Fenton reagent and titanium dioxide nanoparticles as antifungal agents to control leaf spot of sugar beet under field conditions

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Abstract: In this study, foliar sprays of Fenton solutions (Fenton reaction, Fenton-like reaction and Fenton complex), titanium dioxide (TiO_2) and the recommended fungicide (chlorothalonil) were estimated in the control of sugar beet leaf spot caused by *Cercospora beticola* under field conditions in two growing seasons. In addition, the impacts of these treatments on some crop characters (leaf dry weight, root fresh weight, soluble solid content, sucrose content and purity of sugar) were examined. Biochemical and histological changes in the livers and kidneys of treated rats compared to an untreated control were utilized to assess the toxicity of the examined curative agents. Overall, chlorothalonil and Fenton complex were the most effective treatments for disease suppression in both tested seasons followed by Fenton-like reagent, Fenton's reagent and TiO_2, respectively. Growth and yield characters of treated sugar beet significantly increased in comparison to an untreated control. There were mild or no (biochemical and histological) changes in the livers and kidneys of treated rats compared to the control. Fenton solutions and TiO_2 may offer a new alternative for leaf spot control in sugar beet.

Key words: control, Fenton, leaf spot, sugar beet, toxicity

Introduction

Sugar beet (*Beta vulgaris* L., Chenopodiaceae) is considered to be one of the main sources of sugar production and in Egypt it is the second largest source after sugar cane (Eweis *et al.* 2006). With increased production due to higher sugar demand, pathogens affecting sugar beet yield have become a major concern.

Cercospora leaf spot (CLS), caused by the fungus *Cercospora beticola* is the major foliar pathogen of sugar beet around the world (Holtschulte 2000) and its occurrence decreases gross sugar yield by 42% (Shane and Teng 1983) which prompts issues (e.g. less extractable sugar) at sugar industrial facilities and low profits for farmers. In the Netherlands, CLS has spread from the southeastern part (the territory of Limburg), where it has been infecting plants for a long time, to the whole country in just three years. The pathogen reduces root size what results in lower sucrose yields, higher content of impurities and higher losses (Lamey *et al.* 1987; Lamey *et al.* 1996). A loss in recoverable sucrose as high as 30% is normal under substantial disease conditions and income reduction as high as 43% has been recorded (Lamey *et al.* 1987 and Lamey *et al.* 1996).

Frequent application of chemical treatments leads to the development of pesticide resistant pathogen strains, causes environmental pollution and can be harmful to humans and animals because of toxicity. Numerous microorganisms considered as plant pathogens as well as insects have developed resistance against chemical pesticides (May 1985; Urech *et al.* 1997; Williams and Heymann 1998; Witte 1998). This phenomenon drastically affects crop and disease management. To minimize the risk of resistance development and reduce dependence on chemicals, new and unconventional techniques for pathogen control have been researched. The objective has been to find alternative treatments that would allow decreasing usage of pesticides.

Recent developments in nanotechnology, especially the ability to prepare highly structured nanoparticles with various sizes and shapes, have resulted in improved new biocidal agents. The first research work on the antimicrobial activity of titanium dioxide (TiO_2) was carried out with *Escherichia coli* (Matsunaga *et al.* 1988) and since that time more research has frequently been utilized against a wide spectrum of microorganisms including viruses, bacteria, fungi, algae and cancer cells (Blake *et al.* 1999; Makowski and Wardas 2001).

The release of reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) is one of the defense strategies of plants against pathogen attack (Vanacker *et al.* 2000). Hydroxyl radical can be formed through a cycle of reactions involving nitric acid (HNO_3), nitrous acid (HNO_2), and hydrogen peroxide. Organic compounds are also sources of

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hydroxyl radical, in the natural aqueous phase, and the major source of hydroxyl radical, production in the aqueous phase comes from the interaction of hydrogen peroxide, iron (Fe), and oxalic acid ($C_2H_2O_4$) via photo Fenton reactions (Arakaki and Faust 1998).

