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Role of hydrogen peroxide in management of root rot and wilt disease of thyme plant

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Abstract

Thyme (*Thymus vulgaris*) is an aromatic medicinal plant cultivated in many countries, including Egypt. The plant has many medicinal benefits that make it an important medical crop. In 2017, root rot and wilt symptoms were detected on thyme plants at different locations of Giza governorate and samples were collected from the infected plants. Seven fungi were isolated from the infected plants (*Pythium* sp., *Fusarium solani*, *Rhizoctonia solani*, *Macrophomina phaseolina*, *F. oxysporum*, *Botryodiplodia* sp., *Alternaria tennis*). Pathogenicity tests of the isolated fungi proved that all of them were pathogenic to thyme. The effect of hydrogen peroxide at different concentrations (0.25%, 0.50%, 1 % and 2%) on linear growth of *Botryodiplodia* sp., *F. oxysporum*, *F. solani*, *M. phaseolina*, *Pythium* sp. and *R. solani* was evaluated *in vitro*. All concentrations significantly reduced the fungal linear growth of all the tested fungi. However, the concentration of 2% was completely inhibited the fungal growth of *R. solani*, *Pythium* sp. and *F. solani*. Evaluation of hydrogen peroxide application as seed treatment and soil drenching for controlling root rot and wilt disease of thyme was also performed under greenhouse conditions. A remarkable reduction in pre- and post- emergence damping off as a response to hydrogen peroxide was detected with all the tested fungi. Furthermore, the treatment of hydrogen peroxide yielded serious increasing in plant survival with all tested fungi compared with untreated plants.

Keywords: *Thymus vulgaris*, root rot, wilt, hydrogen peroxide, damping-off.

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Introduction

Thymus vulgaris is an aromatic medicinal plant belonging to the family Lamiaceae; it is spread entirely over the world (Hosseinzadeh et al., 2015). The plant has been cultivated in several countries, including Egypt, for medicinal and culinary uses (Stahl-Biskup & Venskutonis, 2012; Stahl-Biskup & Sáez, 2002). It is considered an important export crop in Egypt, the average dry material yield of thyme is about 800 kg/ acre which produce about 16 kg essential oil. Its medical uses include therapy for chest infections, sore throat, cough, it has antifungal, antiseptic, and antibiotic properties (Ekoh et al., 2014). Additionally; it has other properties, include appetite stimulant effect; improvement of liver function; therapy of urinary and bronchial infections (Reddy et al., 2014). *T. vulgaris* is attacked by certain soil borne fungi causing root rot disease (Carr, 1987). Many studies reported that *Rhizoctonia solani*, *Fusarium* spp., *Macrophomina phaseolina*, *Alternaria* spp., *Colletotrichum* spp., *Botrytis cinerea*, *Stemphylium botryosum*, *Cladosporium cladosporioides* and *Phoma multirostrata var. macrospora* are the most important plant pathogens in thyme plant , causing root, stem and foliage diseases (Palacioğlu et al., 2017). Machowicz-Stefaniak et al. (2002) reported that the bases of roots and stems of thyme were colonized by many pathogenic fungi include; *R. solani*, *Fusarium* spp., *Alternaria* spp., *Colletotrichum* spp. and *Thielaviopsis basicola*. Root rots disease caused by many soil borne fungi, however, the poor conditions of the soil, such as the high proportion of the ground water and poor drainage is considered an important factor for disease infection. Alternative control methods are being considered because of the high costs for continues production of new chemicals to overcome the development of pathogen resistance to fungicides (Kotan et al., 2009; Laitila et al., 2002). Additionally, fungicides are harmful to human, living organisms and the environment (Jarvis, 1988). Reactive oxygen species (ROS) are considered the toxic side effects of O_2, used for respiration and energy supply in aerobic organisms. These chemical compounds include hydrogen peroxide (H_2O_2), hydroxyl radical (OH), singlet oxygen ($^1O_2$), superoxide anion (O_2^-) and alpha-oxygen. ROS have harmful effects on DNA, lipids and proteins; it is also related to plant defense due to their antimicrobial activity (Daub et al., 2013; Fridovich, 1998). Hydrogen peroxide is a reactive oxygen species that occurs in all aerobic organisms as a metabolite. It is considered a cytotoxic at high concentrations, due to conversion to the stronger oxidant, hydroxyl radical OH (Apel & Hirt, 2004). It inhibits, with other reactive oxygen species, the development of microbes and contributes in various protection responses (Garcia-Brugger et al., 2006; Baker & Orlandi, 1995). Hydrogen peroxide also has multiple models of oxidative structural and different stages of oxidation to amino acids and proteins, and variations in its activity against a microbial enzyme (Finnegan et al., 2010). The present work aimed to evaluate hydrogen peroxide, as a reactive oxygen species, against the mycelial growth of root rot and wilt fungi isolated from infected *T. vulgaris*. Moreover, hydrogen peroxide application in controlling root rots and wilt disease of *T. vulgaris* under greenhouse conditions was also evaluated.

