Egypt. J. Phytopathol., Vol. **45**, No. 1, pp. 45-56 (2017)

Management of Poinsettia Leaf Blight caused by *Botrytis cinerea* using Fenton-based Solutions

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The present work was carried out to determine the effect of certain Fenton-based solutions, *i.e.* Fenton, Fenton like and Fenton oxalate complex compared to chlorothalonile fungicide on poinsettia leaf blight. *Botrytis cinerea* was isolated and identified morphologically and confirmed at the molecular base using specific primer pairs (C729+) and (C729-). DNA fragment of 700 bp was detected in the genome compared to the control. Moreover, spraying plants with the tested solutions 24hr after inoculation with the pathogen and two times later every seven days in the greenhouse decreased the disease severity. Chlorothalonile followed by Fenton oxalate complex were more effective than Fenton like and Fenton, being 14.45, 24.44, 30.0 and 38.89%, respectively compared to the control showing 54.44%. Furthermore, increasing in some plant growth parameters (Plant height "cm"), number of branches/plant, number of bracts/plant, number of leaves/plant, fresh weight of bracts (g) and fresh weight of leaves (g) and total chlorophyll, as well as oxidative reductive enzymes showed that Fenton oxalate complex was superior in these regards. In conclusion, Fenton based solutions such as Fenton oxalate complex may be used as an alternative to fungicides to manage the poinsettia leaf blight caused by *B. cinerea*.

Keywords: *Botrytis cinerea*, fenton based solutions, leaf blight, molecular identification, morphological identification and oxidative enzymes.

Poinsettia (*Euphorbia pulcherrima* Will. ex. Klotzsch) is flowering potted shrub belongs to the *Euphorbiaceae*. Its cultivation in Egypt has started since 1860 by Egyptian campaign during their return from Mexico, where the origin of these plants (Anonymous, 2011).

Despite the economic importance of poinsettia plants, there is no available information about its production in Egypt. Globally, the biggest producers of poinsettia are the USA and Europe, producing respectively 50 and 100 million plants yearly (Lütken *et al*., 2012) and is being exported between European countries in large quantities, for its cyathia and bracts (Islam and Joyce, 2015).

Poinsettias are subjected during the growing season to different diseases (Margery-Daughtrey and Chase, 2016), among which Botrytis blight caused by *Botrytis cinerea* is being the most important (Pritchard *et al*., 1996 and Margery-Daughtrey *et al*., 2000). The infected plants show tan to brown lesions on leaves,

stems, and bracts. Extensive tan cankers can form on stems when *B. cinerea* enters via blighted petioles or shoots. Lesions begin at the margins of bracts, turning darker as they expand (Margery-Daughtrey *et al*., 2000).

The morphology and the cultural characteristics of *Botrytis* species are useful for detecting certain species (Kumari *et al*., 2014) which are being time consuming and deceptive (Rigotti *et al*., 2002 and Kumari, *et al*., 2014). Consequently, Rigotti *et al*. (2002) designed a species specific primer pairs (C729+/–) sensitive to detect DNA fragment of 0.73 kb in the genome of *B. cinerea*, that allowed rapid detection of the fungus inside the plant tissues (Gindro *et al*., 2005).

Different methods can be followed to control the poinsettia leaf blight such as obtaining resistant cultivars, spraying of effective fungicides and provoking of natural plant defense metabolites (Kutek and Floryszak-Wieczorek, 2002).

The production of superoxide (O_2^-) and hydroxyl radical (•OH) (Vanacker *et al*., 2000 and Hamza *et al*., 2016a and Hamza *et al*., 2016b) is one of the earliest aspects of defence strategy against plant pathogens attack. The hydroxyl radical is the most reactive oxygen radicals (Das and Roychoudhury, 2014). It could be formed upon direct reaction of H_2O_2 and O_2^- (Haber–Weiss reaction) and through the cycle of reactions involving oxidation of transition metals, such as Fe_2^+ or Cu^+ (Fenton reaction) (Wojtaszek, 1997). Fenton reaction is considered the major source of the OH (Arakaki and Faust, 1998).

The production of OH in quantitatively high production from common Fenton based solutions needs pH 2-3 and exposure to Ultraviolet (UV) lamps. This high acidity and the UV light are harmful to plants. Thus, Fenton based solutions with higher generation rates of hydroxyl radicals under natural sunlight are desired for controlling the plant pathogens without the recognized phytotoxic effect (Wojtaszek, 1997).