Common Fenton solutions (or photo Fenton solutions) need high acidity (low pH) to obtain high generation rates of hydroxyl radicals. On the other hand, this high acidity may induce phytotoxicity to plants via the destruction of cell walls and the release of nutrients such as calcium (Ca) and magnesium (Mg). Ultraviolet (UV) lamps are usually used to enhance Fenton solutions in order to obtain higher rates of hydroxyl radicals. However, UV light is harmful to plants. Therefore, Fenton solutions with high generation rates of hydroxyl radicals under natural sunlight (not harmful to plants) are in demand for efficient control of plant pathogens without toxic effects. The effects of Fenton solutions under natural sunlight against strawberry powdery mildew (*Sphaerotheca aphania* [Wallroth] Braun var. *aphanis*) have been examined. The results showed that these solutions protected plants against infestation with a conidial suspension and also suppressed conidial germination with 20 min of exposure of the solution to sunlight (Sakugawa 2008).

In our studies the antifungal activity of Fenton types with different hydroxyl radicals generating sources (Fenton, Fenton-like and Fenton complex) and TiO_2 nanoparticles (were assessed as foliar sprays against *C. beticola*, the causal agent of leaf spot of sugar beet and compared to the recommended fungicide (chlorothalonil) under field conditions. Furthermore, the effect of the examined applications on some growth traits and yield characters of sugar beet i.e. leaf dry weight, root fresh weight, soluble solid content, sucrose content and purity of sugar were estimated. In addition, the possible toxic effect of these products on public health was evaluated by examining biochemical and histological changes in the livers and kidneys of treated rats compared to the control.

Materials and Methods

Tested materials

Ferric chloride ($FeCl_3 \cdot 6H_2O$), ferrous sulfate (H_2SO_4), anhydrous oxalic acid and hydrogen peroxide were obtained from Nas Trading Agents Hadaek El Ebour, Salah Salem Road, Nasr City, Cairo, Egypt. Titanium dioxide nanoparticles (TiO_2 NPs) were obtained from Egypt Nanotech Company Limited, Dreamland, El-Wahaat Road, 6th October, Giza, Egypt with a purity of 99.99%. Chlorothalonil with trade of Bravo® 50% and produced by Syngenta Agro S.A.E., 6th October, Giza, Egypt was used in this study.

Treatments

The following treatments applied at 0.1 mg · l^{-1} were used in the studies: common Fenton's reagent (50 mM H_2O_2 and 0.7 mM Fe^{2+}, pH 5.5), Fenton-like reagent (50 mM H_2O_2 and 0.7 mM Fe^{3+}, pH 5.5), a new type of Fenton's reagent using a Fe^{3+}-oxalate complex (Zuo and Hoigne 1992; Faust and Zepp 1993) (50 mM H_2O_2, 0.7 mM Fe^{3+}, and 3 mM oxalic acid, pH 5.5), TiO_2 NPs (0.5 g · l^{-1}) commercial fungicide (chlorothalonil). Pure water (pH 5.5) was the control. The prepared solutions had pH 5.5 using H_2SO_4 to enhance survival of microorganisms since the microbial population would not survive under pH 5 longer than 48 h. The Fenton solutions had pH 5.5 in order to eliminate the risk of fungus death which might result from acidity of the water solutions (Rodríguez-Chueca *et al.* 2011). Moreover, mixing fungicides with too acidic or alkaline water can reduce fungicidal activity.

Fenton solutions phytotoxicity

Prior to analyzing the impact of Fenton solutions on leaf spot of sugar beet, a test assessing proper fixation levels of Fenton solutions and their effects on sugar beet plants was carried out. Infected sugar beet leaves were sprayed with various concentrations of hydrogen peroxide (10, 15, 20, 25, 50 and 100 mM), while maintaining the level of Fe^{3+} at 0.7 mM and oxalic acid at 3 mM (Sakugawa *et al.* 2012).

Application

This study was carried out at the Research Experimental Farm of Plant Pathology Research Institute, Sakha Station, Kafr-El-Sheikh, Egypt in two seasons (2013–2014/2014–2015) using a randomized complete block design with three replicates. Each replicate consisted of 6 rows; 900 cm long and 60 cm wide. Furthermore, each row contained 45 hills 20 cm apart. All culture practices were performed according to recommendations. Plants were sprayed with Fenton's reagent, Fenton-like reagent, Fenton complex, TiO_2 and chlorothalonil three times at 10 day intervals.