Materials and methods

Isolation and identification of the causal fungi: Thyme plants showing root rot and wilt disease symptoms (include yellowing, dryness and wilt) were collected from different locations of Giza governorate during 2017. The infected roots were thoroughly washed under running tap water then cut into small pieces with sterilized scalpel, and surface sterilized in 1% sodium hypochlorite for 2 minutes, then rinsed several times in sterilized distilled water. The sterilized pieces were then dried between folds of sterilized filter papers and placed on potato dextrose agar. The inoculated plates were incubated at 27^{o} C for seven days and checked daily for fungal development. The developing fungi were purified using the hyphal tip or single spore technique. Identification of the isolated fungi was carried out at the department of mycology and plant disease survey research, Plant Pathology Research Institute., ARC, Giza, Egypt, according to description given by Gilman (1957), Barnett and Hunter (1972) and Nelson et al. (1983). Isolation frequency of the developing colonies of each fungus was calculated as percentage of the total developing colonies.

Preparation of inoculum: Inoculum of obtained fungi (*Botryodiplodia* sp., *Fusarium oxysporum, F. solani, Macrophomina phaseolina, Pythium* sp. and *Rhizoctonia solani*) was separately grown on corn sand meal medium in 500 ml glass bottles. Bottles were incubated at 27°C for 20 days (Ahmed et al., 2016).

Pathogenicity tests: This experiment was carried out under greenhouse conditions. Sterilized clay sand soil (1:1 w/w) was individually infested with the tested fungi at the rate of 2% (w/w) in formalin sterilized pots (20 cm diam.). The pots were watered for one week to enhance growth and distribution of the fungal inoculum. Then, twenty seeds/ pot were planted and three replicates were used for each fungi treatment. Percentages of pre- and post-emergence damping-off were recorded after 15 and 30 days from planting date, respectively. Pathogenicity of isolated fungi to 30 and 60 days-old thyme plants was also tested in infested pots. Each pot was planted with four unrooted stem tip cuttings. Four replicates were used for each treatment. Percentages of wilted thyme plants were recorded after 30 and 60 days from planting date as follows:

$$\text{Disease incidence (\%)} = \frac{\text{No. of infected plants}}{\text{Total No. of plants}} \times 100$$

Effect of different hydrogen peroxide concentrations on linear growth of the tested fungi: The effect of hydrogen peroxide (El Nasr Company for Intermediate Chemicals, Alexandria Desert Road km 28, Industrial Area, Abou-Rawash, Giza, Egypt) on linear growth of the tested fungi was carried out *in vitro*. Potato dextrose agar (PDA) medium was autoclaved at 121°C for 15 min. Hydrogen peroxide (50) was added to PDA with different concentrations (0.25%, 0.50%, 1% and 2%) directly before poured into 9 cm diameter sterilized Petri dishes. Three replicates for each concentration were used and plates containing only PDA medium were used as control. All plates were then inoculated individually by the tested fungi using 5 mm fungal growth discs taken from 14 days old cultures and incubated at 27°C. The plates were checked daily and the experiment was terminated when the mycelial growth of control covered the medium surface. The fungal growth diameter of all treatments and control was measured and percentage of reduction in mycelial growth was calculated using the formula suggested by Ahmed (2005) as following:

$$\text{Reduction in linear growth (\%)} = \frac{G1 - G2}{G1} \times 100$$

Where, G1 = Linear growth of fungal control. G2 = Linear growth of fungal treatment.

