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Influence of Hydrogen Peroxide (H_2O_2) on *Trichoderma harzianum* Potentiality to Control Sunflower Root/collar Rot Pathogen *Sclerotium rolfsii*

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In *vitro* studies showed significant inhibitory effects of hydrogen peroxide (H_2O_2) on growth of either sunflower root/collar rot pathogen, *Sclerotium rolfsii* or the biocontrol agent *Trichoderma harzianum*. H_2O_2 provided synergistic effects with *T. harzianum*, since the antagonistic potentiality of *T. harzianum* against *S. rolfsii* was increased with increasing H_2O_2 concentration. Sunflower root/collar rot severity was significantly reduced when seeds were singly treated by H_2O_2 or *T. harzianum*. H_2O_2 appeared to be the most effective to prevent *S. rolfsii* infectivity followed by *T. harzianum*. The present study provided synergistic effects for H_2O_2 to improve biocontrol capacity since H_2O_2 at 1.2g/l with *T. harzianum* achieved sunflower protection by 78% against *S. rolfsii* infection.

Keywords: Hyrdrogen peroxide, *Sclerotium rolfsii*, sunflower root/collar rot, *Trichoderma harzianum*.

Sclerotium rolfsii Sacc., teleomorph, *Athelia rolfsii* (Okabe and Matsumoto, 2003) is a soil borne pathogen that causes root and collar rot disease on plants. It primarily attacks host organs including stems, roots, fruits, petioles and leaves under favourable conditions. It commonly occurs in various warm temperature regions of the world, and can infect more than 500 plant species (Rajeev and Mukhopadhyay, 2002 and Fu *et al*., 2002). However, breeding for resistance to *Sclerotium rolfsii* has been hampered due to lack of germplasm with immunity or resistance to this pathogen. Furthermore, the use of fungicides to protect the roots of sunflower from soil borne infections is neither practical nor economical (Steadman, 1979). Accordingly, other approaches for controlling phytopathogenic microbes become a good target for minimizing disease incidence and severity with least cost and without environmental pollution (Elad, 1992; Galal and Abdou, 1996; Quintanilla and Brishammar, 1998; Abdou *et al*., 1999 and Woo *et al*., 2006). *Trichoderma* spp. are agriculturally important for their beneficial effects on plant growth and development and for their capability to induce plant defense responses against pathogens (Harman *et al*., 2004 and Waghunde *et al*., 2016). Trichoderma are being tested as alternatives to chemical fungicides. (El-Mougy *et al*., 2012 and Guzman-Valle *et al*., 2014). Oxidative burst, mediated by H_2O_2, has been recognized as a key component of plant defense response during an incompatible interaction. Elevated levels of H_2O_2 also activated the expression of several defense genes to both bacterial and fungal pathogens (Kachroo *et al*., 2003). H_2O_2 is reported to inhibit biotrophic but benefit necrotrophic pathogens. Infection by necrotrophs can result in

a massive accumulation of H_2O_2 in hosts. The hemibiotroph, Septoria tritici, infecting wheat is inhibited by H_2O_2 during the biotrophic phase, but a large H_2O_2 accumulation occurs in the host during reproduction (Shetty *et al*., 2007). H_2O_2 participates in many resistance mechanisms, including reinforcement of the plant cell wall, phytoalexin production, and enhancement of resistance to various stresses (Quan *et al*., 2008).

The present study was planned to gain an informative statements towards interaction effects of bioagent *T. harzianum* and hydrogen peroxide in sunflower *Sclerotium rolfsii* relationship.

Materials and Methods

1-Causal agent:

The most aggressive *Sclerotium rolfsii* isolate Sr1 which was isolated from sunflower basal stem rot by Ismail et al. (2006) was used throughout this study.

2-Bioagent, Trichoderma harzianum:

Trichoderma harzianum isolate No. A1 d was purchased from Fitogen Plant Diseases lab. Varsak Zeytinlik, Kepez, Antalya, Turkey and was used throughout this study.

