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COMPARATIVE STRUCTURAL AND BIOCHEMICAL STUDY ON CALCIUM EFFECTS ON CERCOSPORA LEAF SPOT DISEASE OF SUGAR BEET

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ABSTRACT

This study was designed to investigate the effect of calcium chloride and calcium chelate on the interaction between sugarbeet plants and Cercospora leaf spot (CLS) disease as well as its direct effect on the pathogen using scanning electron microscope. It was clearly observed that, both calcium salts significantly reduced CLS disease severity with calcium chelate being more effective in CLS disease control. The levels of chitinase, β1,3 glucanase and Polyphenol oxidase (PPO) of the salt-treated plants were significantly increased as compared to the untreated control. Thus, both calcium chelate and chloride have a role in triggering the induction of plant resistant. The increase of chitinase, β1,3 glucanase and PPO was correlated with the reduction in disease severity**.** Scanning electron microscopy showed that, calcium treatments decreased the density of emerging conidiospores compared to those of untreated infected control. The stomatal guard cells of the calcium-treated leaves appeared closure than in the untreated control. This study concludes that, the two tested calcium salts have a role in inducing disease resistance as well as stomatal functioning. They may also have a fungistatic effect on the pathogen.

Keywords: *Cercospora beticola,* chitinase , β1,3 glucanase, Polyphenol oxidase, induce resistance, scanning electron microscope.

INTRODUCTION

Cercospora beticola, causal agent of cercospora leaf spot (CLS) on *Beta vulgaris* L. (sugar beet), is a serious problem wherever this crop is grown. It is one of the most common and destructive sugar beet diseases, affecting more than a third of all fields worldwide (Shane and Teng 1992 and Holtschulte, 2000). *C. beticola* is highly successful, not only because of their widespread distribution, high dispersal and sporulation, but because commercial cultivars are often highly or moderately susceptible to these diseases (Daub and Ehrenshaft 2000). Considering the recent concern regarding pesticide residues and its hazard effect on the environment, there is a need for alternative CLS management practices that will reduce risk to environment, human and livestock. Increasing the calcium content in fruits and vegetables with calcium salts has increased storage life, mainly as a result of the role of calcium in changing physiological and reducing pathological disorders (Conway *et al.* 1992). We previously reported that calcium compounds could reduce the severity of CLS of sugar beet (Esh, 2005).

The mechanisms of calcium salts involved in disease reduction of CLS of sugar beet in the laboratory or growth-chamber experiments are due to

multiple effects of direct suppression on spore germination and fungal growth in combination with increased calcium uptake by plants. High levels of calcium solutions significantly inhibited mycelial growth sporiolation and cercosporin production (Esh, 2005 and Esh and Elkholi, 2006). On the other hand, the regulatory activity of Ca^{2+} might have importance in the mechanism of elicitor action. Elicitors such as chitinase activate ß-1,3-glucan syntheses resulting in the leaves which is important in resistance of plants to fungal attack. This process appears to be dependent on an influx of Ca^{2+} into the cell. . The performance of plant cell membranes under the stress of pathogens attack might depend on the Ca^{2+} status of the membranes and the availability of Ca^{2+} inside and outside the cell (Gurr *et al.* 1992 and Xing *et al.* 1996).

This study was designed to investigate the effect of calcium compounds on the host pathogen (*C. beticola*) interaction through the Pathogenesis related proteins (PR-Proteins) as well as its direct effect on the pathogen using scanning electron microscope.

MATERIALS AND METHODS

Effect of Calcium chelate and calcium chloride on CLS severity under field conditions

Source of Cercospora isolate

A virulent isolate of *C. beticola* (isolated from Kafr El-Sheikh) was obtained from the Cercospora collection in the of Sugar Crops Pest and Diseases Research Dept., Sugar Crops Research Institute, ARC, Giza.

Spore production and infection of sugar beet plants

Cercospora beticola inoculum was prepared according the method described by Vereijssen *et al.* (2003) and modified by Esh (2005). In briefly, colonies of *C. beticola* (14-days old culture) were flooded with 10 ml sterile distilled water and rubbed with a glass rod. Half milliliter of this suspension was added on the surface of sugar beet leaf agar (SBLA) then incubated at 26°C under a 16-hr photoperiod for 7 days. Conidia were then harvested in 20 ml sterile distilled water amended with 0.3% Tween-80 by gently rubbing the plate surface with a glass rod. The spore suspension was diluted and adjusted to 2×10^5 / ml using a hemacytometer.