Effect of hydrogen peroxide as seed treatment and soil drenching on pre- and post- emergence damping-off under greenhouse conditions: Hydrogen peroxide was tested for controlling root rot and wilt disease caused by *Botryodiplodia* sp., *F. oxysporum*, *F. solani*, *M. phaseolina*, *Pythium* sp., *R. solani* in pot experiment. Sterilized clay sand soils (1:1 w/w) were mixed separately with each fungal inoculum at 2% (w/w). Formalin sterilized pots (20 cm diam.) were packed with sterilized soils and watered for 7 days to enhance growth and distribution of the fungal inoculum. Twenty seeds/ pot were soaked in hydrogen peroxide with concentration of 2 % for 20 min before planting. Three replicates were used for each fungi treatment. After 15 days, the pots were irrigated with hydrogen peroxide (with concentration of 2%). Control pots were left without addition of hydrogen peroxide.

Statistical analysis: The present study was carried out with three replicates as designed factorial experiment in a complete randomized design (Snedecor & Cochran, 1980). MSTAT-C statistical package version (4) was used for analysis using Least Significant Difference (L.S.D) test at 0.05.

Results

Isolation and identification of the causal fungi: Data in Table (1) showed that seven fungi (*Alternaria tennis*, *Botryodiplodia* sp., *Fusarium oxysporum*, *F. solani*, *Macrophomina phaseolina*, *Pythium* sp., *Rhizoctonia solani*) were isolated from plants collected from Giza governorate. *Pythium* sp. was the most frequently isolated fungi (31.25%) followed by *F. solani* (22.32%), *R. solani* (16.96%) and *M. phaseolina* (11.61%), while *A. tennis* (3.57%), *Botryodiplodia* sp. (5.36%) and *F. oxysporum* (8.93) showed the lowest isolation frequency.

Pathogenicity tests: Data in Table (2) showed the pathogenicity of six fungi as percentages of pre- and post-emergence damping-off as well as those of survival seedlings. All the tested fungi were able to infect the plants and cause pre- and post-emergence damping-off. However, *Pythium sp.* followed by *M. phaseolina* recorded the highest percentages of pre-emergence damping-off (45% and 41.7, respectively). Moreover, *F. solani* followed by *M. phaseolina* recorded the highest percentages of post- emergence damping-off (55% and 36.7%, respectively). Subsequently, they recorded the highest percentages of reduction in survivals which recorded 85 % and 78.4 %, respectively (Figure 1). In contrast, *Botryodiplodia* sp. recorded the lowest percentages of pre- and post-emergence dumping-off (30% and 18.3%, respectively). Subsequently, it recorded the lowest percentage of reduction in survivals (48.3 %).

Table 1: Frequency percentages of the isolated fungi from diseased thyme plants collected from Giza governorate, Egypt.

Fungi	Number of isolates	Frequency (%)	Symptoms
Alternaria tennis	4	3.57	Root rot
Botryodiplodia sp.	6	5.36	Root rot
Fusarium oxysporum	10	8.93	Wilt/root rot
F. solani	25	22.32	Root rot
Macrophomina phaseolina	13	11.61	Root rot
Pythium sp.	35	31.25	Root rot
Rhizoctonia solani	19	16.96	Root rot
Total	112	100	Root rot