Consequently, many authors studied the effect of Fenton based solutions under natural light against different plant diseases as cucumber powdery mildew caused by *Sphaerotheca fuliginea* (Sakugawa *et al*., 2012); strawberry powdery mildew caused by *Spaerotheca aphania* [Wallroth] Braun var. aphanis (Sakugawa, 2008); cucumber downy mildew caused by *Pseudoperonospora cubensis* Berk and Curtis (Hamza *et al*., 2016b) and sugar beet leaf spot caused by *Cercospora beticola* (Hamza *et al*., 2016a). They confirmed the potency of these solutions in controlling these diseases under greenhouse and field conditions without any phytotoxic effects.

In the present study, the potentiality of the Fenton based solutions, *i.e.* Fenton, Fenton-like and Fenton oxalate complex in comparison to chlorothalonil fungicide was evaluated against the poinsettia leaf blight disease caused by *B. cinerea* under greenhouse conditions. The effect of the tested materials was examined on some agronomic parameters, *i.e.* plant height, number of branches, number of bracts and leaves, fresh weight of bracts and leaves as well as chlorophyll content. Moreover, the changes in the activity of two oxidative enzymes, *i.e.* polyphenoloxidase and peroxidase due to the tested treatments were evaluated. According to the available

literature and our knowledge, poinsettia leaf blight caused by *Botrytis cinerea* was not previously recorded in Egypt.

Materials and Methods

1. The plant source:

Poinsettia plants (cv. Peterstar Red) were obtained from Mahmoud Helmey Farm at Kafr-Hakim, Imbaba, Giza, Egypt.

2. Isolation of the causal pathogen:

Poinsettia plants with symptoms of botrytis blight were collected from a nursery located at Giza governorate. The leaves were carefully washed with tap water, reasonably sliced; surface sterilized by immersing in 2% sodium hypochlorite for 2min, rinsed in sterile water and aseptically transferred onto potato dextrose agar (PDA) medium in Petri dishes, incubation was made at 20±1ºC for 7 days. The growing fungus was purified and identified according to its morphological and molecular bases.

3. Pathogenicity test:

The pathogenicity test was carried out in the greenhouse of the Plant Pathology Dept., Fac. of Agric., Cairo Univ., by spraying conidial suspension of the tested fungus (2×10^6 spores/ml) on the plant leaves. All plants were covered with plastic bags for 24hr. to keep high humidity. Plants sprayed with water were prepared as a control.

4. Identification of the causative fungus according to morphological and molecular bases

4.1. Morphological characteristics:

To study the morphological characteristics of the pathogen isolate, the fungus was grown in 9cm Petri dishes containing PDA medium and incubated at 20±1°C for 7 days. The length and width of 30 conidia were measured microscopically at × 40 magnification. Cultural characteristics such as the conidial shape and color were also examined and compared with Barnett and Hunter (1972) for species description.

4.2. Molecular test:

4.2.1 DNA extraction

B. cinerea isolate was grown in 100-ml Potato dextrose broth, at 20±1°C. for 7 days until complete growth. The fungus mycelial mat was harvested by filtration and was grounded in liquid nitrogen using a mortar and pestle. The procedure for extracting DNA was modified from the CTAB method (Rigotti *et al*., 2002). DNA pellets were dissolved in 50 µl of distilled water and stored at -20°C according to Khazaeli *et al*. (2010).

4.2.2 PCR amplification

Botrytis cinerea isolated from poinsettia was identified at the molecular level using species specific primer pairs (C729+) and (C729-) as described by Rigotti *et al*. (2002). Amplification was performed in a 25 µl of Polymerase chain reaction (PCR) consisted of 2 µl genomic DNA (50 ng), 0.5 µl of each primer "Bio-search" Technologies (10 pmol), 12.5 µl Red PCR master mix (Bio-line) and 9.5 µl ddH_2O. Primers sequences were 5'AGCTCGAGAGAGATCTCTGA-3' (C729+) and 5'-

CTGCAATGTTCTGCGTGGAA3'(C729-). The amplification was performed in a thermocycler (Techne-Progene). The PCR programmed was performed as the following steps: Initial denaturation 94°C for 4 min followed by 35 cycles of denaturation 94°C for 30s, annealing 55°C for 30s, extension 72°C for 90s and a final extension step at 72°C for 10 min. The PCR product was then kept at 4°C. PCR amplicon was separated by electrophoresis on a 1.5% agarose gel staining with Red Safe dye in 0.5x TBE buffer and visualized under UV light. A 100bp DNA marker (Bio-line) was used as a molecular size standard.