Disease severity and disease reduction percentages were recorded according to Shane and Teng (1992) 100 days after planting. Leaf dry weight and root fresh weight as well as total soluble solid (TSS%) were assessed in sugar beet fresh roots using a hand refractometer according to McGinnis (1982). The percentage of sucrose was determined by adding 173 ml (3%) lead acetate to 26 g of plant tissue samples collected from the root center (Helrich 1990). After filtration, sucrose was measured with a saccarometer and the purity percentage was determined as described by Carruthers and Oldfield (1961).

Toxicity assessment

Adult Wistar male rats (*Rattus norvegicus*), 8-weeks old and weighing 80–100 g were obtained from the Faculty of Medicine, Tanta University. Rats were housed in cages with free access to water and food under conditions that met all the regulated guidelines for the environment, housing, and management of laboratory animals used for research. The rats received a standard diet as described by Romestaing *et al.* (2007). Animals were allowed to adjust to their new conditions two weeks before the study. The animals were randomly separated into five groups; with three rats in each. Four groups were used for the treatment with the examined Fenton solutions and TiO_2

(30 days) and the fifth one was a control. The studied products were provided orally to rats at a dosage of 500 mg · kg^{-1} body weight. In the control treatment rats were given an equivalent volume of water.

Biochemical parameters

Collected blood samples after centrifugation at 4,500 rpm for 15 min at 4°C were used to evaluate the glutamic pyruvate transaminase (GPT) and creatinine as described by Reitman and Frankel (1957) and Barham and Tinder (1972), respectively.

Histology test

The histopathology test was performed at the Histopathology Laboratory, Department of Histopathology, Faculty of Veterinary Medicine, Kafr-El-Sheikh University according to Bancroft and Stevens (1996).

Statistical analysis

Data were analyzed statistically by the analysis of variance test (ANOVA) and the means were significantly compared by Duncan's multiple range test (Duncan 1955).

Results

Fenton solutions phytotoxicity

The results revealed that 50 mM H_2O_2 was efficient in the control of leaf spot (information not published) and did not affect the health conditions of sugar beet plants. However, lower doses of H_2O_2 did not reduce the severity of the disease, while higher levels harmed leaf tissues.

Efficacy of the tested treatments against *Cercospora beticola* under field conditions

Disease severity and efficiency of examined products against *C. beticola* under field conditions in two growing seasons (2013–2014/2014–2015) are presented in Table 1 and Figure 1. Disease severity of *C. beticola* was clearly reduced in every single treatment compared to the control in both vegetation seasons. Chlorothalonil and Fenton complex showed the highest effectiveness against leaf spot of sugar beet followed by Fenton like reagent, Fenton reagent and TiO_2, separately in both growing seasons. The performance of the applied products against *C. beticola* in the first season was higher than in the second one.

Effect of the tested treatments on sugar beet growth characters

The information in Table 2 demonstrates the impact of the examined products on leaf dry weight and root fresh weight of sugar beet under field conditions in both growing seasons. Leaf dry and root fresh weights of sugar beet were significantly increased in treated plants compared to the control in both growing seasons. Chlorothalonil and Fenton complex provided the best control against leaf spot of sugar beet followed by Fenton like reagent, Fenton reagent and TiO_2, respectively, in both vegetation seasons. The efficiency of the tested products against *C. beticola* in the second season was higher than in the first season.

Table 1. The severity of sugar beet leaf spot disease in different treatments in both growing seasons

Treatments	Disease severity	
	1st season	2nd season
$FeSO_4 + H_2O_2$	18.33±2.8 de	14.33±1.15 cd
$FeCl_3 + H_2O_2$	23.30±2.8 bc	16.0±1.73 c
$FeCl_3 + H_2O_2$ + oxalic acid	20.0±5.0 cd	10.0±0.21 de
Titanium dioxide	28.30±2.8 b	30.0±5.0 b
Chlorothalonil	8.30±2.8 f	6.0±1.0 ef
Control	65.0±5.0 a	61.0±2.8 a

Means in a column followed by the same letters are not significantly different using Duncan's multiple ranges test