Calcium treatments

Sugar beet variety Raspoly (recorded as a susceptible variety, EL-Kholi 1995) was used in this experiment during the season 2007-2008. A field experiment was carried out in an experimental field located at Sakha Research Station, Kafr El-Sheikh Governorate. Plot area was 21 m^2 with five rows each. The experimental design used was complete randomize design with three replicates (plots) for each treatment. The results only recorded for the inner three rows of each plot. Before the inoculation with *Cercospora* sugar beet plants were sprayed twice 12 and 14 weeks after cultivation with the two tested calcium compounds Ca-chelate (Calcium EDTA) and Ca-chloride at the concentration 12000 ppm (12 g/l). Tween-80 was used as a surfactant in all the treatments as 0.5 ml/liter according to Esh (2005).

Inoculation:

Sugar beet plants were inoculated 2 weeks after the last calcium treatment. conidial suspensions was atomized onto sugar beet leaves from all directions until runoff. After inoculation, plants were irrigated and left for the natural environmental conditions. The results of Cercospora severity (DS_{AGR}, Agronomica diagram) was calculated according to Battilani key (0-5 scale) of severity (Battilani *et al.* 1990).

Determination of PR proteins:

Samples were extracted according to the method described by Malik and Singh, (1990). The enzyme extract was prepared by grounding 5 g leaves in 0.1 M sodium phosphate buffer pH 7 (2 ml / g fresh weight), then centrifuged at 6000 rpm for 30 min at 4 ^{o}C. The clear extract was collected, completed to 15 ml volume using phosphate buffer and used as crude enzyme source.

Determination of chitinase:

A mixture of crude enzyme source (1 ml) and suspension of colloidal chitin (1 ml, 0.1% in 50 mM sodium acetate buffer, pH 5) was incubated at 38°C in a water bath with constant shaking. After 1 hr, the release of *N*-acetylglucosamine in the reaction mixture was estimated by the method of Reissig *et al.* (1955). The enzyme activity was determined using *N*-acetylglucosamine (Sigma) as the standard. Absorbance at 660 nm was measured using a Milton Roy Spectronic 1201. One unit of chitinase is defined as the amount of enzyme producing 1 µmol *N*-acetylglucosamine/min in 1 ml of reaction mixture under standard assay conditions. Specific activity was expressed as µg of glucose released / ml /min.

Determination of β-1,3 glucanase:

The reaction mixture was the substrate laminarin (Sigma-Aldrich) (2.5% w/v) in 10 mM ammonium acetate, pH 6.0, and 1 mM DTT. The reaction incubated at room temperature for 1h. Samples were assayed for the release of reducing sugars according to the Somogyi-Nelson method Nelson (1944) modified by (Naguib 1964 and 1965). Absorbance was measured at 660 nm using a Milton Roy Spectronic 1201. Standard curve of glucose was used as reference (Lim *et al.* 1991). β 1,3 glucanase activity was determined as µg of glucose released / ml /min.

Determination of Polyphenol oxidase (PPO):

The reaction mixture contained 0.2 ml of crude enzyme source, 1 ml of phosphate buffer pH 7, 1 ml of 10^{-3} M catechol and complete with distilled water up to 6 ml .The reaction was incubated for 30 min at 30 ^{o}C. One unite of polyphenol oxidase was expressed as the change in absorbance at 420 nm / 30 min / 1 g fresh weight (Matta and Dimond, 1963).

Scanning Electron microscope studies

Leaf samples were taken from treated and non-treated infected and uninfected plants at 7 and 16 days after inoculation. Specimens were cut, fixed in 2.5 % glutaraldehyde for 24 h at 4^{o}C, then post fixed in 1 % osmium tetroxide for 1h at room temperature (Harly and Fergusen, 1990). The specimens were then dehydrated with ascending concentrations of acetone, critical point dried, and finally sputter coated with gold. The examination,

measurements and photographing were done through a Jeol Scanning Electron Microscope (JSM- T 330A) equipped with image recording and processing system (SemAfore) at Faculty of Agriculture, Ain Shams University, Egypt.

RESULTS AND DISCUSSION

Effect of Calcium on disease severity.