5. Disease management:

5.1. Preparation of Fenton-based solutions:

Chemicals used in the experiments including, hydrogen peroxide (H_2O_2), iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), iron (III) chloride ($FeCl_3$), anhydrous oxalic acid ($H_2C_2O_4$), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were provided by Dr. Aly Derbalah, Professor of Pesticides Chemistry and Toxicology, Faculty of Agriculture, Kafr-El-Sheikh University, Egypt.

The Fenton-based solutions were prepared according to Sakugawa *et al.* (2012) as follows:

1) Fenton reagent (50 mM H_2O_2 + 0.7 mM $FeSO_4 \cdot 7H_2O$ + 1000 ml deionised water, pH 2.5)

2) Fenton-like reagent (50 mM H_2O_2 + 0.7 mM + $FeCl_3.6H_2O$ + 1000 ml deionised water, pH 2.5)

3) Fenton oxalate complex (50 mM H_2O_2 + 0.7 mM $FeCl_3.6H_2O$ + 3 mM oxalic acid + 1000 ml deionised water, pH 4)

5.2 The effect of Fenton-based solutions in control of poinsettia leaf blight in greenhouse:

This experiment was carried out in the greenhouse of the Plant Pathology Department, Faculty of Agriculture, Cairo University, in 10th December 2016. A randomized complete block design with three plants per treatment was designed. Plants were sprayed with each of the tested Fenton-based solution in concern 24hr after inoculating the plant leaves with the pathogen and two times later every seven days. Fenton-based solutions and chlorothalonile fungicide as positive control at the rate of 1.2 g/l were applied to plants as a mist using atomizer in the morning.

5.3 Disease assessment:

Plants were examined for disease symptoms and disease severity was determined using proposed scale (1-10) suggested by Mary-Hausbeck and Harlan (2011), where 1=healthy, 2=small isolated lesions, 3=moderate-sized isolated lesions, 4=numerous moderate-sized lesions, 5=large necrotic areas, 6=large necrotic areas with 30-50% defoliation, 7=large necrotic areas with 51-70% defoliation, 8=large necrotic areas with 71-90% defoliation, 9=>91% defoliation, 10=plant death. The disease severity was recorded using the following formula:

$$\text{Disease severity (\%)} \ \frac{\Sigma \text{ nxv}}{10 \text{ N}} \times 100$$

Where: n = Number of investigated leaves in each category. v = Numerical values of each category. N = Total number of investigated leaves.

6. Determination of total chlorophyll content:

Chlorophyll content was determined using a portable chlorophyll meter (Minolta SPAD-502, Japan), as SPAD unit at the end of the experiment according to Guo (2014).

7. The effect of the tested treatments on the plant growth parameters:

Plant height, number of branches, number of the bracts and leaves as well as fresh weight of bracts and leaves were determined 60 days after spraying with the tested materials.

8. Oxidative reductive enzymes activity:

This experiment was performed to through light on the effect of the tested treatments on the activity of two different oxidative enzymes, *i.e.* polyphenoloxidase (PPO) and peroxidase (POX) in the leaves of poinsettia plants, 24h after spraying with the Fenton based solutions and the fungicide in use.

To determine the activity, 0.5g of treated leaves was homogenized in 1.5ml of 100mM phosphate buffer in a mortar by liquid nitrogen. The homogenate was filtered through four layers of cheesecloth and centrifuged at 6000 rpm for 20 min at 4^{o}C. The supernatant re-centrifuged again at 6000 rpm for 20 min at 4^{o}C prior to determine of PPO and POX activities (Kochba *et al*., 1977).

Polyphenoloxidase activity was measured by following the change of absorption at 495 nm due to enzyme activity as described by Matta and Dimond (1963). Where, 0.1 ml enzyme was added to 1.0 ml sodium phosphate buffer (0.2 M) at pH 7.0 and 1.0 ml of 10^{-3} M catechol and the final volume was brought to 6.0 ml with distilled water.

