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Evaluation of the efficiency of some antioxidant chemical for germination of bean seeds and on the inhibiting growth of the pathogen causing root and stalk rot disease under laboratory

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Abstract

The study was conducted to isolate and identify the fungi associated with bean root rot, testing their pathogenicity and evaluate the activity of some antioxidants: Ascorbic acid, Benzoic acid, Salicylic acid (SA) and Phylax on bean seed germination and against growth of some pathogenic causes . The results of Isolation and identification showed the presence of nine species which are belong to seven genus of fungi, of these fungi, *R. solani* was found the more abundant followed by *F. solani* and *M. phaseiolina*. While the other fungi recorded lower frequency percentage. The Preliminary test of pathogencity for nine isolates showed a significant increase in the percentage of seed infection compared with 0.00% in control. The results showed that all of the chemical with its three concentrations (1500, 2500 ,5000 ppm) effects on seed germination percentage and varying degrees at 1 to 24 hours , SA treatments showed significantly increased germination compared with other treatments while there were no significant differences between the soaking the seeds in SA at concentration 2500 ppm and the sedimentation with water for 24 hours at 100% each , and that there was an inverse relation between concentrations and germination. There were significant differences in the inhibition growth of fungi with the entire chemical to varying degrees compared to the fungus alone, with a percentage of inhibition of 0.0%.SA superiority reduced the rate of inhibition of tested fungi relative to other chemicals. It was observed that there was a positive relationship between concentrations and the percentage of inhibition .

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Introduction

Phaseolus vulgaris L. is considered as the most important leguminous crops that may fertilize soil in many of the world countries (Buruchara, 2006). In the under developing countries, it is the important leguminous crop due to its nutrient value and their dry grains contain high percentages of proteins, vitamins and nutrient fibers or its residues may be used as animal fodder (Nicolai *et al.,* 2015). Common bean plants can be subjected to infect by soil pathogenic organisms *Fusarium oxysporum, F. solani, M. phaseolina, Pythium* spp, *Sclerotium rolfsii* and *Rhizoctonia solani* (Binagwa *et al.,* 2016; Maina *et al.,* 2017), which are considered more importance at local and international levels. They are recorded as pathogenic causes to seeds rot, damping off pre-post emergence and root rot disease and these diseases cause effect on plant growth and its productivity (Matloob, 2012; Valkonen and Marcenaro, 2016). Many researches mentioned to the frequently seen pathogenic causes which were isolated from bean plants and they causes severe losses the *F. solani, R. solani* and *M. phaseolina* (Mwang-ombe *et al.,* 2007). A number of means were used to combat this disease, including the use of chemical pesticides, but repeated use led to soil pollution as well as human health and the environment as a direct result of the residual impact of the chemicals used in the control (Choi and Hwang, 2011). So, safe control ways required safe test and active ways to manage this disease. There are many chemical compounds Ascorbic acid, Benzoic acid, Salicylic acid and Phylax that may be used in the control operation and these chemicals have high inhibition ability against many soil borne fungal pathogens and have positive effect in seed germination percentage increase (Gomah, 2010; Hassan, 2013; Al-Juboory *et al.,* 2016). Hemeda (2009) found when he used acids like Ascorbic acid, Benzoic acid, Salicylic acid at 20 mM that these acids inhibited the fungi growth in which they cause bean roots rot and these were *Alternaria alternata, F. oxysporum f.* sp *phaseoli. M. phaseolina, R. solani*, and when the seeds were immersed in these acids at 1 mM concentration for 2 hours, it was noticed that plants fresh weight increased. The study was aimed to isolate and identify the causal agents of bean root and detection of pathogenic fungi isolates using bean seeds *in vitro*, test efficiency of some antioxidant chemical acids is evaluated on the germination of bean seeds *in vitro* and against some fungal isolates on the PDA media.

Materials and methods

Samples collection

Infected bean plants were collected from Al-Madaen, Al- Dora and Al-Rathwania regions in Baghdad governance during the spring season (from 15-3-2016 to 15/4/2016). These infected plants had disease symptoms such as leaves dryness and yellow color and presence lesions and decay on roots and stem that had brown color and in advanced causes they got plant wilt and death. The infected plants were pulled out soil and put into polyethylene bags and then they were transferred to the laboratory for isolation.