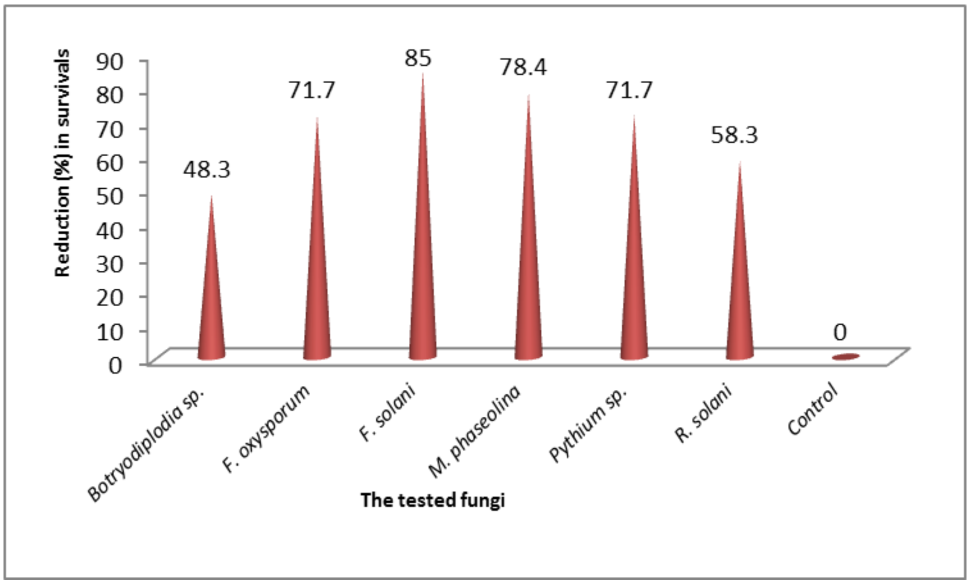

Figure 1: Reduction percentages in survivals of thyme plants in response to the infection with the tested fungi.

Table (3) showed that all the tested fungi were significantly pathogenic to thyme plants causing root rot or wilt disease in different degrees compared with the control treatment. Moreover, percentages of disease incidence positively correlated by the time elapse from 30 to 60 days after planting date. *R. solani* was the most virulent fungus as it recorded the highest percentage of disease incidence, 30 after planting date (62.5%), followed by *F. solani* (50%), *F. oxysporum* and *Botryodiplodia* sp. (25%). Furthermore, *F. solani*, *F. oxysporum* and *R. solani* recorded the highest percentages of disease incidence, 60 after planting date (75%). In contrast, *Pythium* sp. was the lowest pathogenic fungi resulting in 25% disease incidence after 60 days from planting date.

Table 2: Percentages of pre- and post- emergence damping-off infection caused by the tested fungi, 15 and 30 days after planting date, respectively.

Fungi	Pre- emergence damping off after 15 days (%)	Post- emergence damping off after 30 days (%)	Survivals (%)
Botryodiplodia sp.	30.0	18.3	51.7
Fusarium oxysporum	40.0	31.7	28.3
F. solani	30.0	55.0	15.0
Macrophomina phaseolina	41.7	36.7	21.6
Pythium sp.	45.0	26.7	28.3
Rhizoctonia solani	38.3	20.0	41.7
Control	0.0	0.0	100.0
L.S.D at 5%	3.9	5.1	-

Table 3: Percentages of wilted thyme plants, 30 and 60 days after planting date in infested soil with the tested fungi under greenhouse conditions.

Fungi	Disease incidence after 30 days (%)	Disease incidence after 60 days (%)
Botryodiplodia sp.	25.0	37.5
Fusarium oxysporum	25.0	75.0
F. solani	50.0	75.0
Macrophomina phaseolina	18.8	62.5
Pythium sp.	12.5	25.0
Rhizoctonia solani	62.5	75.0
Control	0.0	0.0
L.S.D at 5%	8.05	13.80

Effect of hydrogen peroxide on root rot and wilt disease of thyme plants *in vitro*: The mycelium linear growth of the tested fungi was significantly affected ($P \leq 0.5$) with hydrogen peroxide treatments compared to the control (Table 4). A positive correlation between hydrogen peroxide concentration and reduction of fungal liner growth was detected. The tested fungi were varied in their response to hydrogen peroxide. *R. solani* was the most sensitive to hydrogen peroxide at all concentrations except the concentration of 1%. In contrast, *M. phaseolina* was the lowest sensitive to hydrogen peroxide at all concentrations. The concentration of 2% was completely inhibited the fungal growth of *R. solani*, *Pythium* sp. and *F. solani*.