3- Laboratory studies:

3-1. Effect of H_2O_2 on the mycelial dry weight of Sclerotium rolfsii and T. harzianum:

The toxicity of hydrogen peroxide with different concentrations on growth of *S. rolfsii* or *T. harzianum* isolate in liquid nutrient broth medium (NB) was studied. Equal disks (10 mm in diameter) were taken from actively edge of 7 days old cultures of the tested *S. rolfsii* isolate grown on nutrient agar medium at 20°C and used for inoculation of 250 ml Erlynmeyer flasks containing 50 ml sterilized nutrient medium amended with tested concentration of H_2O_2. Concentrations were prepared in sterile distilled water and aliquots were pipetted to NB medium to obtain final concentration of 0.2, 0.4, 0.8 and 1.2 g/l hydrogen peroxide. For control treatment, conical flasks containing medium without the tested chemical were inoculated similarly to be taken for comparison.

Each treatment was incubated at 20°C (Abdou and Galal, 1997) for 10 days. Mycelial dry weight (MDW) of different treatments was measured (mg MDW per 100 ml liquid medium) after separation of fungal mass by filtration through Whatman No-1 filter paper and dried at 60°C for 48 hrs. The following equation was used to calculate percentage inhibition of fungal growth. (Sutton and Starzyk, 1972).
Inhibition%=MDWof the control –MDW of the treatment / MDWof the control x100

3-2. Effect of H_2O_2 on some growth parameters of Sclerotium rolfsii and T. harzianum:

H_2O_2 was tested at concentrations *i.e.*, 0.2, 0.4,0.8 and 1.2 g/l. ,each was added to autoclaved nutrient agar medium in conical flasks and then dispensed to Petri dishes (15 ml medium/plate) and left to solidify. Plates were then inoculated with *S. rolfsii*

by placing cork porer made agar disks (10 mm) taken from the periphery of fungal colonies grown 7 days after incubation. Plates containing nutrient agar medium without H_2O_2 were inoculated similarly to be taken for comparison (Abdou and Galal, 1997).

Three plates for each treatment were used then incubated at 20° C. Data were recorded by measuring the diameter of the fungal growth (two diameters) and were determined when the mycelia growth of any dish reached the edge of the plate. When the sclerotia covered the control plates, after 14 days, the number of sclerotia in Petri plates was assayed as follows:

Equal 3 disks (1 cm2) were taken from a constant distance., 3 cm from the center, and the number of sclerotia on each disk was counted and the means for three plates were determined (Moussa and Rizk, 2003). As for *T. harzianum* sporulation, the number of spores was calculated from 7 day old cultures by gently brushing spores from the surface of the plate with a fine paintbrush into 5-10 ml distilled water containing 0.05% tween. Each suspension was stirred vigorously by vortex for 1 min to break up spore aggregates. For each suspension, the number of spores was counted using haemocytometer (Morris and Nicholls, 1978)

3-3- Interaction effects of H_2O_2 and Trichoderma harzianum on Sclerotium rolfsii growth in vitro:

H_2O_2 was tested at 0.4, 0.8 and 1.2g/l. concentrations, each was added to autoclaved nutrient agar medium in conical flasks and then dispensed to Petri dishes (15ml medium/plate) and left to solidify. Plates were then inoculated with *S. rolfsii* by placing cork porer made agar disks (10 mm) taken from the periphery of fungal colonies grown for 7 days after incubation at 20oC.The mycelial disks of 10 mm diameter of *Trichoderma harzianum* strain No. A1 d and *Sclerotium rolfsii* were placed opposite to each other in Petri plates containing different concentrations of H_2O_2. Plates containing nutrient agar medium without H_2O_2 were inoculated similarly to be taken for comparison. The data were recorded regularly on the growth of the pathogen and bioagent isolate No. A1 d. Percentage of mycelial growth inhibition (MGI %) was calculated according to the formula MGI% = (dc - dt) ×100/dc

Where, dc= fungal colony diameter in control sets, dt= fungal colony diameter in treatment sets.