Isolation and identification of the pathogen

The infected bean plants that had disease symptoms (lesion and decay) were cut at 5 cm height from crown region, and were washed carefully under running tap water to remove the soil and the impurities, Roots and the crown of each plant was cut into approximately 0.5 cm and surface sterilized in 1% sodium hypochlorite(6% - free chloride) for 2 minutes after surface sterilization it rinsed with sterile water and dried on sterilized filler papers. Four pieces of surface sterilized plant materials were separately plated in each petridish of 9 cm diam containing 15-20 ml of Potato Dextrose Ager (PDA) supplemented with 100 mg/L chloromphenicol. which was sterilized in autoclave at 121 C^{o} and 1.5 kg/ cm-2 pressure for 15 minutes after 4 days of incubation at 25°C single spore isolation from each developing colony was done to have pure culture.. All the pieces were examined under the high and low power of a compound microscope. Isolates were identified to species level according to their cultural and morphological features (Parmeter and Whitney, 1970; Barnett and Hunder, 1972; Leslie and Summerell, 2006).

Fungus species were recognized till the species level by Dr. Hadi Mahdia Aboude/agricultural research department/Ministry of sciences and technology depending on the adopted taxonomic keys. The isolation frequency of the species was calculated as follows:

$$\text{Frequency (\%)} = \frac{\text{Number of pieces containing the fungus}}{\text{Total number. of pieces}} \times 100$$

(Juber *et al.,* 2016)

Pathogenicity Tests

Pathogenicity of the pathogenic fungi using bean seeds in laboratory

This test was done in mycotoxin laboratory which is belong to plant protection department/College of Agriculture, University of Baghdad. , 14 isolations that were obtained by isolation operation and it had high presence ratios were tested. Three fungus isolation to *F. oxysporum*, three to *F. semitectum* , three to *F.solani*, two fungus isolations *to M. phaseolina* and three isolates to *R. solani* on the sterilized (WA) having Tetracycline in 9 cm - diameter petri dishes. Bean seeds were planted after their sterilization in sodium hypochlorite for two minutes, In the center of the dish, a 5mm diameter disc is taken from the edge of the pure fungal colony of the above mentioned isolates, that were grown on PDA at 5 days age. The petridishes were incubated at 25 ±2 C° for 3 days. Bean seeds were planted after their sterilization in sodium hypochlorite for two minutes, the seeds of the beans were then sterilized in sodium hypochlorite solution for two minutes, they were washed by distilled water and dried on filter paper , they were put at 1 cm far from petri dish edge circular at average of 10 seeds per petri dish .

Five petri dishes were used for each isolation as replicates beside presence of control treatment which did not have pathogenic fungus. The petridishes were incubated at 25±2 °C. The design of the experiment was CRD and the data were taken after 10days from planting by estimating the infection percentage using the following equation.

$$\%\text{Infection} = \frac{\text{No. of the infected seeds}}{\text{Total No. of seeds}} \times 100$$

Al-Juboory *et al.,* 2016)

Effect of chemical (acids) on germination of bean seeds in laboratory

This experiment was carried out to test the effectiveness of chemicals and the effective concentration of Ascorbic acid, Benzoic acid, Salicylic acid and Phylax compound on germination of bean seeds. The seeds were sterilized by sodium hypochlorite and washed by sterilized distilled water and then were dried on sterilized filter papers. The seeds were soaked individually inside three concentrations (1500, 2500 and 5000 ppm) of these chemical materials for one hour and 24 hours. The seeds were distributed on a layer of wet and sterile cotton in in 9cm -diameter petri dishes. Beside presence of control treatment. The seeds were planted after water soaked for one and 24 hours by five seeds per each petri dish (Gomah, 2010). Five petri dishes were used for each concentration and time. They were incubated at 25±2 °C. After 10 days, the seeds germination percentage was recorded.

Effect of some chemicals against in the inhibition growth of pathogenic fungus on the PDA medium

To choose the effective material and the suitable concentration (1500, 2500, 5000 ppm) of the chemical materials Ascorbic acid , salicylic acid ,benzoic acid and Phylax against growth of the diseased fungus isolations, *F. oxysporum* (Fox2) (*F. semitectum* (Fse1), *F. solani* (Fso2), *M. phseolina* (Mp1) and *R. solani* (Rs1 ,Rs2 , Rs3) by using the toxic PDA . A 500 ml glass flask was prepared for each concentration of the three concentrations and for each chemical mentioned above, containing the sterilized PDA medium, leaving a second flask containing only the middle of the plant (without addition) three glass flasks were prepared to each of the above chemical materials . The flasks contained the sterilized PDA and to each flask one of the three concentrations of each of the chemical acid (1500, 2500 and 5000 ppm) were added with presence of control treatment (PDA only).