Data in Table 1 show that CLS severity on sugar beet plants treated with the two tested calcium compounds were significantly decreased as compared to those untreated plants. Calcium compounds were sprayed as 12000 ppm based on former studies on the effect of several calcium compounds on CLS (Esh, 2005).

Table(1): Effect of spraying sugar beet plants with 12000 ppm of calcium compounds twice before infection on CLS incidence, under field conditions.

Calcium compounds		2 weeks after infection		4 weeks after infection	
		Disease severity	% of reduction	Disease severity	% of reduction
Calcium chelate	*infected*	1.06 c	52.25	2.33 c	50.00
	uninfected	0.20 d	-	0.33 d	-
Calcium chloride	*infected*	1.46 b	34.23	2.64 b	43.34
	uninfected	0.00 d	-	0.34 d	-
Control	*infected*	2.22 a	-	4.66 a	-
	uninfected	0.33 d	-	0.49 d	-
LSD at 0.05		0.35	-	0.28	-

Disease severity recorded 2 weeks after infection showed a significant decrease in diseases severity of treated plants (34.23% calcium chloride and 52.25% calcium chelate) as compared to those of untreated infected plants. The percentage of infection showed a significant decrease in plants treated with calcium chelate as compared to that control plants.

However, disease severity recorded 4 weeks after infection, showed a significant difference between calcium chelate and calcium chloride. Calcium chelate significantly decreased disease severity as compared to calcium chloride treated and untreated infected plants. Calcium chloride also showed a significant decrease of disease severity compared to untreated infected control. The reduction of disease severity resulted from calcium chelate treatment was 50% while in case of calcium chloride it was 43.34%.

The increase in calcium availability may enhance the resistance of cell wall to pathogen penetration. Tobias *et al.* (1993) reported that at least 60% of the calcium present in the cells is located in the cell wall. According to Tobias *et al.* (1993) most of the calcium, infiltrated by pressure into apple fruits, accumulated in the region that corresponds to the middle lamella. Here, calcium forms ionic bonds inside and between pectin polymers and other components of the middle lamella forming a structure named ‘‘egg box’’. They

explained the resistance of the cell wall by reducing the capacity of some fungal enzymes to degrade cell wall components. On the other hand, the *in vitro* studies showed that, some calcium compound found to have suppressive effect on the cercospora growth, spore germination and cercosporin production (Chung 2003 and Esh and Elkholi 2006).

Determination of PR proteins:

Data in table 2 show that the levels of chitinase , β1,3 glucanase and PPO in the treated plants significantly increased compared to the untreated control. It is clear that calcium chelate and chloride has a role in triggering the induction of plant resistant. The level of chitinase was increased about two folds in the uninfected calcium treated plants as compared to those of untreated control. As for treated infected plants, the enzyme level was increased two folds than those of treated uninfected plants. In case of β1,3 glucanas it is noticed that it acted the same way as in case of chitinase. It is worthy to mention that the increase in chitinase , β1,3 glucanase correlated with the decrease in disease severity.

Foliar application of calcium chloride has been also reported to delay ripping and control mould disease in many fruits and vegetables (Conway *et al.* 1994 and Cheour *et al.* 1991). Calcium chloride and calcium chelate treatments, in present study, could reduce CLS disease severity and increase the activity of chitinase and ß-1,3-glucanase enzymes. In this respect, Conway *et al.* (1994) reported that calcium enhanced tissue resistance against fungal infection by stabilizing or strengthening cell wall, thereby making them more resistant to harmful enzymes produced by fungi and that it also delays aging of fruits. Calcium ions can form salt-bridge cross-links, it makes the cell wall less accessible to the enzymes and control ripening softening as well as plant diseases (Sams *et al.* 1993). Several studies have demonstrated that over expression of chitinases and ß-1,3-glucanase in plants is associated with enhanced resistance to various fungal pathogens (Chen *et al.* 1999 and Datta *et al.* 2001 and El-Gamal *et al.* 2007).

Table 2 shows that, a significant increase in PPO activity after spraying with the tested calcium salts compared to the untreated control. The increase of the PPO level correlated with the decrease in CLS severity. Wei *et al.* (2002) stated that Ca^{2+} increased the activity of PPO and Peroxidase (0.022%-0.0342% respectively) and enhanced the carnation's resistance to leaf spot disease. This activation of PPO by Ca^{2+} has been described elsewhere, and it has been reported that Ca^{2+} acts on PPO, which normally is found in its latent form, modifying the conformational state of this enzyme and thus boosting its activity (Soderhall, 1995 and Ruiz *et al.* 2003).