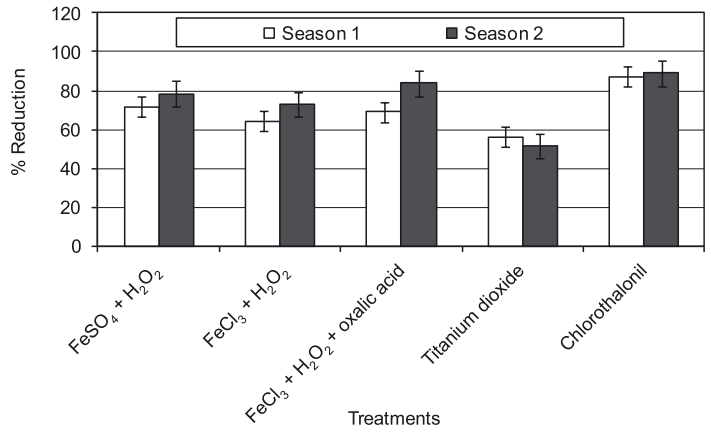

Fig. 1. Reduction of sugar beet leaf spot disease in different treatments in the two growing seasons

Table 2. Leaf dry and root fresh weight of sugar beet plants as affected by different applied treatments in both growing seasons

Treatments	Leaf dry weight [g]		Root fresh weight [kg]	
	1st season	2nd season	1st season	2nd season
$FeSO_4 + H_2O_2$	18.76±0.25 b	19.9±0.10 c	10.8±0.20 b	11.93±0.30 b
$FeCl_3 + H_2O_2$	18.26±0.25 cd	19.63±0.15 cd	10.4±0.36 bc	11.33±0.28 bc
$FeCl_3 + H_2O_2$ + oxalic acid	20.33±0.15 a	20.5±0.3 b	11.06±0.9 b	12.2±0.26 a
Titanium dioxide	18.10±0.17 cd	18.50±10 e	9.60±.28 cd	10.5±.50 d
Chlorothalonil	20.66±0.28 a	21.33±0.3 a	12.0±0.50 a	12.5±0.43 a
Control	17.63±0.15 e	18.03±0.49 ef	9.0±0.15 de	9.8±0.15 e

Means in a column followed by the same letters are not significantly different using Duncan's multiple ranges test

Impact of the tested treatments on some quality parameters of sugar beet

The information in Table 3 and Figure 2 demonstrates the relative impact of the examined products on TSS, sucrose and sugar purity under field conditions in both growing seasons. The total soluble solids and sucrose percentage as well as sugar purity were essentially increased in sugar beet plants treated with the tested products compared to the un-treated plants (control) in both growing seasons. In addition, chlorothalonil and Fenton complex provided the highest qualities among the examined products followed by Fenton-like reagent, Fenton's reagent and TiO_2 NPs, individually in both growing seasons. The efficacy of the studied products in the first growing season was higher than in the second one in regard to the discussed parameters.

Table 3. Percentages of total soluble solids (TSS) and sucrose in sugar beet root extracts from different treatments in the two growing seasons

Treatments	TSS [%]		Sucrose [%]	
	1st season	2nd season	1st season	2nd season
$FeSO_4 + H_2O_2$	20.66±0.28 b	20.2±0.26 bc	17.0±0.5 bc	17.4±0.17 c
$FeCl_3 + H_2O_2$	20.43±0.40 b	19.93±0.11 cd	16.83±0.28 cd	16.43±0.15 b
$FeCl_3 + H_2O_2$ + oxalic acid	21.66±0.15 a	20.56±0.40 ab	17.5±0.3 ab	17.86±0.57 a
Titanium dioxide	19.83±0.28 c	19.83±0.15 cd	16.36±0.11 de	16.63±0.57 b
Chlorothalonil	22.0±0.20 a	20.83±0.28 a	18.0±0.26 a	18.10±0.10 a
Control	19.66±0.28 c	18.96±0.50 de	16.13±0.11 ef	15.86±0.11 c

Means in a column followed by the same letters are not significantly different using Duncan's multiple ranges test