Each concentration was added before solid of the media on the flask and good shaking was used to ensure homogenous distribution and them the media were distributed in 9cm- diameter plastic dishes and inoculated by five (5 mm- diameter) discs that were taken from edges of the pure pathogenic fungus colonies grown on the PDA media for five days individually. Three replicates were used to each concentration beside control treatment for each fungus. All petridishes were incubated at 25±2 ^{o}C and when the fungus cultures growth in control treatments dishes was completed the final results were recorded by estimating media of two orthogonal diameters of the fungus colony.

The inhibition percentage was estimated according to the following equation.

Inhibition (%) = Fungal growth diameter in control – fungal growth diameter in treatment /fungal growth diameter in control ×100

Result and discussion

Isolation and identification of infected plants

Results of isolation and identification showed presence nine species belong to seven genus associated to the root rot and stem bases (Table 1) which had rot and lesion symptoms disease and the *R. solani* fungus had the priority with variance presence percentage between the regions ranged between 75 -100% followed by *F. solani* with percentage ranged between 40.5- 65.2%, while the *M. phaseolina* presented between 9.5-35.5%.

Table 1. Fungi accompanying to the infected roots of bean plants and ratios and regions of them.

Fungi	Frequency %			
	Al-Maden	AL-Dora	Al-Rathwania	Average
Alternaria alternata (Fres) Keisler	6.8	14.5	10.6	10.63
Drechslera australiensis (Bugnicourt) Subram and Jain	6.5	3.8	5.00	5.10
Fusarium oxysporum Schlecht.	25.4	35.50	22.65	27.85
F. semitectum Berk. and Rav.	30.7	20.6	25.5	25.60
F. solani (Mart.) Sacc.	55.0	40.5	65.2	53.56
Macrophomina phaseolina(Tassi) Goid.	25.7	35.5	9.5	23.56
Rhizoctonia solani Kuhn	75.0	90.8	100.0	88.6
Trichoderma harzianum Rifai	3.2	2.61	4.11	3.30
Ulocladium atrum Preuss	2.1	3.00	0.00	1.70

These results were in agreement with El-Mougy *et al.* (2007) and Valkonen and Marcenaro (2016). Previous studies which mentioned that *R.solani , F.solani and M. phaseolina* fungi were the main causes of roots rot disease and agreement with Mwang-ombe *et al.* (2007) of the spread of a soil borne fungal pathogen in the bean fields of the fungal *F.solani, M. Phaseolina* and *R. solani* by surveying the root rot of bean for ten fields in Kenya. They found that the main cause of root rot disease of beans is *R. solani* followed by the *Fusarium* with an appearance ranging from 5.6-65.2%. Roots rot disease spreads in many region in Babel governance and *F. solani* is the more spread fungus and it was founded in most of the tested samples with different percentage ranged between 14-65% followed by *R. solani* and *M. phaseolina* at presence ratio 28.1 and 22.6% respectively(Matloob, 2012). Timothy *et al.* (2013) mentioned that the most important causes of root rot disease are *M. phaseolina* and *Fusarium* spp and they appeared in most samples which were collected from Latin America, Middle America and Carbine sea fields, *F.oxysporum* and *F. semitectum* appearance in Al-Maden , AL- Dora and Al-Rathwania regions samples ranged between 22.65-35.5% and 20.6-30.7 % respectively.

Table 2. Effect of pathogenic fungi isolates on percentage of bean seed infection on the Agar water medium.

SL. No.	Treatments	% Infection
1	Control without fungi	*0
2	*Fusarium oxysporum* (Fox1)	56
3	*F. oxysporum* (Fox2)	68
4	*F. oxysporum* (Fox3)	28
5	*F. semitectum* (Fse1)	60
6	*F. semitectum* (Fse2)	52
7	*F. semitectum* (Fse3)	36
8	*F .solani* (Fso1)	68
9	*F .solani* (Fso2)	84
10	*F .solani* (Fso3)	62
11	*Macrophomina phaseolina* (Mp1)	76
12	*Macrophomina phaseolina* (Mp2)	60
13	*Rhizoctonia solani*(Rs1)	100
14	*R. solani* (Rs2)	96
15	(Rs3) *R. solani*	100
	LSD at P ≤ 0.05	

These results agreed with many studies that explained that species of *Fusarium* spp such as *F. oxysporum*, *F. proliferatum, F. semitectum* and *F. solani* were from the main causes of root rot in some field of vegetables crops (Salari *et al.,* 2012; Zakaria and Sasetharan, 2014).