4- Interaction effects of H_2O_2 and T. harzianum on S. rolfsii infectivity:

The present study was carried out in a greenhouse located at Plant Pathol. Dept., Fac. Agric., Minia Univ., ELMinia, Egypt. Effect of bioagent *T. harzianum* and/or H_2O_2 at concentrations of 0.8 and 1.2 g/l on resistance of sunflower cv. Sagha 53 to root/collar rot was studied. Hundred seeds for each treatment were wetted by 5ml of each tested treatments for 12 hours overnight while seeds wetted by 5ml distilled water were used for control then treated seeds were coated by Trichoderma *harzianum* suspension plus 1 ml tween 20 five hours before planting then seeds were planted in five replicates (4 pot replicates, 5 seed per each pot) .Soil was artificially infested with *S. rolfsii* grown in barely grains for 10 days and mixed with soil at 2.5%w/w and transferred into 15 cm diam. Pots, 5 kg soil per pot. Sterilized soil not

infested with *S. rolfsii* served as control. Seeds of sunflower were seeded in separate sets, 5 seeds per pot. Soil moisture was adjusted to 50% water hold capacity (Keen and Raczkowski., 1921) and amount of water loss was restored after each 24 hrs.

5 -Disease assessement:

The arbitray (0-5) disease scale described by Abd Elrazek *et al*., (1974) was used to measure the disease severity in which. 0= no infection, 1= 1- 20 % infection; 2 =21-40 % infection; 3= 41-60% infection; 4 = 61- 80 % infection; 5= 91-100% infection. The following equation was used to calculate percentage of disease severity.

Disease severity= 0A+1B+2C+3D+4E+5F/5T X 100

Where A, B, C, D, E and F are the numbers of plants corresponding to the numerical grades 0, 1, 2, 3, 4 and 5 respectively, and 5T is the total number of plants (T) multiplied by maximum disease grade 5 (Sharma *et al*., 2006).

6- Statistical analysis:

Data were subjected to statistical analysis using analysis of variance and means were compared using least significant difference at 0.05 confidence test as described by Gomez and Gomez (1984).

Results

1-Effect of H_2O_2 on Sclerotium rolfsii growth :

Growth parameters of *S. rolfsii*, *i.e.* radial growth, number of sclerotia and MDW were variously affected by H_2O_2 concentrations tested (Table 1). Regarding radial growth and MDW, antifungal activity of H_2O_2 was pronounced even at the lowest H_2O_2 concentration (0.2g/l) that inhibited radial growth by 33.3 % and MDW by 55.6 inhibitory effect. Increasing H_2O_2 concentration enhanced inhibitory effect and the highest linear growth suppression was expressed at 1.2g/l concentration (up to 76% inhibitory effect). H_2O_2 caused significant reduction in sclerotia production and its effect was increased with increasing its concentration (Table 1 and Figure 1B). Concentration 1.2 g/l H_2O_2 completely inhibited sclerotia formation.

Table 1. *In vitro* effect of hydrogen peroxide (H_2O_2) on *Sclerotium rolfsii* growth after incubation for 7days at 20°C, radial growth and mycelial dry weight, number of sclerotia

Treatment and Conc. (g/l)	Radial growth (mm)	Inhib. %	No. sclerotia per plate	Inhib. %	Mycelial dry weight (mg/50 ml liquid media)	Inhib. %
Untreated	80.00	0.0	130	0.0	932	0.0
H_2O_2 0.2	53.33	33.3	86	33.8	413	55.6
0.4	46.67	41.6	77	40.7	257	72.4
0.8	33.33	58.3	45	65.3	203	78.2
1.2	18.67	76.6	0	100	127	86.3
Means	46.4	41.9	67.6	47.9	386.4	58.5
L.S.D at 0.05	4.33	-	4.64	-	54.7	-

