect of cystatins against phytopathogenic fungi has been amply demonstrated with the use of transgenic plants that overexpress these genes. Munger *et al*. (2012) reported a significant decrease in the severity of damage caused by *Botrytis cinerea* in transgenic potato plants (*Solanum tuberosum*) that expressed the gene of a corn cystatin (CCII). It was recently shown that transgenic tomato plants expressing the gene of a multidomain wheat cystatin (TaMDC1) showed a significant reduction in the damage caused by *B. cinerea* and *Alternaria alternata* in separate leaf bioassays inoculated with the respective pathogens (Christova *et al*., 2018).

Thus, a differential protective effect of cystatins has also been reported when evaluated under *in vitro* and *in vivo* conditions when expressed in transgenic plants. Carrillo *et al*. (2011) reported that barley cystatin (HvCPI-6) in *in vitro* tests showed high effectiveness in inhibiting the growth of the phytopathogenic fungi *Magnaporthe grisea*, *Plectosphaerella cucumerina*

and *Fusarium oxysporum*. However, the transgenic Arabidopsis plants that expressed the gene for said cystatin (HvCPI-6) did not show differences in the damage caused by these fungi, with respect to the control plants.

As far as is known, there is only one report in which a cystatin has been directly applied to control fungal diseases. Popovic *et al*. (2012) reported that the direct application of kiwi cystatin (1.1 µg wound^{-1}) in apple and carrot fruits, prevented the infection and appearance of symptoms produced by *Botrytis cinerea* and *Alternaria radicina*, respectively. The results obtained in the present work indicate that the application of amaranth cystatin prevents and controls the development of early blight in tomato plants.

Tomato plants sprayed with the highest concentration of amaranth cystatin (335 µg plant^{-1}) reduced the severity of damage caused by *Alternaria alternata* by 95% (Figure 2). Our results appear to be similar to those reported in cystatin gene overexpressing transgenic plants. Munger *et al*. (2012) observed in potato plants transformed with the corn cystatin gene (CCII) a reduction of 90% in the severity of damage caused by *Botrytis*. *cinerea*, compared to the wild line used as a control.

Preventive effect of amaranth cystatin on *Alternaria alternata* infection

In this test healthy tomato plants of the Brandywine, Cherokee Purple and Striped German varieties previously sprayed with cystatin (335 µg plant^{-1}) were infected with *A. alternata*. After 12 days it was found that the control plants sprayed with water and that were subsequently infected with *A. alternata* showed different susceptibility to infection. The Cherokee Purple and Striped German varieties were more tolerant to *A. alternata* infection and showed levels of damage severity of 0.02% and 0.1%, respectively, while the Brandywine variety was more susceptible with values of 0.53% (Figure 3).

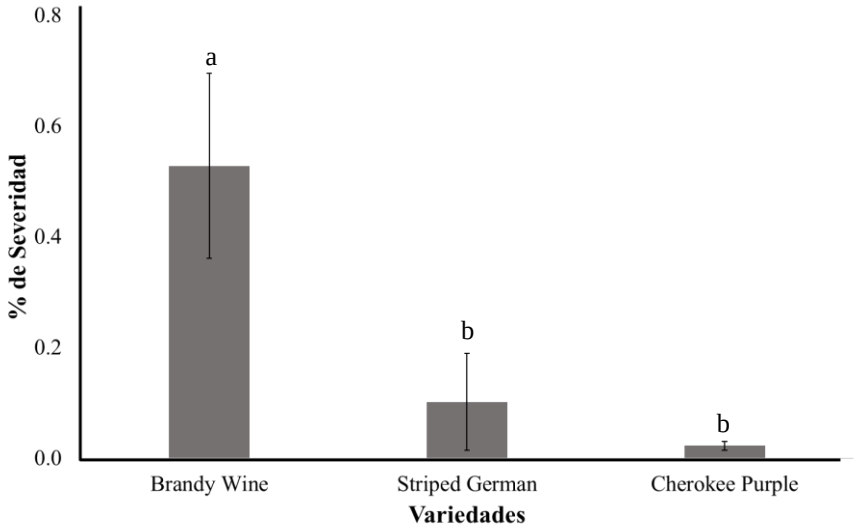

Figure 3. Severity of the damage produced by *Alternaria alternata* in tomato plants var. Brandywine, Cherokee Purple and Striped German. Damage was evaluated 12 days after infection in tomato plants sprayed with water and subsequently inoculated with *A. alternata*. The bars above the columns indicate the standard error (n= 9). Different letters indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