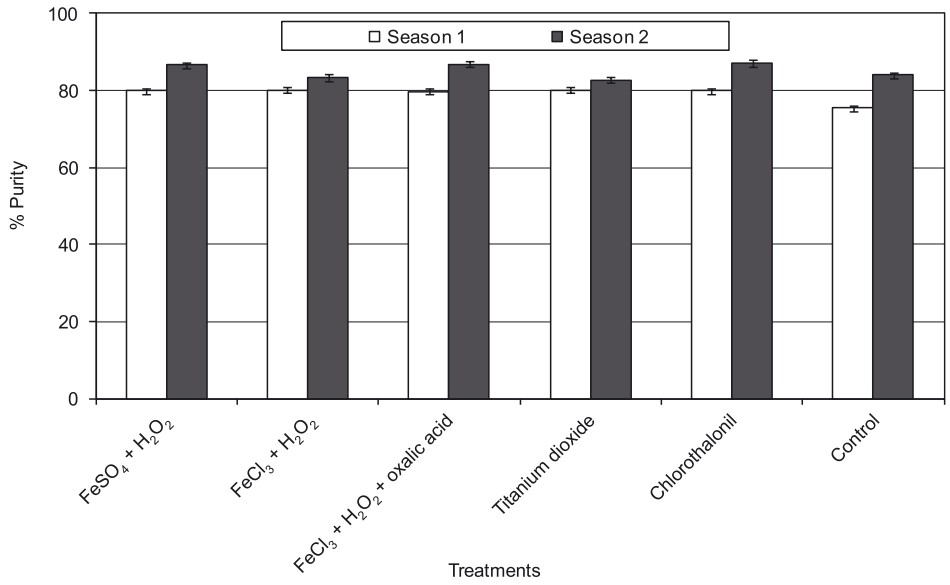

Fig. 2. Percentages of sucrose purity in sugar beet root extracts from different treatments in the two growing seasons

Toxicity evaluation

Biochemical parameters

The information in Table 4 shows that there were no considerable changes in GPT and creatinine levels in rats treated with the Fenton solutions and TiO_2 NPs compared to the untreated control. The lack of significant differences in GPT and creatinine levels between the control and treated rats suggested ordinary liver and kidney functions.

The histological variation in the liver

The ordinary structure of liver tissue is presented in Figure 3A. The tissues of the rats treated with Fenton solutions and TiO_2 NPs were normal. They looked the same as in the control with mild histopathological changes, such as a slight hydropic degeneration of hepatocytes (Figs. 3B and D) and Kupffer cells initiation (Fig. 3C).

The histological variation in the kidney

The rats treated with Fenton solutions and TiO_2 (Figs. 4B–E) showed normal kidney tissues like in the control (Fig. 4A) without any histopathological changes.