Table(2): Effect of spraying sugar beet plants with 12000 ppm of calcium compounds twice before infection on CLS incidence, under field conditions on chitinase, β1,3glucanase and polyphenol oxidase.

	Treatments						LSD at (0.05)
	Calcium chelate		Calcium chloride		Control		
	Inf.	*Uninf.*	*Inf.*	*Uninf.*	*Inf.*	*Uninf.*	
Disease severity [1]	1.12	0.2	1.46	0	2.22	0.33	**0.35**
Chitinase [2]	66.3	32.6	78.4	29.7	54.2	14.3	**1.73**
β1,3 glucanase [2]	75.6	26.4	75.1	18.5	40	13.2	**1.35**
Polyphenol oxidase [3]	0.69	0.36	0.87	0.31	0.62	0.16	**0.19**

1: Battilani key (0-5 scale) of severity ***Inf.*** **: infected**
2: µM of glucose released / ml /hr. ***Uninf.*****: uninfected**
3: unite / 30 min./1 g fresh weight

In addition, the regulatory activity of Ca^{2+} might have importance in the mechanism of elicitor action. Elicitors such as chitinase activate ß-1,3-glucan syntheses resulting in the leaves which is important in resistance of plants to fungal attack. This process appears to be dependent on an influx of Ca^{2+} into the cell. Phytoalexin production in carrots, potato tubers, and soybean has been shown to be dependent on the maintenance of an external Ca^{2+} supply. The performance of plant cell membranes under the stress of pathogens attack might depend on the Ca^{2+} status of the membranes and the availability of Ca^{2+} inside and outside the cell (Gurr *et al.* 1992 and Xing *et al.* 1996). Raz and Fluhr (1992) reported that calcium is necessarily involved in the ethylene-mediated pathogenesis response. Blocking calcium fluxes with chelateors inhibited ethylene-dependent induction of chitinase accumulation, but not ethylene independent induction. Artificially increasing cytosolic calcium levels by treatments with the calcium ionophore ionomycin or the calcium pump blocker thapsigargin stimulated chitinase**.**

Scanning electron microscope (SEM).

Samples of all treatments were investigated using scanning electron microscope. In general, it was obviously noticed that, calcium treatments decreased the density of the emerging conidiospores as compared to those of infected treated control ones. Both upper and lower surfaces of the infected leaves were covered by a multitude of conidiospores emerging from the stoma (Figs. 1 and 2). The SEM micrographs show the fungal mycelium on the surface of the necrotic areas and additionally, inter- and intracellularly in leaf tissues (Fig 2). Micrographs of Figure (2) revealed severe damage occurred in the mesophyll tissues of infected leaves. Calcium treatment reduced the damage effects on both palisade and spongy tissues.According to Steinkamp *et al.* (1979), penetration is followed by ramification and intercellular growth of fungal hyphae in the parenchymous tissue of the epidermis. Lartey *et al.* (2007) reported that cercospora emerged from the necrotic splits and stomatal tissue of sugar beet.

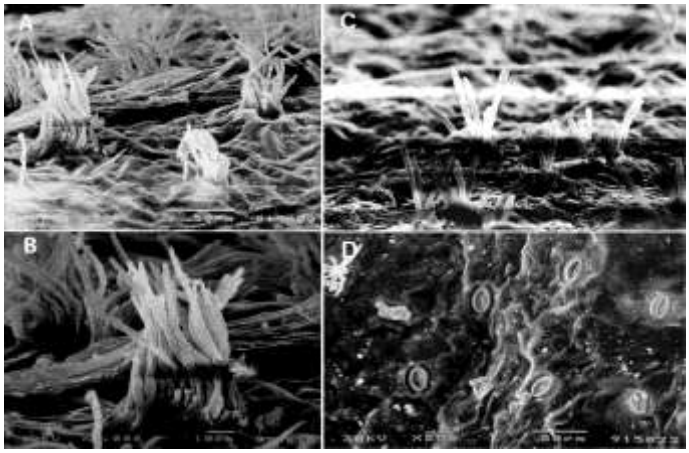

Fig.1: Scanning electron micrographs showing the surface of leaves treated with calcium chloride (D), calcium chelate(C) as compared to treated the untreated control (A).