Effect of hydrogen peroxide on root rot and wilt disease of thyme plants under greenhouse conditions: This experiment was performed to test the efficiency of hydrogen peroxide as seed treatment and soil drenching for controlling root rot and wilt disease caused by the tested fungi shown in table 5. The experiment was carried out in pots (20-cm diam.) under greenhouse conditions. Hydrogen peroxide treatment yielded a remarkable reduction in pre- and post- emergence damping off with all the tested fungi. The percentages of reduction were varied depending on the response of each fungus to hydrogen peroxide. Regarding the reduction in pre- emergence damping off, *Pythium* sp. followed by *F. oxysporum* were the most sensitive to hydrogen peroxide, 92.7% and 92.1%, respectively, while, *R. solani* recorded the lowest percent, 54% (Figure 2).

Table 4: The *in-vitro* effect of different hydrogen peroxide concentrations on linear growth of the tested fungi.

Fungi	Concentrations of hydrogen peroxide: Control (%)	0.25 %	0.50 %	1 %	2 %
Botryodiplodia sp.	(9.0) [a]	(4.3) [a] (52.2%) [b]	(3.7) [a] (58.9%) [b]	(2.7) [a] (70%) [b]	(1.6) [a] (82.2%) [b]
Fusarium oxysporum	(9.0) [a]	(5.6) [a] (37.8%) [b]	(6.6) [a] (26.7%) [b]	(2.5) [a] (72.2%) [b]	(1.0) [a] (88.9%) [b]
F. solani	(9.0) [a]	(6.5) [a] (27.8%) [b]	(5.1) [a] (43.3) [b]	(3.6) [a] (60%) [b]	(0.0) [a] (100%) [b]
Macrophomina phaseolina	(9.0) [a]	(8.2) [a] (8.9%) [b]	(7.8) [a] (13.3%) [b]	(6.7) [a] (25.6%) [b]	(5.6) [a] (37.8%) [b]
Pythium sp.	(9.0) [a]	(4.7) [a] (47.8%) [b]	(3.3) [a] (63.3%) [b]	(1.3) [a] (85.6%) [b]	(0.0) [a] (100%) [b]
Rhizoctonia solani	(9.0) [a]	(4.0) [a] (55.6%) [b]	(2.0) [a] (77.8%) [b]	(1.5) [a] (83.3%) [b]	(0.0) [a] (100%) [b]

L.S.D 5 %: Fungi (F) = 0.5, Concentration (C) = 0.3, Interaction (F) x (C) = 0.7. [a] mean of linear growth (cm) of three replicates. [b] Reduction percentage of fungal growth compared to the control.