Peroxidase activity was measured by following the change of absorption at 436 nm due to guaiacol oxidation as described by Putter (1974). The reaction solution composed of 50 mM phosphate buffer (pH 7.0), 20.1 mM guaiacol, 12.3 mM H_2O_2 and 0.1 ml enzyme extract.

9. Statistical analysis:

Data were analyzed using CoStat software (version 6.4, CoHort Software, USA) according to Gomez and Gomez (1984). The differences between means were compared at 5% level using the least significant differences (L.S.D.) according to Fisher (1948).

Results

1. Morphological and Molecular identification of the pathogen:

The fungus colony was initially white and then turned grey on PDA medium. The fungus formed black and irregular sclerotia impeded into the medium. The conidiophores are dichotomously branched bear hyaline, ellipsoid to ovoid conidia (7–16 × 5–9 µm) on clusters like. Accordingly, the fungus was morphologically characterized as *B. cinerea*. The initial identification was confirmed using *B. cinerea* specific primer pairs C729+/729− and an expected DNA fragment of 700 bp was detected (Fig. 1).

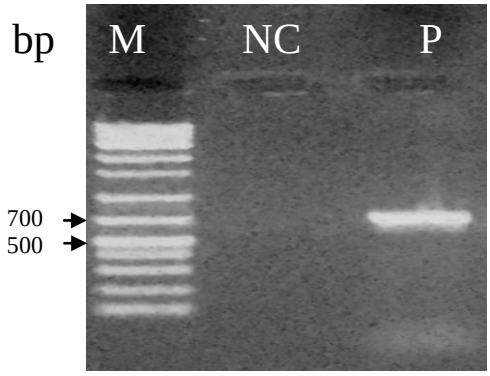

Fig. 1. Agarose gel showing a single PCR amplicon 700 bp. The gel was stained with ethidium bromide and visualized by ultraviolet radiation. Left lane (M) , marker is 1Kb DNA ladder. NC, negative control, lane (1) *B. cinerea* isolate.

2. Effect of treatments on disease severity (%)*:*

In this experiment, poinsettia plants were sprayed with Fenton preparations 24hr. before inoculation with *B. cinerea* and 7 days later. The disease severity was decreased by the treatments. The lowest disease severity values, being 14.45 and 24.44% were recorded by Chlorothalonil fungicide and Fenton oxalate complex, respectively. Meanwhile, Fenton recorded the highest disease severity, being 38.89%. The untreated inoculated control recorded 54.44% (Fig 2).

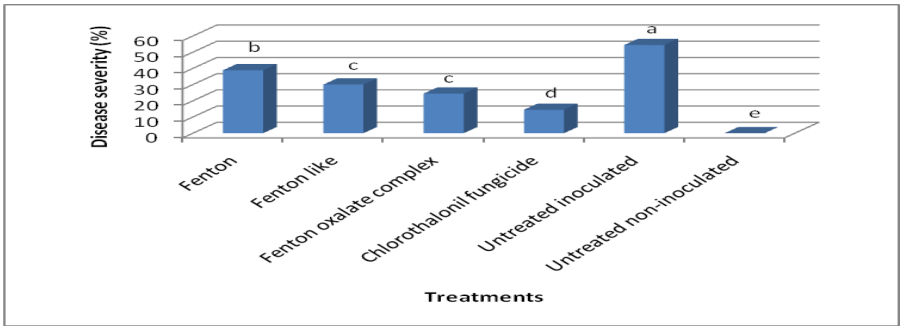

Fig. 2. Effect of the tested treatments on the occurrence of poinsettia leaf blight caused by *B. cinerea*. The bars with the same letters are not significantly different ($p<0.05$).

3. Effect of treatments on the total chlorophyll content:

Data presented in Table 1 show that the examined treatments significantly changed the total chlorophyll content in the bracts and leaves compared to the control. The highest value of chlorophyll content in bracts was 17.43 SPAD unit due to spray with Fenton. While the lowest value was 5.93 SPAD unit in the negative control (untreated and un-inoculated plants). In leaves, the highest value of total chlorophyll content was 59.23 SPAD unit in the negative control followed by 58.33, 55.03 and 54.13 SPAD units in plants sprayed with Chlorothalonil, Fenton oxalate complex and Fenton like, respectively with significant differences ($p<0.05$). The lowest values were 45.80 and 35.70 SPAD unit in the Fenton and positive control (untreated and inoculated plants), respectively with significant difference ($p<0.05$).