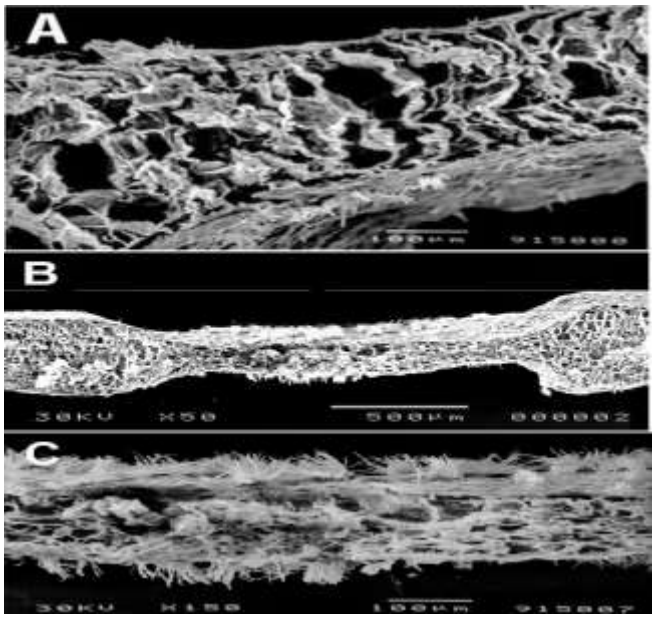

Fig. 2*:* Scanning electron micrographs of infected leaves treated with Calcium chloride before infection (A) and untreated infected control (B&C). Notice the severe reduction in the mesophyll tissues of the untreated infected samples.

Following the primary infection and establishment of infection, conidia are produced on conidiophores, mostly on the abaxial surface of the infected leaves. Subsequently, conidia spread by many factors, wind, rain… ect., to initiate secondary infection (Weiland and Koch, 2004). The conidiophores development must commence with reemergence of *C. beticola* from lesion tissue. In this work we found that the pathogen emerged from both stomatal and necrotic tissues of sugar beet leaves this in agreement with those findings by Latery *et al.* (2007) who reported that *C. beticola* ruptures the epidermis of sugar beet leaves and these points of emergence appear to be foci of later epidermal tearing.

On the other hand, in a former work we observed negative effect of different calcium salts on liner growth, sporulation an cercosporin production of *C. beticola* (Esh and ElKholi, 2006). It seems that some calcium salts has a role in inducing plant resistance and also has a fungistatic or fungicidal effect on *C. beticola*. Many reporters reported the fungistatic effect of calcium compounds on certain diseases such as (Ko and Ching, 1989, Conway *et al.* 1992, Biggs, *et al.* 1993, Beresford *et al.* 1995, Smilanick and Sorenson, 2001 and Brecht *et al.* 2003).

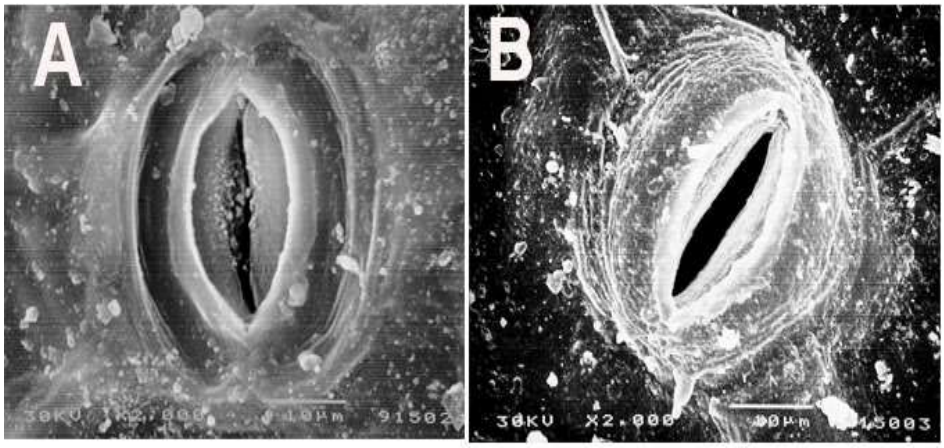

Fig 3: Scanning electron micrograph showing the stoma of sugar beet leaves treated with calcium compounds (A) as well as that of the untreated control (B).