These results agreed with Al-Juboory *et al.* (2016) that isolates of *M. phaseolina*, which were isolated from watermelon plants grown with root rot disease, significantly reduced the germination rate of the seeds on the WA plant ranging from 0 to 65%.

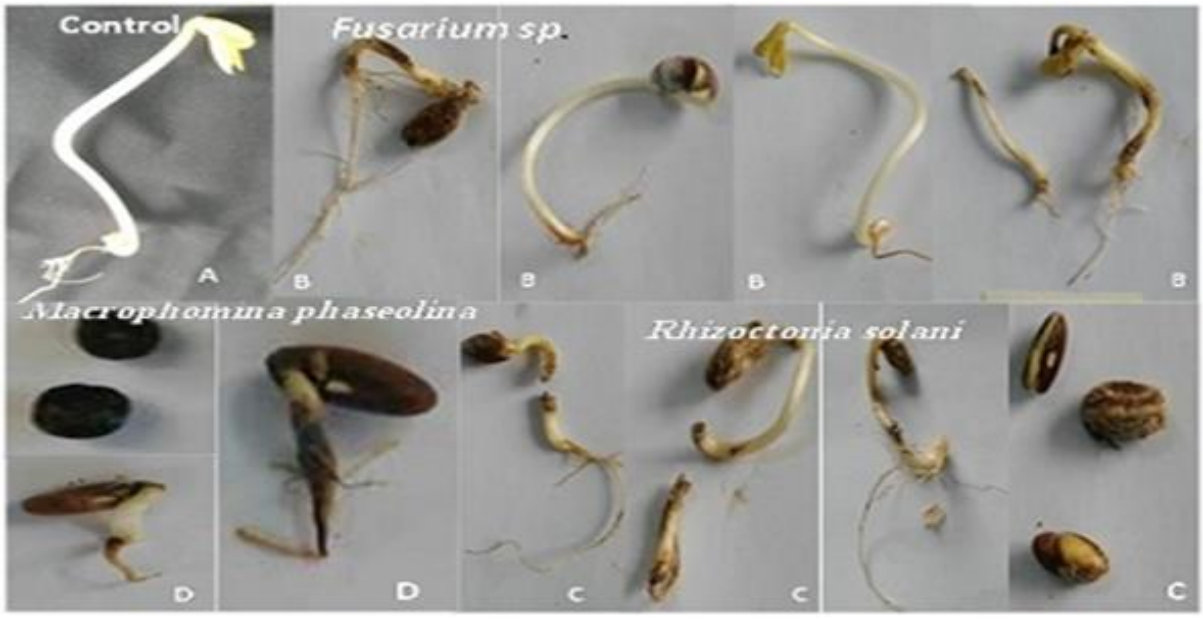

Fig. 1. Effect of some pathogenic fungi isolates on percentage of bean seed and seedlings infection, A-healthy seedling , B- infected seedlings by *Fusarium* sp., C- seeds and seedling infected by *R. solani* fungus, D- seeds and seeding infected by *M. phaseolina*

These results agreed with finding of each of Al-Juboory (2002), Matloob (2012), Al-Mosawe (2012), that the *F. oxysporum*, *F. semitectum* and *F. solani* species were caused that caused root rot disease in some plants of Family Leguminosae. The results of microscopic examination revealed a number of fungi associated with the roots and bases of bean stalks such as *Alternaria alternata*, *Drechslera australiensis* and *Ulocladium Atrum*, with ratios of less than 10.63, 5.10 and 1.7% respectively. Presence of these fungi may be attributed to their high ability in productive units production and to tolerance of the inconvenient condition and to their ability to stay live with host presence and their family range is wide and their large competition ability against the other soil organisms.