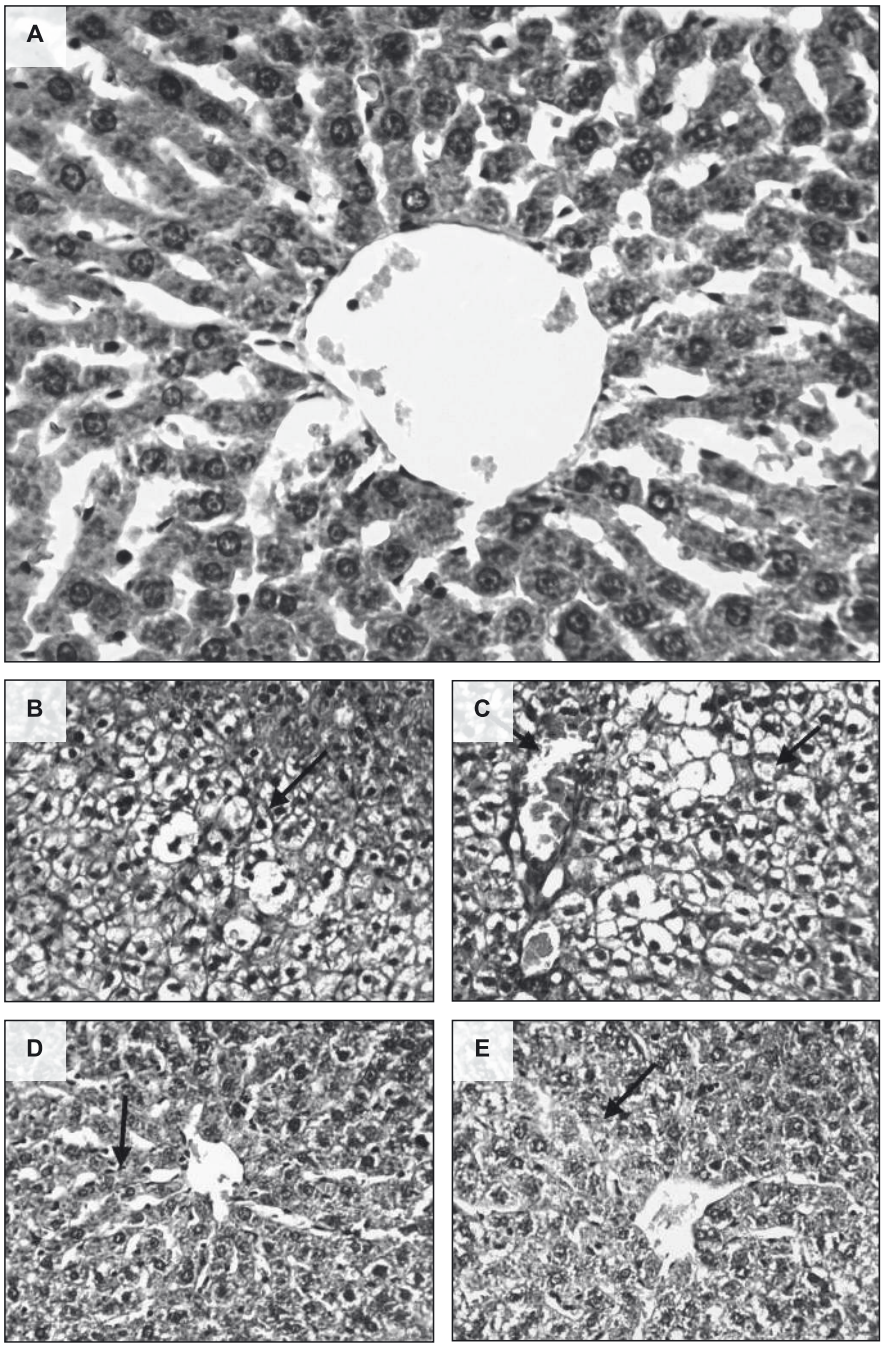

Fig. 3. Sections in the liver of rats treated with TiO_2 (B); H_2O_2/Fe^{2+} (C); H_2O_2/Fe^{3+} (D); and a new type of solution, H_2O_2/Fe^{3+}/oxalic acid (E) compared to the control (A)

