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THE BIOLOGICAL STERILIZATION EFFICIENCY TEST IN CONTROLLING PATHOGENIC FUNGUS *RHIZOCTONIA* SPP. WHICH INFECTS POTATOES (*SOLANUM TUBEROSUM*)

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Abstract

Soil samples and infected potato plants were collected from potato growing areas from several areas in Baghdad province (Yusufiya, Abu Ghraib, Taji and Nahrawan), where the results of laboratory isolation showed that 14 isolates had been obtained from the fungi *Rhizoctonia* spp. Furthermore, there was a variation in the pathogenicity of fungi isolates ranged from highly pathogenicity and pathogens isolates, as the disease index average of the fungi isolates has reached between (3.55-5.00). The results of nuclei dyeing showed that the isolates cells *Rhizoctonia* spp. possessed multinucleate ranged between 3-10 nuclei except the two isolates R5 and R6, which were unique in having two nuclei in each cell of hyphae. The results showed that the nucleotide sequences of the Internal Transcribed Spacer (ITS) site for the tested isolates were 99-98% compatible with the global isolates. Therefore the diagnoses of these isolates was confirmed as *R. solani*, while the R5 and R6 isolates was matched with the global isolate AB196640 by 99%, that belonged to the group BNR AG-A binucleate. Moreover, the results showed that the experience of the biological sterilization efficiency test of the soil, which was carried out within the pots, showed that the infection severity for all the treatment decreased with a significant difference from the treatment of the pathogenic fungus (comparison). It was observed that the treatment of the wheat bran treatment was superior with a significant difference from all the treatments and the infection severity of the pathogenic fungus in the biological sterilization experiment for two weeks was 6.66, 0.01, 0.01% for the concentrations of 250, 500, 1000 g/m^2 soil, respectively. As well as, it was observed when testing the period of biological sterilization for a month within the pots, all treatments had a significant difference in reducing the infection severity of the pathogenic fungus *R. solani*. The treatment of wheat bran was significantly superior in reducing infection severity of the pathogenic fungus by the same tested concentrations which reached 0.010% for all concentrations compared with the of the pathogenic fungus treatment (comparative) at 100%. Finally, the biological sterilization efficiency of the soil in the field experiment showed that the infection severity was reduced in all treatments with a significant difference. As well as, the treatment of wheat bran was significantly superior to the pathogenic fungus treatment, comparative by (86%), where it reduced the infection severity of pathogenic fungus which was 6.66, 0.01 and 0.01% for concentrations of 250, 500 and 1000 g/m^2 soil respectively.

Key words: Stem canker and Black scurf, *Rhizoctonia solani*, *Rhizoctonia* spp. (BNR), Bio-sterilization of the soil.

Introduction

The Potato (Solanum tuberosum) belongs to the Solanceae family, one of the world's most important crops, which ranks fourth in the world after wheat, corn and rice (Bowen, 2003). The potato crop is exposed to many fungi diseases, including stem canker and black scurf caused by *Rhizoctonia* solani, which is one of the most important pathogens found in the soil and attacks the potato crop causing a significant damage and loss of production in quantity and quality (Larkin, 2001). Biological sterilization method was used as an alternative to methyl bromide because it is effective in eliminating soil pathogens and is environmentally safe and economical (shennan *et al*., 2014). This method depends on adding organic matter to the soil as a source for Carbon to encourage the rapid growth of microorganism. As several materials were used, including rice bran, wheat bran, then soil watered to fill soil pores, reduce oxygen and then the soil is covered with a polyethylene covering to prevent oxygen penetration into the soil surface (Shennan *et al*., 2014). This method aims to create an anaerobic environment for stimulating anaerobic microorganisms to grow, including *Clostridium* spp., *Enterobacter* spp. bacteria. Which uses carbon as an energy source and

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produces many gases, including Carbon Dioxide CO_2, Hydrogen Sulfide H_2S, Ammonia NH_3, Methane CH_4, in addition to many organic acids, aldehydes, alcohol, ammonia, mineral ions and organic compounds that are toxic to many pests and pathogens (Momma, 2008). Some studies have indicated the efficacy of the biological sterilization method using wheat bran in reducing the disease percentage of Fusarium oxysporum, causing wilt disease on Tomato (Lycopersicon esculentum mill) (Momma *et al*., 2006), also in reducing the numbers of *Ralstonia solanacearum* bacteria that causes bacterial wilt of tomato (Momma *et al*., 2007). As well as, the efficiency of biological sterilization using rice bran to reduce the disease percentage of Verticillium dahlia, which affects strawberry (fragaria ananassa duch) (Shennan *et al*., 2010). Therefore, the aim of this study was to evaluate the biological sterilization efficiency method using three local organic materials in controlling the pathogenic fungus *Rhizoctonia* solani on the potato crop.