Table 1. Effect of the tested treatments on the chlorophyll content

Treatment	Total chlorophyll content (SPAD unit) in	
	Bracts	Leaves
Fenton	17.43	45.80
Fenton like	8.80	54.13
Fenton oxalate complex	7.47	55.03
Chlorothalonil fungicide	6.90	58.33
Untreated inoculated	11.03	35.70
Untreated non-inoculated	5.93	59.23
Mean	9.59	51.37
$L.S.D_{0.05}$	0.67	0.87

4. Effect of the tested treatments on plant growth parameters:

Data presented in Table 2 show that the tested treatments significantly affected the plant growth parameters, *i.e.* plant height (cm), number of branches, bracts and leaves as well as the fresh weight of bracts and leaves (g). Fenton oxalate complex gave the highest plant height (32.0 cm), greater number of branches (6 branch/plant) and fresh weight of bracts (28.65g). On the other hand, Chlorothalonil fungicide gave the highest number of bracts (76.0 bract/plant) and leaves fresh weight, being 13g. Despite the high efficiency of Fenton oxalate complex and Chlorothalonil fungicide in the improvement of the plant height, number of branches, as well as fresh weight of bracts and leaves, they caused the lowest number of leaves, being 4 and 3 leaf/plant, respectively. Otherwise, Fenton followed by Fenton like gave the highest number of leaves, being 6 and 6.67 leaf/plant, respectively. But they also gave the lowest plant height (29.10 and 29.0 cm, respectively), number of branches (4 and 3.67 branch/plant, respectively), number of bracts (30.0 and 45.33 bract/plant, respectively) and fresh weight of bracts (10.73 and 22.44 g, respectively).

Table 2. Effect of treatments on some plant growth parameters

Treatment	Plant height(cm)	No. of:			Fresh weight (g) of	
		branches	bracts	leaves	bracts	leaves
Fenton	29.10	3.67	30.00	6.00	10.73	12.26
Fenton like	29.00	4.00	45.33	6.67	22.44	9.88
Fenton oxalate complex	32.30	6.00	70.33	4.00	28.65	6.56
Chlorothalonil fungicide	30.00	6.00	76.00	3.00	25.02	13.00
Untreated inoculated control	28.00	3.00	22.67	16.33	16.75	7.62
Untreated non-inoculated control	29.53	6.00	83.00	16.00	33.34	9.91
$LSD_{0.05}$	1.50	1.33	5.53	2.48	3.16	1.74

5. Estimation of oxidative-reductive enzymes:

Data presented in Table 3 show the changes in the activity of polyphenoloxidase (PPO) and peroxidase (POX) due to spraying Euphorbia plants with the chemicals in concern compared with the control. Results indicated considerable increase in the activity of the two enzymes in the leaves of all sprayed plants compared with the control. Plants sprayed with Fenton oxalate complex recorded the highest activity of polyphenoloxidase (0.056 unit.min^{-1}) and peroxidase (0.0129 unit. min^{-1}) followed by those sprayed with Fenton like then Fenton, being 0.055 and 0.0127 unit. min^{-1} and 0.053 and 0.124 unit. min^{-1}, respectively. Meanwhile, the activity of the two enzymes in untreated leaves (control) recorded the lowest activity, being 0.036 and 0.116 unit.min^{-1}.

Table 3. Effect of the tested chemicals on the oxidative-reductive enzymes

Treatment	PPO (Δ OD_{495}. min^{-1})	POX (Δ OD_{436}. min^{-1})
Fenton	0.053	0.124
Fenton like	0.055	0.127
Fenton oxalate complex	0.056	0.129
Chlorothalonil fungicide	0.041	0.119
Untreated inoculated control	0.036	0.116

D i s c u s s i o n

Poinsettia leaf blight caused by *B. cinerea* significantly impairs the beauty of the plant. Thus significantly reduces the final yield (Kutek and Floryszak-Wieczorek, 2002). The management is urgently needed and the accurate identification of the pathogen is of paramount importance the first step in the disease management (Khazaeli *et al*., 2010).