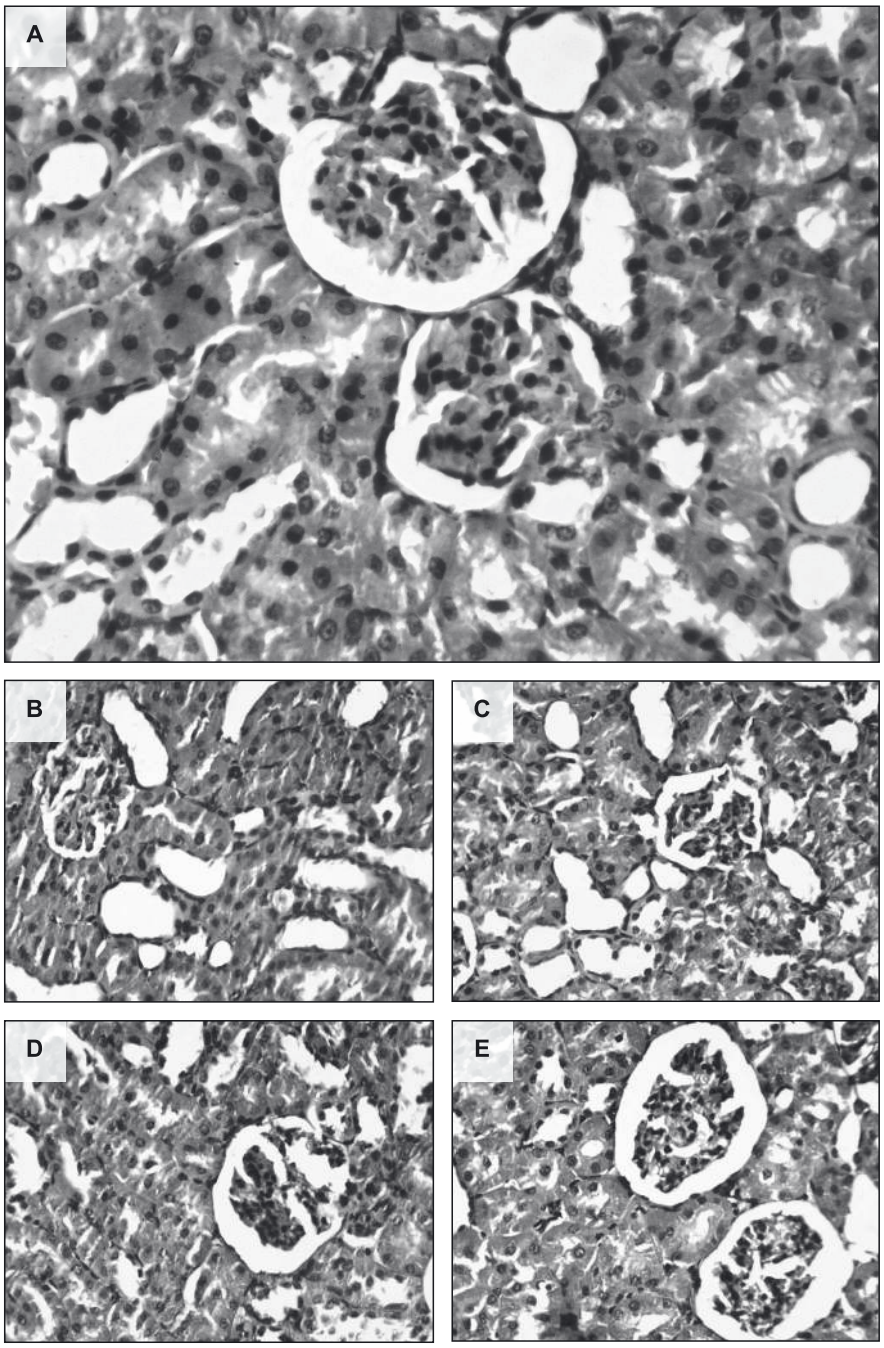

Fig. 4. Sections in the kidney of rats treated with TiO_2 (B); H_2O_2/Fe^{2+} (C); H_2O_2/Fe^{3+} (D); and a new type of solution, H_2O_2/Fe^{3+}/oxalic acid (E) compared to the control (A)

Table 4. Effect of Fenton solutions and titanium dioxide nanoparticles on two biochemical parameters glutamic pyruvate (GPT) and creatinine of treated rats

Treatments	GPT [U · l^{-1}]	Creatinine [mg · dl^{-1}]
$FeSO_4 + H_2O_2$	59.00 a	0.386 a
$FeCl_3 + H_2O_2$	58.20 a	0.386 a
$FeCl_3 + H_2O_2$ + oxalic acid	58.00 a	0.387 a
Titanium dioxide	58.20 a	0.385 a
Control	58.10 a	0.388 a

Means in a column followed by the same letters are not significantly different using Duncan's multiple ranges test

Discussion

The results of this study demonstrate that Fenton-like reagent, Fenton's reagent and Fenton complex controlled leaf spot infection on sugar beet plants and the acidity was too low to harm the plants. The antifungal effect of Fenton solutions likely happened due to its high $^{\cdot}OH$ photoformation rate under normal daylight conditions. The data showed that the Fenton complex was potentially active against leaf spot pathogen more than Fenton and Fenton-like reaction. This might be a direct result of the fact that the Fe^{3+}-organo-complex retains light from the sunlight and is steady at natural pH. Also it maintains an essential separation from the pH dependence of traditional Fenton. Strong and photoactive Fe^{3+}-complexes are made by means of carboxylate or polycarboxylates groups (e.g. oxalate, malonate and citrate), while the Fe^{3+}-complex is active in the presence of light under normal pH (Spuhler *et al.* 2010).

Hydrogen peroxides as ROS are considered to be one of the chemical defense strategies of plants against the infection of fungal and bacterial pathogens (Apostol *et al.* 1989; Mehdy 1994). Plant tissues produce the ROS in highly variable amounts (nM–mM levels) (Schopfer 1994; Papadakis and Roubelakis-Angelakis 1999). In this study, the Fenton solutions applied onto the leaves of sugar beet plants as exogenous ROS could induce its action against *C. beticola* by the same mechanism as endogenous ROS in plant tissues. Therefore, it might be considered as an alternative treatment to chemical fungicides in the control of leaf spot disease.