These results coincide with that reported by Smith and Kotcon (2002), who when evaluating resistance to early blight in different tomato varieties, both heirloom and commercial hybrids, found that the Brandywine variety turned out to be one of the most susceptible to infection by *A. alternata*.

Despite the low incidence of early blight, in tomato plants var. Brandywine infected with *A. alternate*., it was observed that the application of cystatin prevented the appearance of symptoms in these plants, compared to the controls used in the trial (Figure 4). Damage severity was similar between plants previously sprayed with water and uninduced cell lysate, while the application of cystatin significantly reduced the appearance of disease symptoms by 96%. These results indicate that cystatin prevented the development of early blight in tomato plants of the Brandywine variety.

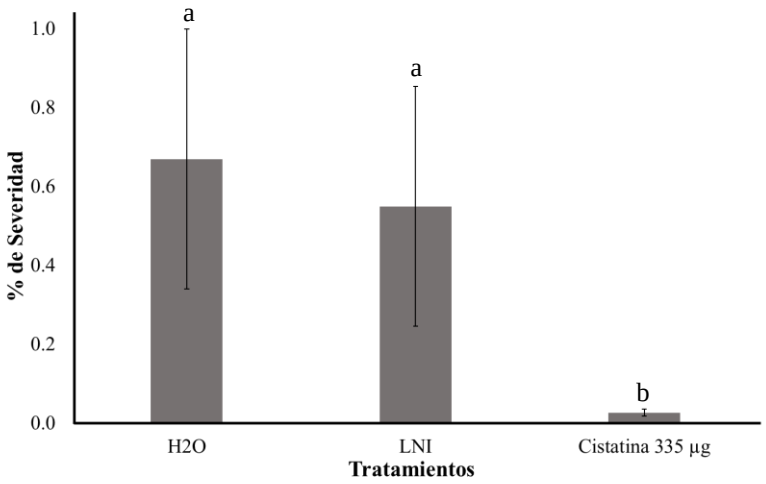

Figure 4. Preventive effect of cystine on the development of early blight in tomato plants var. Brandywine. Tomato plants previously treated with cystatin were infected with *A. alternata* and after 12 days the damage was evaluated. Plants sprayed with water (H_2O) and *E. coli* cell lysate in which cystatin production was not induced (LNI) were used as controls. The bars above the columns indicate the standard error (n= 5). Different letters indicate significant differences between treatments according to the Tukey test ($p \leq 0.05$).

Curative and preventive effect of cystatin in greenhouses of tomato growers

These trials were carried out in Agro Invernaderos Gasca SPR of RL tomato producers of the varieties Brandywine, Cherokee Purple and Striped German. The results obtained suggest that the application of cystatin in tomato plants in greenhouses in production also prevents and controls the development of early blight. Firstly, the curative effect of cystatin was evaluated in tomato plants of the mentioned varieties that showed early blight symptoms and that was confirmed to be caused by *A. alternata*.

After three applications of cystatin at different doses, the evolution of the damage was evaluated and compared with diseased plants that had been treated with a conventional method based on the use of copper salts. As shown in (Figures 5 to 7), a greater number of necrotic leaves and chlorotic spots were observed in plants treated with the conventional chemical control compared to those treated with different doses of cystatin.

In contrast, the cystatin-treated plants showed new shoots without symptoms of the disease (green, without chlorotic or necrotic spots). Brandywine plants more susceptible to early blight showed a better appearance with the application of cystatin than with the conventional control method, despite the fact that intermediate doses of cystatin were applied (Figure 5).

Figure 5. Tomato plant variety Brandywine treated with a) conventional chemical control; and b) with cystatin (168 µg plant^{-1}).