Material and Methods

Isolation of pathogenic fungus *Rhizoctonia* spp. from the soil and infected potato plants

Soil samples and infected potato plants were collected from potato growing areas from several areas in Baghdad province (Yusufiya, Abu Ghraib, Taji and Nahrawan) district. At 200 grams were weighted of each soil sample and placed in glass flasks, then the volume completed to 1 liter and after 10 minutes, the sample passed through a sieve measuring 60 mesh, while the plant residues were collected from the sieve and washed with running water. Furthermore, he potato-infected parts (roots and stolon) were cut into 0.5 cm small pieces and then the residues of isolated plants from soil and the plant parts infected were sterilized with sodium solution hypochlorite NaClO (1% chlorine free) for 2 minutes. Subsequently, it was washed with sterile distilled water and dried on filter paper and then transferred to a petri dish containing on a (Potato Dextrose Agar) PDA sterile medium by 4 pieces/dish. Finally, the dishes incubated at a temperature of 2 ± 25°C for 3 days and the growing fungal colonies then tested around the plant pieces and the pathogenic fungus then purified in Petri dishes contained on the PDA medium.

Pathogenicity test of the pathogenic fungus *Rhizoctonia* spp. at laboratory

The pathogenicity of pathogenic fungus isolates was tested using local radish seeds by preparing a medium water agar (WA). The medium of the fungi been pollen with a piece of 0.5 cm diameter of *Rhizoctonia* spp. and incubated at 25°C for 48 hours. Radish seed was planted about six seeds/dish with a distance of 1 cm from the edge of the fungal colonies by four replicates/isolation. Additionally, four dishes were planted with radish seeds only without the presence of pathogenic fungi as a comparison and after 6 days of planting, length of the colored area in radish seedlings was calculated as a result of Pathogenic fungus infection and calculation the infection severity index for each isolate (Sneh *et al*., 2004).

Dyeing the nuclei

The average of nuclei count was determined in each cell of the *Rhizoctonia* spp. using Aniline blue dye as described by (Herr, 1979). At the rate of four replicates for each fungal colony and each replicate 10 fungal cells were then tested by microscope under X400 magnification.

Molecular Diagnosis

The use of general primer (TCC GTA GGT GAA CCT GCG G) ITS1 and ITS4 (TCC TCC GCT TAT TGA TAT GC) for diagnosis of fungi isolates. DNA for fungi isolates was extracted using the extraction kit produced by Microgene (South Korea). The DNA was doubled in the polymerase machines (PCR) according to the following program: Pre-Denaturation at 95°C for 5 min, Denaturation at 95°C for 30 seconds, Annealing at 60°C for 30 seconds, Extension at 72°C for 45 seconds. A final extension at 72°C for 5 min and the nucleotide sequences were read and compared with those in the gene bank (https: // www.ncbi.nlm.nih.gov).

The biological sterilization efficiency test within plastic pot and the field

The experiment was carried out within pot in the plastic house belonged to the Plant Protection Department. Where Two periods have been adopted, which they are 2 weeks and 4 weeks to demonstrate the efficacy of the biological sterilization method in inhibiting the pathogenic fungus in the industrial polluted soil. A silty soil free of impurity was prepared and sterilized for two consecutive times at the Autoclave at 121°C and 15 bar pressure for one hour at a time. The soil was distributed according to the treatments in polyethylene bags measuring 50 × 80 cm and supported by three types of organic materials wheat bran, wheat straw and the cutoff cynodon dactylon residues in three concentrations. Each treatment polluted with the pathogenic fungus grown on sterile wheat seeds by 1g / 1kg soil, the soil was watered well to the field capacity. The field experiment was carried out at the experiment station B of the college of Agricultural Engineering Sciences - Plant Protection Department during the spring season 2018-2019, where the field soil was prepared by plowing it twice in an orthogonal way, softened and leveled well. The biological sterilization treatments were added with the addition of