Fenton solutions mode of action is not clearly understood. Based on the results showing that treatment with Fenton solution for only three weeks successfully suppressed the disease it can be concluded that Fenton solutions are directly involved in suppression and inhibition of the fungus. Another theory is that the antifungal activity of Fenton solutions resulted from Fenton solutions entering fungal cells (Polo-Lopez *et al.* 2011) by the diffusion of H_2O_2 through cell membranes. Once hydrogen peroxide enters the fungal cell, several oxidative reactions may occur in the cell. Moreover, the iron normally found in cells can be discharged from the chelating atoms inside and produce hydroxyl radicals under the radiation impact by means of the Fenton/Habere Weiss Cycle (Imlay *et al.* 1988). At that point the produced hydroxyl radicals induced the disintegration of polysaccharides as reported by Hammel *et al.* (2002) who demonstrated that the hydroxyl radicals abstracts hydrogen ion as from sugar subunits of polysaccharides that cleavage of the polysaccharide chain.

The treatment with Fenton reagents is cost effective, biodegradable and utilizes safe reagents. It might be an alternative to chemical fungicides to control pathogens infecting sugar beet plants. The development of fungus resistance to fungicides will not happen with Fenton's reagents since it is difficult for fungi to avoid the attack of hydroxyl radicals due to its high reactivity to various organic and inorganic compounds (Sakugawa *et al.* 2012).

Several theories have been proposed to explain the impact of TiO_2 NPs on the development of fungi. One hypotheses is that TiO_2 NPs on fungi might distort and damage the sporangial membrane, resulting in a spillage of intracellular substances and finally the death of fungal cells (Maness *et al.* 1999; Sunada *et al.* 2003). This could be explained by the fact that reactive oxygen species, such as $^{\cdot}OH$, O_2^{-}, and H_2O_2 generated on the TiO_2 surface in the presence of light, lead to peroxidation of polyunsaturated phospholipids in the cell wall. Lipid peroxidation causes a breakdown of the cell membrane structure and therefore its associated functions and finally cell death. All life forms have a cell membrane made up of a variety of lipids with various degrees of unsaturation and rely on their structures to carry out essential functions. Thus, the proposed killing mechanism is applicable to all cell types (Maness *et al.* 1999; Sunada *et al.* 2003). The releasing of reactive oxygen species depends on the surface area of the semiconductor, which results in more oxygen species at the surface and higher hydrogen peroxide production which leads to high antimicrobial activity of the smaller TiO_2 nanoparticles (Oshira *et al.* 2008). In spite of this high oxidizing power, TiO_2 appears to be safe on plant surfaces (Frazer 2001).

Secondly, if nanoparticles of TiO_2 are mixed with water and applied on plants, the water will evaporate with the progression of time and the remaining unabsorbed TiO_2 will stay on the surface of the plants as solids. The unabsorbed TiO_2 was found to make plants resistant to external stress. Likewise, a portion of the TiO_2 particles provides supplements and essential substances to plants and increases the ability of plants to use solar energy in photosynthesis, which subsequently increases plant growth and yield.

It is speculated that physically TiO_2 NPs, as nanocides, enter into the cells by crossing the cell layer and can defeat the resistance issue of microorganisms to fungicides. It is not expected that the fungi will become resistant to such a physical mechanism (Hamza *et al.* 2015, 2016).

The results showed an increase in sugar beet yield collected from plants having had different chemical treatments as compared to the control in both growing seasons. This may have been due to the reduction in disease severity caused by *C. beticola* as a result of these chemical treatments which reflected on the plant health and increased crop yield (Hamza *et al.* 2015).

The biochemical and histological tests for all examined Fenton's reagents and TiO_2 NPs showed no essential modifications in the kidneys or livers in rats, which is important for public health. Moreover, the observed changes in the examined tissues were for the most part uncorrelated with the dosage, which proves the safety of the tested chemicals for public health.

This study is considered to be the first step toward more studies about using these effective, alternative products for controlling plant pathogens which help reduce environmental pollution and side effects on public health induced by fungicide application.

Conclusions

Fenton's reagents and TiO_2 NPs could give practical control of sugar beet leaf spot under field conditions. These Fenton's reagents and TiO_2 NPs had mild or no toxicity

compared to the high dose offered orally to the treated rats and it is expected that people will not be exposed to this level under any conditions. The physical mode of action of Fenton's reagents and TiO_2 NPs against *C. beticola* might be utilized in the control of plant pathogens.

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