In the Cherokee Purple variety, the cystatin-treated plants showed fewer necrotic leaves compared to the plants treated with the conventional chemical control, despite the fact that in these plants the cystatin concentration was lower compared to the plants of the Brandywine variety (Figure 6).

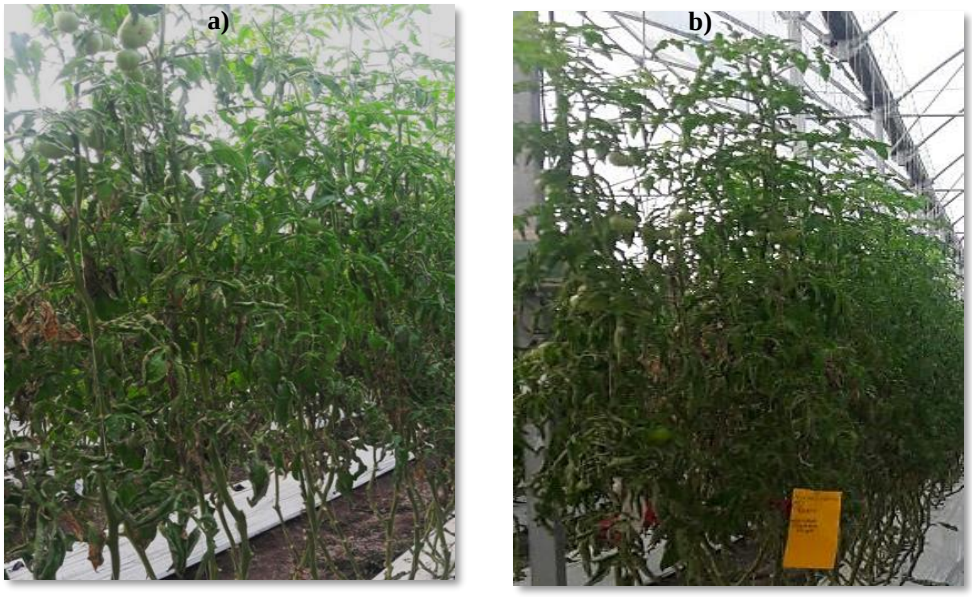

Figure 6. Cherokee Purple variety tomato plant treated with a) conventional chemical control; and b) with cystatin (84 µg plant^{-1}).

Regarding the Striped German variety, the plants treated with cystatin presented a greater number of healthy leaves (without necrotic or chlorotic areas) compared to plants treated with conventional chemical control (Figure 7).

Figure 7. Striped German variety tomato plant treated with a) conventional chemical control; and b) with cystatin (335 µg plant^{-1}).

Regarding the cystatin preventive effect test, it was observed that two applications of cystatin to healthy plants of the Brandywine variety were sufficient to prevent the appearance of early blight that affected the rest of the plants in the greenhouse. In the cystatin-treated plants, the leaves did not show necrotic borders compared to the plants treated with the conventional chemical control (Figure 8).

Figure 8. Tomato plant variety Brandywine treated with a) conventional chemical control; and b) with amaranth cystatin (335 µg plant^{-1}).

The results obtained raise the possibility of using amaranth cystatin in the prevention and control of early blight in tomato. So far there are no reports of the use of phytocystatins directly to prevent or control diseases caused by phytopathogenic fungi. Although it is still necessary to make a larger-scale analysis of the effect of cystatin in tomato, as well as exploring the possibility of using it to control other fungal diseases that affect other crops of agronomic importance, the results obtained suggest its potential use in the control of diseases, with the advantage that cystatin as a biological product degrades, does not contaminate, or represents any potential health risk.

Conclusions

The results of this work demonstrated that amaranth cystatin can prevent and control early blight development in tomato plants infected with *A. alternata*. Although the dose to use will depend on the susceptibility of the variety to the attack of the pathogen. These results are very promising since they demonstrate the biotechnological potential of amaranth cystatin, which can be used for the biocontrol of phytopathogenic fungi that affect crops of economic importance.

Acknowledgments

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