In the present work, the fungus was identified morphologically as *B. cinerea* and confirmed with specific primer pairs (C729+/-), according to Rigotii *et al.* (2002). Where, DNA fragment of 700 bp was detected in 25 µl of PCR reaction mixtures consisted of 2 µl genomic DNA (50 ng) compared to the control. The result is in agreement with that obtained by Rigotti *et al*. (2002) who detected a single band of 0.7 kb in 13 strains of *B. cinerea* and 0.6 kb in *B. fabae* using C729+/- primer pairs. Moreover, Gindro *et al*. (2005) found that the DNA fragment of 0.7 Kb in the *B. cinerea* genome was amplified in reactions containing no more than 1x 10^5 to 1 single conidium, and Khazaeli *et al*. (2010) reported that the C729+/- primers successfully confirmed the morphological diagnosis, which performed on 30 *B. cinerea* isolates. A single DNA band of 0.7 kb was detected from the genome (Khazaeli *et al*., 2010).

The plants have different internal weapons to protect themselves against the plant pathogens attacking. Among these weapons are the formations of reactive oxygen radicals which obstruct the invasion of fungal and bacterial pathogens into plant cells (Mehdy, 1994 and Sakugawa *et al.*, 2012). Moreover, the action of exogenous ROS-generators may be similar to the endogenous ROS, which formed normally in plant tissues during the self defense (Sakugawa *et al*., 2012).

Consequently, spraying the leaves of poinsettia plants with Fenton based solutions, *i.e.* Fenton, Fenton like and Fenton oxalate complex reduced the severity of poinsettia leaf blight caused by *B. cinerea*. The Fenton oxalate complex was more effective than Fenton and Fenton like. These results are in agreement with those obtained by Sakugawa *et al*. (2012) who reported that the Fenton oxalate complex was more effective than Fenton and Fenton like in reducing powdery mildew of cucumber plant. Also, Hamza, *et al*. (2016a) found that the Fenton oxalate complex was superior in reducing sugar beet leaf spot disease caused by *Cercospora beticola*. The high effect of the Fenton oxalate complex may be due to its high hydroxyl radical (OH) (Sakugawa *et al*., 2012).

The mode of action of Fenton based solutions is not understood, but it may be involved in direct inhibition of the fungus by the diffusion of hydrogen peroxide (H_2O_2) through fungal cell membranes where several oxidative reactions may occur in the cell (Polo-Lopez *et al*., 2011 and Hamza, *et al*., 2016a) or indirectly by inducing the pathogenesis related proteins in the plants. Where, an increase in the activity of oxidative enzymes, *i.e*. PPO and POX was recorded in plants treated with the tested solutions in the entire work.

Moreover, the tested solutions caused positive changes in the total chlorophyll content in the leaves and bracts. Furthermore, an increase in agronomic parameters was recorded in the treated plants compared to the control. This may be due to the decrease in disease severity caused by *B. cinerea* as a result of these chemical treatments which reflected in the plant health (Hamza *et al*., 2016b).

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(Received 22/02/2017;
in revised form 23/03/2017)

مكافحة مرض لفحة أوراق بنت القنصل باستخدام محاليل الفنتون

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استهدفت الدراسة اختبار تأثير بعض محاليل الفنتون وهي Fenton, Fenton like and Fenton oxalate complex مقارنة بالمبيد الفطري كلوروثالونيل على مكافحة مرض لفحة أوراق نبات بنت القنصل المتسبب عن الفطر بوترايتس سينريا. تم عزل الفطر المسبب للمرض وتعريفه مورفولوجياً وجزيئياً باختبار عديد البلمرة المتسلسل بواسطة زوجين من البادئات المتخصصة وهما +C729 و-C729 حيث تم الحصول على جزء من الدنا بحجم ٧٠٠ قاعدة نيتروجينية. أدي رش الأوراق بالمحاليل المختبرة إلى خفض شدة الإصابة بالمرض، وكان مبيد الكلوروثالونيل متبوعاً Fenton oxalate complex أكثر كفاءة في خفض شدة الإصابة مقارنة بباقي المحاليل المختبرة. كما لوحظ زيادة في محتوى النبات من الكلوروفيل الكلي وبعض القياسات النباتية الأخرى مثل ارتفاع النبات (سم) وعدد الأفرع/نبات وعدد الأوراق الخضراء/نبات وعدد الأوراق الحمراء/نبات وكذلك الوزن الطري لكل من الأوراق الخضراء والحمراء بالجرام لكل نبات، كما حدثت زيادة في نشاط كل من انزيم البيروكسيديز والبوليفينول اوكسيديز وكان مركب Fenton oxalate complex هو الأكثر كفاءة في هذا الشأن.