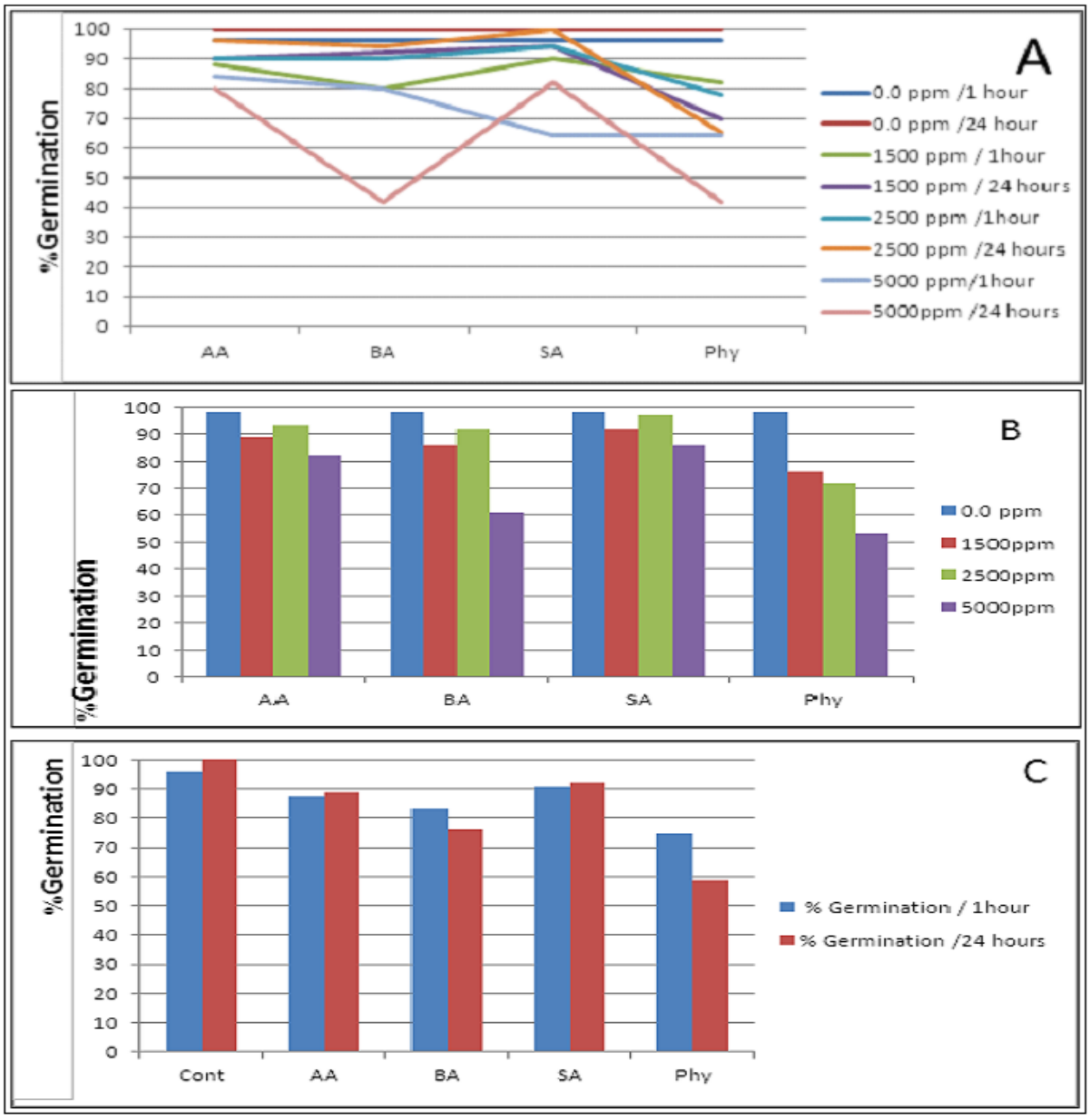

Fig. 2. Effect of *some chemicals* on germination of bean seeds after soaked for 1 and 24 hours , A Effect of acids concentrations and soaking periods interaction , B- effect of acids and concentrations interaction , c- effect of acids and seeds soaking period interaction. LSD (p=0.05) Acids=0.98 – LSD (p=0.05) Conc.=0.85- LSD (p=0.05) Time =0.69 – LSD (p=0.05) Acids × Conc=1.70 LSD (p=0.05) Acids×Time =1.39 – LSD (p=0.05) Conc × Time =1.20 – LSD (p=0.05) Acids × Time × Conc =2.41.

There are some factors that contribute in rising the infection ratio of roots rot disease such as continuation on using inconvenient agricultural systems, soil fertility levels decline, use seeds collected for the same previously cultivated farms, repeating crop cultivation yearly and use sensitive species to roots rot disease infection. The incidence and severity of the disease varies according to environmental conditions and soil conditions, such as the number and type of pathogens present under certain conditions (Morris, 2017).