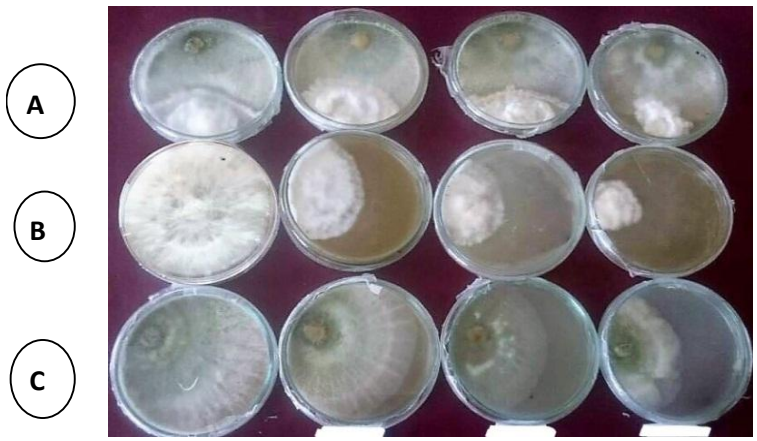

Fig. 1. Antagonistic activity of *T. harzianum* against *S. rolfsii* as influenced by different concentrations (g/l) of H_2O_2 (A). Growth of *S. rolfsii* as influenced by H_2O_2 concentrations (B) and *T. harzianum* growth at H_2O_2 supplemented medium (C).

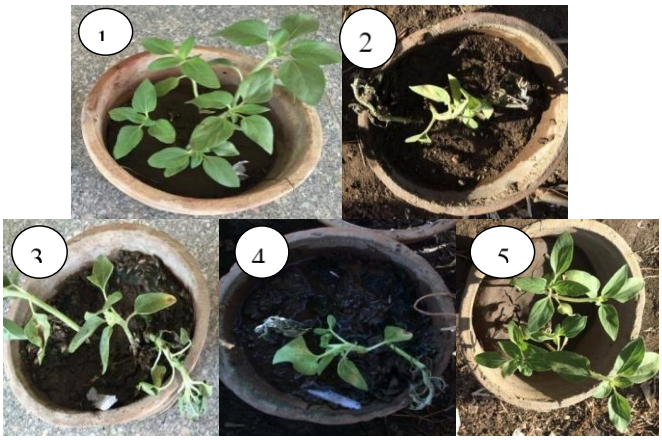

Fig. 2. Twenty one days old sunflower plants c.v. Sakha 53 growing from untreated seeds in non infested soil (1), untreated seeds planted in *S. rolfsii* infested soil (2),*S. rolfsii* infested soil and *T. harzianum* treated seeds (3), *S. rolfsii* infested soil and H_2O_2 treated seeds (4) and *S. rolfsii* infested soil H_2O_2 + *T. harzianum* treated seeds (5).

2- Effect of H_2O_2 on T. harzianum isolate NO A1 d growth:

Generally, growth of the bioagent *T. harzianum* isolate NO. A1 d was variously affected by H_2O_2 concentrations (Table 2 and Fig. 1C). Obvious antifungal activity was pronounced by H_2O_2 towards the linear growth at the lowest concentration tested (0.2g/l). A significant linear growth suppression was provided due to 0.4 g/l concentration. Increasing the concentration of H_2O_2 increased the inhibitory effect, since 1.2 g/l H_2O_2 concentration revealed a great reduction in the linear growth and MDW. Regarding spore production, H_2O_2 significantly inhibited spore formation and its inhibitory effect was increased with increasing H_2O_2 concentration.

Table 2. *In vitro* effect of hydrogen peroxide (H_2O_2) on *Trichoderma harzianum* growth parameters after incubation for 7-days, at 20°C

Treatment and Conc.(g/l)	Radial growth (mm)	Sporulation (10^4/ml)	Mycelial dry weight (mg/50ml) liquid media
Untreated	80	153	943
H_2o_2 0.2	71.66	109	876
0.4	55	99	786
0.8	43.33	74	701
1.2	40.33	45	560
Means	58.06	96	773.2
L.S.D at 0.05	4.69	4.41	79

3- In vitro effect of H_2O_2 on T. harzianum antagonistic potentiality against S. rolfsii growth:

Radial growth and sclerotial formation by sunflower root/ collar rot -caused by *S. rolfsii* were strongly inhibited by H_2O_2 and *T. harzianum* when they were tested individually or in combinations (Table 3 and Fig.1A). Antagonistic potentiality of *T. harzianum* was pronounced, since it exhibited significant reduction in *S. rolfsii* radial growth (72.92%) and sclerotial formation (54%). Antagonistic potentiality of *T. harzianum was* significantly enhanced when growth medium was supplemented by H_2O_2 .However, antagonistic activity of *T. harzianum* against *S. rolfsii* growth was increased by increasing H_2o_2 concentration. Highest radial growth suppression (98%) and sclerotial formation (90% reduction) were pronounced at 1.2 g/l H_2O_2 followed by 87.5 and 85.2 % radial growth reduction and 80 and 60% reduction in sclerotial formation at 0.8 and 0.4 g/l H_2O_2, respectively.

Table 3. Effect of hydrogen peroxide (H_2O_2) and *Trichoderma harzianum* isolate NO. A1 d each alone or in combination on *Sclerotium rolfsii* radial growth and production of sclerotia

Treatment	Radial growth (mm)	Inhibition %	Sclerotia formation per plate	Inhibition %
*S. rolfs*ii (control)	80	0.0	50	0.0
Trichoderma harzianum	21.66	72.92	23	54
Tri+H_2O_2 0.4 g/l	11.66	85.42	20	60
Tri+H_2O_2 0.8 g/l	10	87.5	10	80
Tri+H_2O_2 1.2g/l	1.6	98	5	90
Means	24. 8	68.76	21.6	56.8
L.S.D at 0.05	5.143	-	4.997	-

4. Interaction effects of H_2O_2 and T. harzianum on the severity of sunflower root /collar rot infection:

Presowing soaking seeds in H_2O_2 solution resulted in increasing resistance of sunflower plants against *S. rolfsii* infection (Table 4 and Figure 2). A significant reduction in disease severity was obtained by 0.8 g/l H_2O_2 concentration (63.66%). This figure was increased with increasing H_2O_2 concentration, since 48.33% protection was provided by 1.2 g/l H_2O_2. Coating seeds with propagule suspension (10^4 colony forming unit/ml) of *T. harzianum* isolate No.1 reduced disease severity on sunflower plants caused by *S. rolfsii* (47.33%). As for interaction effects, all combinations tested provided significant protection enhancement as compared to the untreated control. The highest protection (78% protection) was provided when seeds were treated by (1.2 g/l) H_2O_2 and then coated with *T. harzianum* propagules without significant differences with most of other treatments.

Table 4.Interaction effects of hydrogen peroxide (H_2O_2) and *Trichoderma harzianum* isolate No. A1 d on the severity of sunflower root rot caused by *S. rolfsii* under greenhouse conditions

Treatment	Disease severity (%)	Protection (%)
H_2O_2 0.8 g/l	63.66	30
H_2O_2 1.2 g/l	48.33	46
Trichoderma harzianum	47.33	47
T. harzianum+H_2O_2 0.8 g/l	27.00	70
T. harzianum+ H_2O_2 1.2 g/l	20.33	78
Untreated	91.00	0.00
Means	49.60	45.16
L.S.D at 0.05	10.66	-

Discussion

A non specific phytopathogenic soil borne fungus, *S. rolfsii* that can infect more than 500 plant species, its control is still difficult through various control methods even fungicides which improper for human health and environment. Safety elements became, in most cases, beneficial when used as fungicide alternatives (Abdou and Galal, 1997 and Adam *et al*., 2000). Thus, hydrogen peroxide and biocontrol agent *Trichoderma harzianum* were concerned individually or in combination to minimize *S. rolfsii* growth and infectivity. *In vitro* studies showed that $H_2O_2$was effective to suppress radial growth, mycelial dry weight and sclerotia production which recorded the lowest values, 18.67, 100 and 12.7%, respectively at 1.2 g/l H_2O_2. H_2O_2 reacted as active oxygen species provided antifungal effects towards various phytopathogenic fungi, e.g. *Fusarium oxysporum* and *Fusarium solani* (Abdou and Galal,1997), *Puccinia helianthi* (Abdou *et al.,* 1999), *Verticillium fungicola* (Savoie and Largeteau,2004) and *Septoria tritici* (Shetty *et al*., 2007).