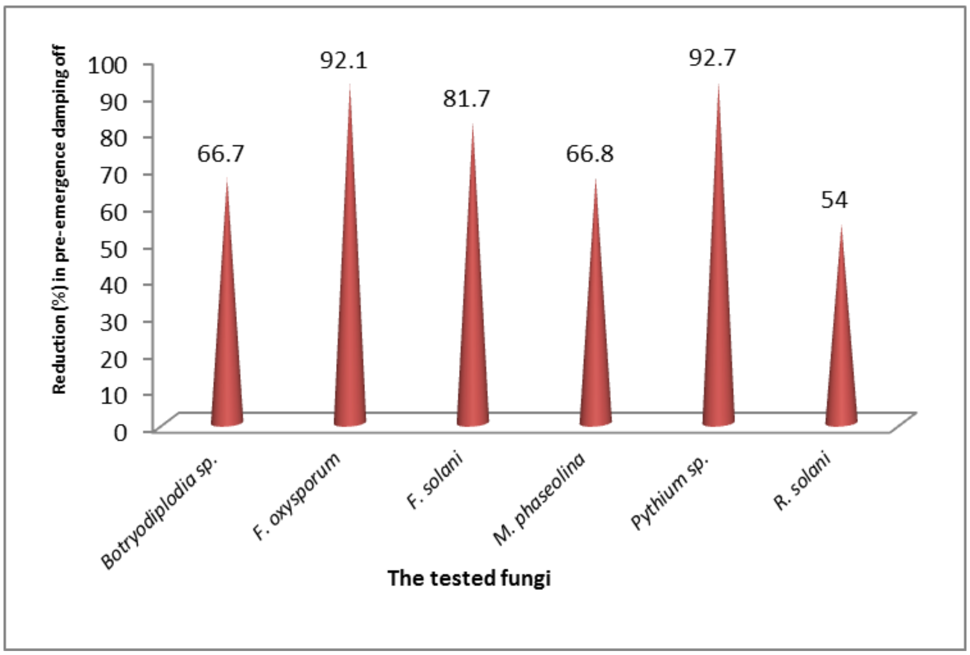

Figure 2: Reduction percentages in pre- emergence damping off in response to hydrogen peroxide treatment.

Furthermore, *F. solani* followed by *Pythium* sp. recorded the highest reduction percentages in post- emergence damping off as a response to hydrogen peroxide treatment (88.3%, 87.6%, respectively), while, *M. phaseolina* recorded the lowest percent, 53% (Figure 3). Overall, the treatment of hydrogen peroxide has yielded serious increasing in plant survivals with all tested fungi compared with untreated plants. The percentages of survival increasing were varied according to the applicable fungus and its response to hydrogen peroxide. The highest percent of survival increasing was recorded on the plants infected with *M. phaseolina*, 81.3%, followed by the plants infected with *F. oxysporum*, 73% (Figure 4). However, the plants infected with *Botryodiplodia* sp. was recorded the lowest percentage of survival increasing (37.9%).

Table 5: Effect of hydrogen peroxide as seed treatment on pre- and post- emergence damping-off, 15 and 30 days after planting date in soil infested with the tested fungi, respectively, under greenhouse conditions.

Fungi	Treatment	Pre- emergence damping off (%)	Post- emergence damping off (%)	Survivals (%)
Botryodiplodia sp.	H_2O_2	10.0	6.7	83.3
	Control	30.0	18.3	51.7
F. oxysporum	H_2O_2	3.3	16.7	80.0
	Control	41.7	36.7	21.6
F. solani	H_2O_2	6.7	3.3	90.0
	Control	36.7	28.3	35.0
M. phaseolina	H_2O_2	13.3	13.3	73.4
	Control	40.0	28.3	13.7
Pythium sp.	H_2O_2	3.3	3.3	93.4
	Control	45.0	26.7	28.3
R. solani	H_2O_2	16.7	13.3	70.0
	Control	36.3	38.3	25.4
L.S.D at 5%		6.9	6.6	-