Rhizoctonia sp., loaded with wheat seeds by 10 g /1 m of planting line, then mixed well according to each treatment as mentioned previously. Moreover, the soil was irrigated to the field capacity and then covered with a polyethylene and keep the sides well closed and the treatments are left for 30 days for the purpose of completing the biological sterilization process. The treatments included: comparison treatment with *Rhizoctonia* sp., comparable treatment without *Rhizoctonia* sp. and treatment of wheat bran at a concentration of 250 g / m^2 with pathogenic fungus. As well as, treatment of wheat bran at a concentration of 500 g / m^2 with pathogenic fungus, treatment of wheat bran at a concentration of 1000 g / m^2 with pathogenic fungus and treatment of wheat straw at a concentration of 250 g / m^2 with a pathogenic fungus. Furthermore, treatment of wheat straw at a concentration of 500g / m^2 with a pathogenic fungus, treatment of wheat straw at a concentration of 1000g / m^2 with pathogenic fungus. Finally, the cutoff cynodon dactylon residue treatment at a concentration of 250g / m^2 with pathogenic fungus, the cutoff cynodon dactylon residue treatment at a concentration of 500g / m^2 with pathogenic fungus, the cutoff cynodon dactylon residue treatment at a concentration of 1000 g / m^2 with pathogenic fungus. After the two biological sterilization periods ending (2 weeks and 4 weeks), the treatment soil was distributed separately for each period of time in plastic pots measuring 10 × 13 × 37 cm and acapacity of 3 kg soil then left for 3 days. Pots were planted with potato tubers of Burren variety by two tuber / pot, the pots were watered when needed until the end of the experiment. After the end of the biological sterilization process in the field, the polyethylene cover was removed and the soil was left for four days. The soil was then divided into a furrow and planted by potato tubers of a Burren variety of 20 tuber per furrow and 20 cm between tuber and another with 4 replicates/treatment. All necessary planting treatments of potato plants were carried out throughout the experiment and the infection severity was calculated based on the disease index and some growth parameters were calculated (number of branches, length of branches, number of tubers, the weight of tubers, the wet and dry weight of the root). The experiment was carried out according to the Completely Randomized Design (CRD) with three replicates/treatment for the pot experiment and Randomized Complete Block Design (RCBD) with three replicates for the field experiment.

Results and Discussion

Isolation of pathogenic fungus *Rhizoctonia* spp. from soil and infected plants

Results of laboratory isolation showed 14 isolates from *Rhizoctonia* spp. that isolated from soil and from infected potato plants (roots and stolon). These isolates have shown a clear difference in the rapidity of their growth and composition of sclerotia and the Mycelium density. As well as, the color of the colonies varies from brown to dark brown and constriction the branching cell in the emergence site and the formation of barriers in the branches near the emergence point also the formation of cells often called short cells, chladospores, or Scterotial cells, which is consistent with what (Parmeter and Whiteny, 1970) described.

Pathogenicity test of the pathogenic fungus *Rhizoctonia* spp. at laboratory

There was a variation in the pathogenicity of the isolates fungus ranged between highly pathogenicity and

Table 1: Pathogenicity test of *Rhizoctonia* spp. isolation on the radish seeds in the laboratory.

Seq.	Isolate code	Isolate site	Rate of * infection severity index	Pathogenicity**	Isolate source
1	Rs1	Abu Ghraib	4.45	highly pathogenicity	Soil
2	Rs2	Taji	4.62	highly pathogenicity	Soil
3	Rs3	Yusufiya	4.75	highly pathogenicity	Soil
4	Rs4	Yusufiya	4.83	highly pathogenicity	Soil
5	R5	Abu Ghraib	5	highly pathogenicity	Soil
6	R6	Abu Ghraib	5	highly pathogenicity	Soil
7	Rs7	Yusufiya	5	highly pathogenicity	Root
8	Rs8	Nahrawan	4.45	highly pathogenicity	Root
9	Rs9	Nahrawan	4.66	highly pathogenicity	Root
10	Rs10	Taji	4.92	highly pathogenicity	stolon
11	Rs11	Yusufiya	5	highly pathogenicity	stolon
12	Rs12	Nahrawan	3.55	highly pathogenicity	Root
13	Rs13	Yusufiya	3.80	pathogens	stolon
14	Rs14	Abu Ghraib	3.90	pathogens	stolon

* Each number in the table represents a rate of 4 replicates and each replicate includes 6 radish seeds.