Biocontrol agent *T. harzianum* was used successfully against plant pathogens (Shaigan *et al.*, 2008, Heydari and Pessarakli, 2010 and Kumar *et al*., 2014).Many researchers used *Trichoderma spp*.for controlling plant diseases caused by *S. rolfsii.* Subsequent experiment was conducted to explore effect of H_2O_2 towards *T. harzianum* growth parameters. H_2o_2 significantly reduced radial growth, sporulation and mycelia dry weight even at 0.4 g/l and its inhibitory effects were increased with increasing H_2O_2 concentration where the highest inhibition percentages were recorded for linear growth, sporulation and mycelia dry weight, at 1.2 g/l H_2O_2. Morever, antagonistic potentiality for *T. harzianum* was affected by H_2O_2. H_2O_2 provided synergistic effects with *T. harzianum* since the antagonistic potentiality of *T. harzianum* against *S. rolfsii* was increased with increasing H_2O_2 concentration. Sunflower root/collar rot severity was significantly reduced when seeds were singly treated by H_2O_2 or *T. harzianum*. The most effective treatment to minimize *S. rolfsii* infectivity was by *T. harzianum* and H_2O_2 together. Data are in line with those reported on plant/pathogen interaction, H_2O_2 (Savoie and Largeteau, 2004) and *T. harzianum* (Vinale *et al.*, 2006).

An obvious synergistic protection effect was recorded with different H_2O_2 combinations tested. The obtained results provided synergistic effects for H_2O_2 to improve biocontrol capacity. Since H_2O_2 at 1.2g/l with *T. harzianum* achieved sunflower protection by 78% against *S. rolfsii* infection. In conclusion, H_2O_2 is benefit to control sunflower root/collar rot infection that caused by *S.rolfsii*. H_2O_2 improves biocontrol activity of *T. harzianum* against *S. rolfsii* infection.

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تأثير فوق أكسيد الأيدروجين على كفاءة الفطر *Trichoderma harzianum* في مكافحة عفن ساق وجذور عباد الشمس المتسبب عن فطر *Sclerotium rolfsii*

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أظهرت الدراسات المعملية تأثير معنوي مثبط لفوق أوكسيد الايدروجين علي قياسات نمو كلاً من الفطر الممرض *Sclerotium rolfsii* المسبب لعفن ساق وجذور عباد الشمس والكائن المضاد *Trichoderma harzianum*. و أظهر فوق أوكسيد الايدروجين تأثير إضافي أدي الي زياده كفاءه فاعليه *T. harzianum* ضد نمو *S. rolfsii*. حيث زادت كفاءة *T. harzianum* بزياده تركيزات فوق أوكسيد الايدروجين المستخدمة. كما انخفضت اصابه عباد الشمس بفطر *S. rolfsii* معنوياً نتيجة معامله البذور بفوق أوكسيد الايدروجين و *T. harzianum* كلا علي حده أو الاثنين معا وأظهر فوق أوكسيد الايدروجين فاعليه في منع الإصابة بـ *S. rolfsii* يليه *T. harzianum* أظهرت الدراسة تأثير اضافي ايجابي لخفض الإصابة عند استخدام فوق أوكسيد الايدروجين مع *T. harzianum* لذلك توصي الدراسة بأن فوق أوكسيد الايدروجين قد يؤدي الي تحسين كفاءة *T. harzianum* في المكافحة الحيوية للإصابة بفطر *S. rolfsii* حيث أن فوق أوكسيد الايدروجين حقق عند تركيز ١،٢ جرام في اللتر مع *T. harzianum* حمايه لنباتات عباد الشمس وصلت الي ٧٨% ضد الإصابة بالفطر *S. rolfsii* موضوع الدراسة.