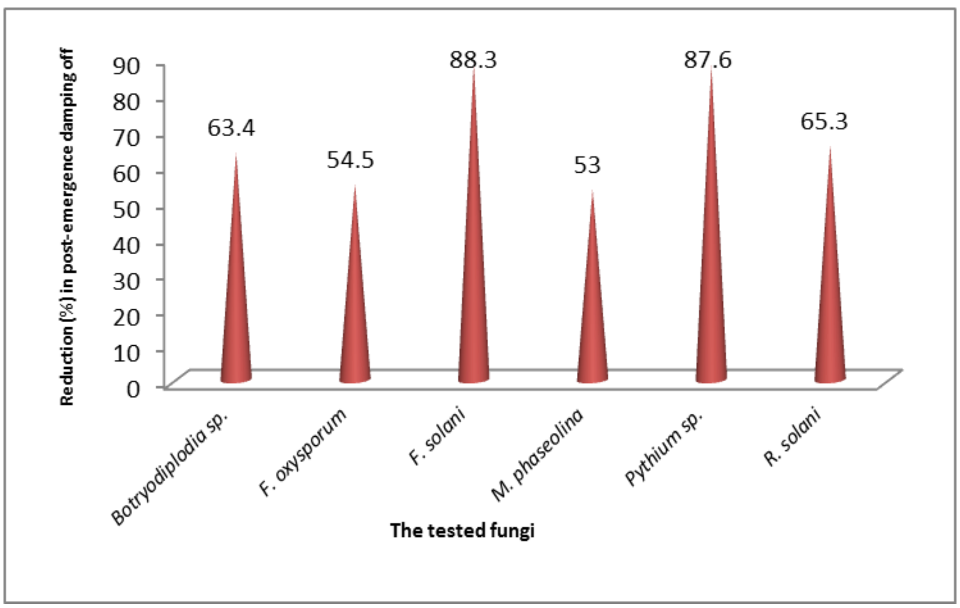

Figure 3: Reduction percentages in post- emergence damping off in response to hydrogen peroxide treatment.

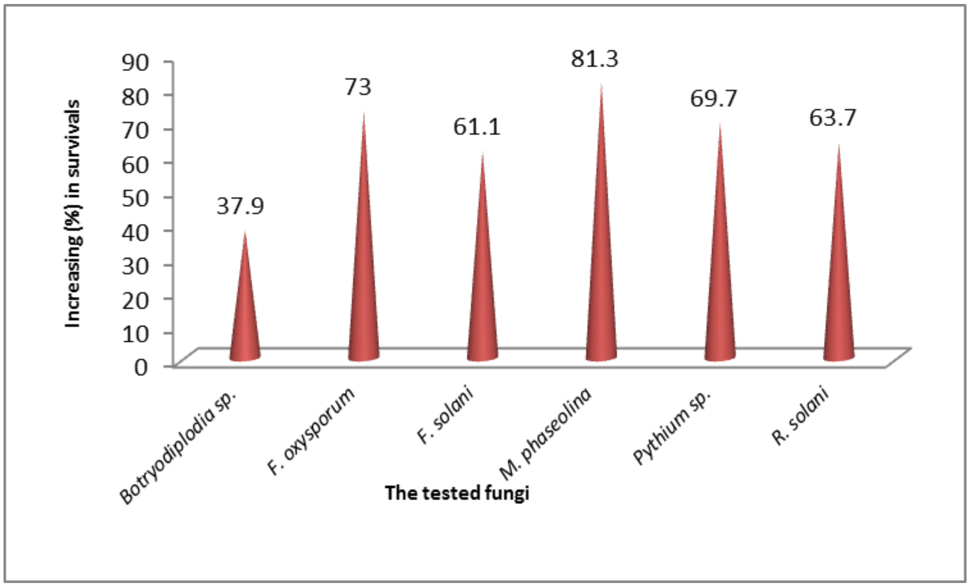

Figure 4: Increasing percentages in survivals of thyme plants in response to hydrogen peroxide treatment.