For studying the effect of calcium treatments on the stomatal tissue and its structure we investigated treated uninfected samples. The inner side of the stomata guarding cells of the treated leaves appears to be closed than in the untreated control (fig 3). On the other hand, the stomata nucleus (opening) appeared wider than in the treated control. Many reporters considered the Calcium role in regulating guard cell turgor and the movements of stomata. Ruiz *et al.* (1993) reported that, the concentrations of calcium ions in xylem sap are often higher than 1 mol m^{-3}, which would be

sufficient to influence, or interfere with, stomatal function if such concentrations were delivered to points of evaporation in the vicinity of the guard cells. The increases in cytoplasmic calcium within the guard cells precede stomatal closure when it is induced by abscisic acid McAinsh *et al.* (1992), and an apparent requirement for calcium when some other agents such as darkness cause stomatal closure Schwartz (1985) suggests that it may be of more general significance.

The Cytosolic Ca^{+2} increases down-regulate inward-rectifying K^{+} channels and activate anion channels, providing mechanisms for Ca^{+2} dependent stomatal closure (Schroeder and Hagiwara, 1989 Blatt, 2000, Schroeder *et al.* 2001 and Klusener *et al.* 2002).

C. beticola have been reported to penetrate leaf tissues only through stomata Solel and Minz (1971) thus, necessitating epiphytic mycelia growth until stomata are encountered. Rathaiah (1976 and 1977) reported that *C. beticola* was capable of penetrating sugar beet leaves through closed stomata at night. An appressorium generally was formed over the stoma when the entry was made by way of a closed stoma, while no appressorium was needed with open stomata. He found that the frequency of penetration was similar in the resistant and susceptible cultivars, but more leaf spots were produced on the susceptible cultivars.

According to these findings, calcium treatment leads to stomata closure. penetration process of the germ tubes penetrating the closed stomata will increase the penetration time. This increase of penetration time would be enough for the plant cells to increase the accumulation of the PR proteins near stomata in defense of plant against the pathogen. This explanation is in agreement with Wubben *et al.* (1993) and supported by data presented in Table 2 that showed calcium treatment increased the accumulation of PR proteins in the treated infected and uninfected plants.

From the present work it would be concluded that, calcium treatment has a role in controlling CLS through different mechanisms: 1- the direct contact to the spores, 2-inducing PR proteins in plant leaves and 3- its effect on Stomata movement and function.

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دراسة مقارنة تركيبيه و بيوكيماويه علي تأثير معاملة بنجر السكر بأملاح الكالسيوم لمقاومة مرض التبقع السيركوسبوري

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تهدف هذه الدراسة إلى بحث تأثير كلوريد الكالسيوم والكالسيوم المخلبي على علاقة العائل بالمسبب المرضي لمرض التبقع السيركوسبوري فضلا عن تأثيرها المباشر على الممرض بإستخدام الميكروسكوب الماسح الإلكترونى. أدت جميع أملاح الكالسيوم المختبره الي خفض شدة الإصابه بالمرض. و قد أظهرت نتائج شدة الإصابه بعد 4 أسابيع من العدوي فرق معنوي بين تأثير كلوريد الكالسيوم و الكالسيوم المخلبي. زادت مستويات إنزيمات البيتا 1- 3 جلوكانيز و الكيتينيز و البوليفينول أكسيديز في النباتات المعاملة و المحقونه و كذلك المعامله و الغير محقونه بالمسبب المرضي زيادة كبيرة مقارنة مع تجربة المقارنه الغير معامله و غير المحقونه. و قد أوضحت النتائج دور كلوريد الكالسيوم و الكالسيوم المخلبي في تحفيز نظم المقاومه في النبات. و قد وجد أن الزيادة في إنزيمات البيتا 1- 3 جلوكانيز و الكيتينيز و البوليفينول أكسيديز ترتبط بإنخفاض شدة المرض. و في دراسات الميكروسكوب الماسح الإلكتروني، لوحظ إنخفاض كثافة التركيبات الفطريه للمسبب المرضي علي سطح الأوراق المعامله بالكالسيوم مقارنة بالأوراق غير المعامله. و قد لوحظ أيضا إنغلاق الخلايا الحارسة للثغور في الأوراق المعامله عنه في النباتات الغير معامله بأملاح الكالسيوم. وتقترح هذه الدراسة دور أملاح الكالسيوم في حث المقاومة في النبات فضلا عن تأثيرها علي عمل الثغور ، وكذلك تأثيرها الكابح للفطر الممرض.

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