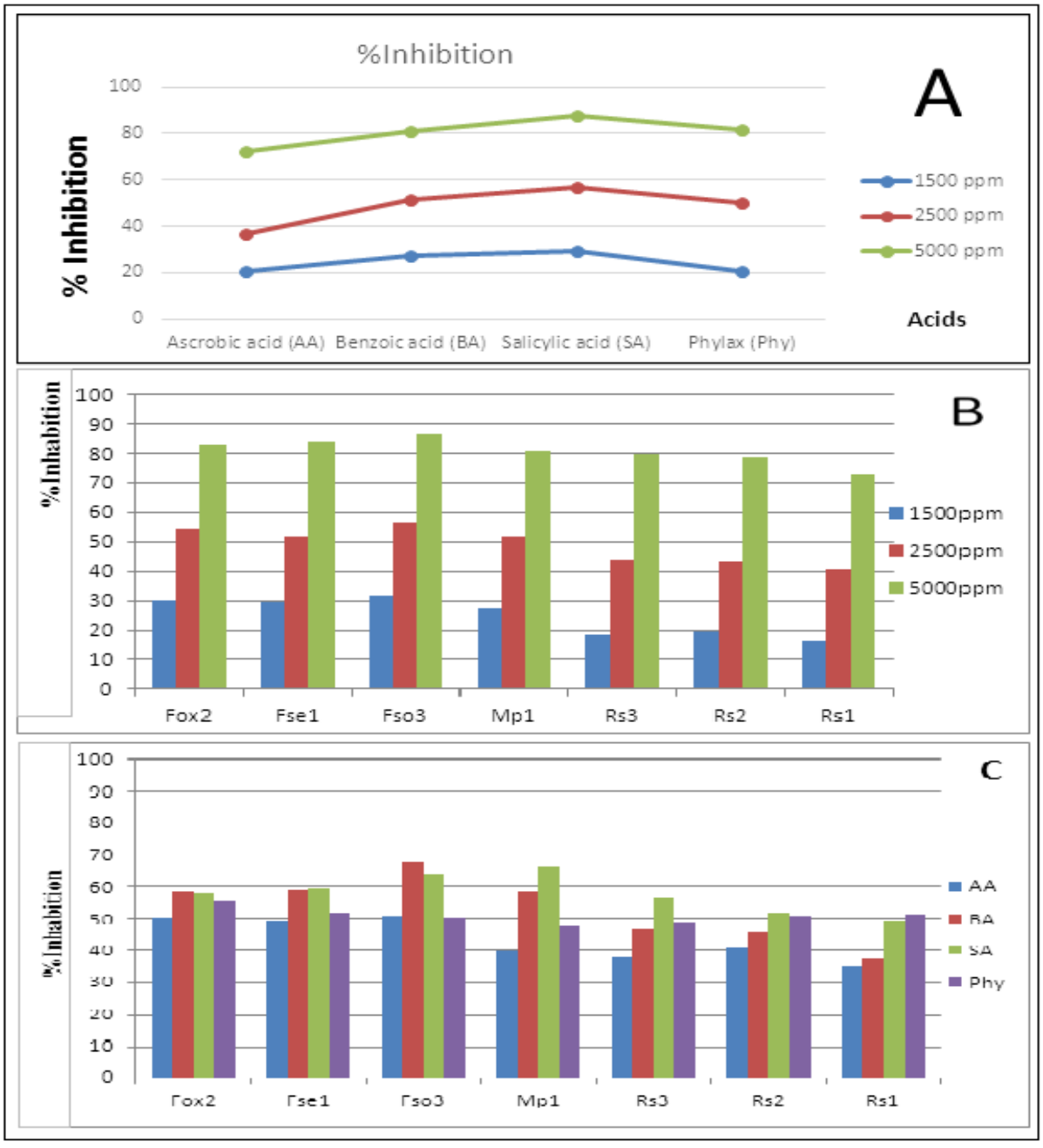

Fig. 3. Effect of some chemicals against in the growth inhibition of pathogenic fungus on the PDA medium:, A- effect interaction of acid and their concentrations ,B- effect interaction of concentration and % inhibition , c- effect interaction of acids and inhibition ratios average. LSD (p=0.05) acids= 1.73 – LSD (p=0.05) Conc= 1.50- LSD (p=0.05) Fungi= 2.29- LSD (p=0.05) acids×Conc = 3.00- LSD (p=0.05) acids×Fungi = 4.59- LSD (p=0.05) Fungi×Conc = 3.97 - LSD(p=0.05) acids×Conc ×Fungi = 7.95.