Discussion

Thyme (*Thymus vulgaris*) is an aromatic plant cultivated for medicinal and culinary uses. Many studies reported that thyme is attacked by some soil borne fungi, causing root rot infections. Seven fungi; *Alternaria tennis*, *Botryodiplodia* sp., *Fusarium oxysporum*, *F. solani*, *Macrophomina phaseolina*, *Pythium* sp., *Rhizoctonia solani* were isolated from the collected thyme plants. The isolated causal agents of root rot and wilt disease are similar to those reported by several studies (Palacioğlu et al., 2017; Machowicz-Stefaniak & Zalewska 2004; Machowicz-Stefaniak et al., 2002). The pathogenicity tests proved that all isolated fungi from the naturally infected samples were able to infect the plants and cause pre- and post-emergence damping-off. However, *F. solani* was the most pathogenic fungi as it recorded the highest percentages of post- emergence damping-off and reduction in survivals. Also, all the tested fungi were significantly pathogenic to thyme plants causing root rot or wilt disease in different degrees, *R. solani* was the most virulent one followed by *F. solani* and *F. oxysporum*, respectively. Similar results reported that *R. solani*, *F. solani* and *F. oxysporum* were the most virulent fungi infect *Coleus forskohlii*, belonging to the family Lamiaceae (Pulla et al., 2013; Singh et al., 2009; Shivkumar et al., 2006). Fungicide treatments are the main control method for these diseases. However, fungicides are harmful to human, living organisms and the environment (Guzzo et al., 1993; Jarvis, 1988). Using of abiotic-agents to give plant acquired resistance against pathogens is considered alternative control methods to fungicide applications. The current study presented a trial to investigate the possibility of minimizing the infection with root rot and wilt disease of thyme using hydrogen peroxide. The effect of hydrogen peroxide at different concentrations on linear growth of the tested fungi was evaluated *in vitro*. The reduction of fungal liner growth was positively correlated with hydrogen peroxide concentration. The concentration of 2% was completely inhibited the fungal growth of *R. solani*, *Pythium* sp. and *F. solani*. These results are in agreement somewhat with Angelova et al. (2005) who reported that exposure of fungal mycelia or spores of 12 fungal species to hydrogen peroxide promoted oxidative stress, as evidenced by inhibition of biomass production and spore germination; accumulation of oxidative modified proteins. Under greenhouse conditions, a remarkable reduction in pre- and post- emergence damping off as a response to hydrogen peroxide treatment was detected with all the tested fungi. However, the percentages of reduction were varied depending on the response of each fungus to hydrogen peroxide treatment. *Pythium* sp., *F. oxysporum* and *F. solani* recorded the highest percentages of reduction in pre- and post- emergence damping off as a response to hydrogen peroxide treatment. Abdel-Monaim et al. (2012) demonstrated that plant resistance, in various plant species, can be induced with elicitors such as salicylic acid and hydrogen peroxide against a wide range of pathogens. Such chemical elicitors activate a wide range of protective mechanisms against pathogen; include the fast production of reactive oxygen species; accumulation of phytoalexins; synthesis of defense proteins and peptides (De Gara et al., 2003; Agrios, 2005; Castro & Fontes, 2005). Previous studies also demonstrated the role of hydrogen peroxide in activation of host defense mechanisms, including stimulate activity of enzymes as chitinase and peroxidase followed by a significant increase in the suberin and lignin content (Quiroga et al., 2000). Additionally, Copes (2009) reported that hydrogen peroxide plays an essential role in lignifications, and a strengthening of cell walls at the site of pathogen attack. The results of the present study revealed serious increasing in plant survival as a response to hydrogen peroxide treatment with all tested fungi compared with untreated plants. This finding is in agreement with Abdel-Monaim (2013) who found that the treatment of hydrogen peroxide as seed soaking under green house and field conditions significantly reduced damping-off and root rot/wilt severity in faba bean. Additionally, the treatment with hydrogen peroxide resulted in an increase of survival plants and fresh/dry weights of the survival plants in pots compared with control. Regarding the effect on seed germination, Wojtyla et al. (2016) reported that the application of hydrogen peroxide as seed soaking lead to improve the seed quality, resulting in better germination performance. Moreover, Lariguet et al. (2013) suggested that hydrogen peroxide considered a gene expression regulator of the gene encoding enzyme hydrolyzing the endosperm, which encourage *Arabidopsis* germination. Also, Barba-Espín et al. (2012) found that treatment of pea seeds with hydrogen peroxide as seed soaking resulting in an increase of seed germination as well as the seedling growth. The authors also proposed that hydrogen peroxide has an important role at the beginning of seed germination, since it could act as a signaling molecule. In conclusion, the present study proved that hydrogen peroxide treatment as seed soaking and soil drenching significantly reduced damping-off disease in *T. vulgaris*, in addition to an increase of survival plants compared with control. These results may help to use hydrogen peroxide for controlling damping-off and root rot/wilt disease in *T. vulgaris*.

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