Pathogenicity of isolated fungi

The results showed that all tested fungal isolates showed a significant increase in the rate of seed infection (Table 2). There was a significant difference in the pathogenicity between the isolates of the fungi. The infection ranged from 28% to 100% compared with zero in control treatment. It caused appearance of roots seed rot and lesion symptoms in seedling stalk region as ring and the infection caused damping off (Fig. 1). Isolations of Rs1 , Rs2 and Rs3 fungus gave high infection reached 100, 96 and 100% respectively at significant difference at the other fungus treatments , followed by *M. phaseolina* (Mp1) and *F. solani* (Fso2) isolations which gave 76 and 84% respectively while the Fox2 fungus isolation gave 68% .The rest isolations ranged in their infection ratio in their treatments between 28 - 62% and this may be due to isolations variance effects on infection percentages to the genetic variance between the fungus isolations which were collected from different regions. These results agreed with the findings of Al-Juboory (2002), Matloob (2012) and Al-Mosawe (2012) in their study pathogenic fungus isolations from infected roots of Faba bean, bean and cowpea. These results also agreed with results of Al-Juboory (2016) who found that *M. phaseolina* isolations that were isolated water melon plants infected by roots rot caused significant decline in germination ratios of water Mellon seeds on the WA media to zero -65 % .

Each value represents the mean of 5 replicates The results(Table 2, Fig. 1) showed that all the tested fungus isolations were pathogen and there was variance in their infection ratio and that may be due to the variance in their ability on produce of pectinase, they have the ability to secretion polygalacturonase enzyme while the non- pathogenic fungus isolations did not have the ability to produce this enzyme or they have low activity in production of this enzyme or to the level of the poison secondary metabolic compounds that cause embryos killing or to the variance in the quantity and quality of these poison materials (Vidhyasekaran, 1997 and Lozovaya *et al.,* 2006).

Many researchers mentioned to the *Fusarium* spp that may excrete number of enzymes such as chitinase, cellulase, protease and polygalcturinase and which have a major role in parasitism on living plant cells (Lozovaya *et al.*, 2006). While the researches indicated that *R.solani* produces many enzymes such as pectin lyase , cellulase, phosphataseor pectinase , Proteinase and pectin methylesterase and these enzymes are responsible of seeds rot occurrence and germination prevent(Bertagnolli *et al.*, 1996), Kumar and Sharma (2013) mentioned that *M. phaseolina* has the ability to produce a Secondary metabolites compounds which may influence on seeds growth such as , Isoasperlin , phaseolinone , and phomenon. Bhattacharya *et al.*(1994) mentioned that fungus isolations variance in their ability to produce phaseolinone passion and isolation fierce depends on the produced poison quantity and seed germination inhibition is correlated with the produced poison quantity.

Effect of the chemicals (acids) on bean seeds germination

The results showed (Fig. 2A) that the Ascorbic acid (AA), Benzoic acid (BA), Salicylic acid (SA) and Phylax (phy) at their three concentrations (1500, 2500, 5000 ppm) affected the bean seeds germination percentage at variable degrees when the seeds were soaked for 1 and 24 hours (Fig. 2A) the Salicylic acid at their concentrations exceeded significantly after 1 and 24 hour soaked and reached 90,94, 94,100, 88 and 82% respectively compared with the other acids treatments while the was non-significant differences between seeds soaked at 2500 ppm concentration treatment and 24 hour water seeds soaked treatment, followed by Ascorbic acid seed soaked in which the germination percentages in its treatments were 88, 90, 90, 96, 84 and 80% respectively. Germination percentages were declined when seeds were soaked in Phylax compound they reached 82,70, 78,65, 64 and 42 % at the three concentrations respectively due to that Phylax compound consists from groups of acids Citric acid, Formic acid, Lactic acid, Ortho-Phosphoric acid, Propionic acid and Sorbic acid and this may influence on embryo vitality.

There was an inverse relation between concentrations and germination ratios (Fig. 2, A and B) and the

germination rates at 1500 ppm for AA, BA, SA and Phy between 76-92% and at 2500 ppm were 71.5-97% and they declined to reach 53-85% at 5000 ppm concentration. It is perhaps increase of acids concentrations and immersion time decrease germination ratios due to photo toxicity, These results agreed with results of Gomah (2010) who mentioned that faba bean seeds germination ratio at 24 hour immersion in AA and SA acids at 2.5 mM concentration was better than treating these seeds with Benzoic acid , Hydroquinone and citric and the germination ratio decreased with increase the concentration due to plant toxicity. The high concentrations of Ascorbic acid may inhibit the germination through destruction of the forms of active oxygen and may result germination start failure (Takemura *et al.,* 2010) . Chemical acids work to regulate certain physiological processes in the seeds, including germination. (Akram *et al.,* 2017).

Effect of some chemicals against in the growth inhibition of pathogenic fungus on the PDA medium

The results indicate (Fig. 3- A, B,C)) Showed significant differences between the inhibition percentage on the mycelial growth of pathogenic fungi isolates (Fox2, Fse1, Fso2, Mp1, Rs1, Rs2 and Rs3) at the studied concentrations(1500, 2500 , 5000 ppm) of the chemical materials AA, BA, SA and Phy in variable degrees compared with control treatment in which the inhibition percentage was 0.00%. SA acid gave at the three concentrations (Fig.3A) significant decline in inhibition percentage rate averages reached 29.58, 57.9 and 87.91% respectively While AA was the least significant reduction in the percentage of inhibition percentage of 20.87, 36.83 and 72.26% at the concentrations of 1500, 2500 and 5000 ppm respectively . The results showed a positive correlation between the tested concentrations and the percentage of inhibition of pathogenic fungi (Fig. 3B) as they increased by increasing these tested concentrations to a maximum of 86.71% for Fso2 at concentration of 5000 ppm, while significantly decreased to 56.29 and 31.66% Concentrations 1500 and 2500 ppm respectively.

The less significant decline of inhibition percentage averages in isolation of *R. Solani* (Rs1) fungus and inhibition percentage were 16.22, 40.54 and 72.99% respectively at the studied concentrations.

The results which are shown in(Fig. 3-C) showed superiority of SA acid on the AA,BA and Phy in reduction the tested fungi inhibition percentage average(Fox2, Fse1, Fso2 Mp1, Rs1, Rs2 and Rs3) and they were 57.89, 59.53, 63.83 , 66.39 , 51.57, 51.73 and 56.44% respectively And significantly different from the percentage of inhibition of the fungi isolates mentioned above at the tested acids The AA was the least significant reduction, with the percentage of inhibition of fungi above 50.05, 49.37, 50.77, 39.90, 34.84, 40.63 and 37.69% respectively. The effect of high concentrations of salicylic acid in inhibiting the growth of pathogenic fungi is attributed to the role of aspirin through its direct effect as an inhibitor of the vital processes required for the growth of pathogens, Which leads to the fold and stop growth (Uquillas *et al.,* 2004). The effect of ascorbic acid in inhibiting the growth of pathogenic fungi may be due to its direct effect as an inhibitor of the vital processes necessary for the growth of pathogens, thus inhibiting their growth and death (Ahmed, 2010). These results support the agreement of many researchers that high concentrations of chemical compounds inhibit the growth of fungi but at the same time reduce the proportion of seed germination significantly (El-Mougy, 2004; Hemeda, 2009; Mbazia *et al.,* 2016), The results of this test at its general scale agreed with finding of Gomah (2010) in when number of acids were used such as Ascorbic acid ,Benzoic acid , Citric acid ,Salicylic acid at concentrations (2.5, 5, 10, 20, 30 and50 mM) and they resulted fungi growth inhibition that cause faba bean root rot *F. oxysporum, F. semitectum, F. subglutinans, M. phaseolna* and *R. solani* and it is noticed that fungi inhibition ratio increased with concentration increase and differed with acid and fungus variance.

The SA inhibited growth of *F. oxysporum* and *F. semitectum* completely at 10mM concentration while the 15mM concentration was active in *R. solani* and *F. subglutinans* fungi inhibition.

These results agreed with findings of Hassan (2007) who found positive relation between five concentrations (10-50 mg.L^{-1}) of SA and inhibition of the pathogenic *Pythium aphanidermatum* As it increases by increasing these concentrations to a maximum of 100% at the concentration of 400mg.L^{-1} and there were significant differences in the pathogenic fungus of rate inhibition. Other studies have shown that chemical compounds have a high ability to inhibit fungi that cause root rot and seedling death The high concentrations of the chemical compounds inhibit fungi growth but in the same time they reduce largely seeds germination percentage (El-Mougy, 2004; Hemeda, 2009).

Recommendations

The use of antioxidant compounds especially Salicylic acid and Phylax in Inhibition of fungal growth And their use in IPM programs effective role in control and not harm the environment.

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