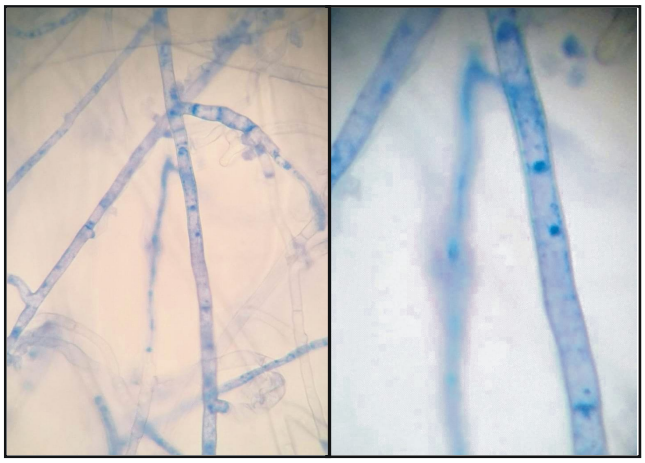

Fig. 1: Binucleate cells of *Rhizoctonia* spp. dyeing by Aniline blue.

pathogens isolates, where the disease index average of the fungi isolates has reached between 3.55-5.00 according to the disease index that described by (Sneh *et al*., 2004), (Table 1). Isolates R5, R6, Rs7, Rs11 isolated from soil and from potato plants prevented germination of radish seeds on PDA medium and their disease index was 5 according to the approved index, while the germination percentage in the comparison treatment was 100%.

Dyeing the nuclei

The results of dyeing the nuclei showed that isolates cells of *Rhizoctonia* spp. having multiple nuclei ranged from 10 to 3 nuclei except isolates R5 and R6, which were unique to having two nuclei in each cell of hyphae as shown fig. 1. Thus, all isolates belonging to the tested fungi except R5 and R6 can be considered as multinucleate, which is due to *R. solani*. While isolating R5 and R6 are binucleate and are considered *Rhizoctonia* sp.

Molecular Diagnosis

The results of the identification of nucleotide of the ITS site for the tested isolates has led to know the isolates sequences of *Rhizoctonia* spp. as shown in table 2. Where it has been found that the two isolates Rs. 1 and Rs. 2 matched with the global isolation of KF712285 by 98%, which back to the *Rhizoctonia* solani anastomosis groups AG-4HGIII. While Rs. 3 and Rs. 4 were 99% matching to the global isolates KJ170346 and KJ170339 belonging to AG-4HGI and the isolates from Rs. 7 to Rs. 14 were 99-98% matching with KX650630. MH113810 respectively, which belonged to the *Rhizoctonia* solani anastomosis groups AG-3PT. Therefore, the diagnosis of these isolates was confirmed as *R. solani*. Furthermore, the isolates of R5 and R6 were matched with the global isolation AB196640 by 99% that belonged to the BNR AG-A binucleate. The results of molecular diagnosis confirmed that the results of dyeing the nuclei and phenotypic diagnosis of fungi isolates, where the results are consistent with (Muzhinji *et al*., 2015) findings. They observed that the AG-4HGIII group caused potato stem canker and the AG-4 HGI group caused stem and stolon canker on potatoes, while the BNR AG-A group caused stem canker and black scurf on potatoes and the AG3-PT group caused black scurf on potatoes. (Silva *et al*., 2010) also found that the AG-4HGI and AG-4HGIII groups caused stem canker on potatoes. Other studies have confirmed that the isolates of *R. solani* that belonged to the AG-3PT group had a high frequency percentage and were the main group responsible for potato infestation in many regions of the world (Compion *et al*., 2003; Woodhall *et al*., 2007; Tsror, 2010; Fiers *et al*., 2011 and Das *et al*., 2014).

The biological sterilization efficiency test of the soil within the pots

The results of the biological sterilization efficiency

Table 2: Molecular matching of Iraqi Isolates of *Rhizoctonia* spp. and the Accession numbers in NCBI.

Seq.	Isolate code	Accession number	Isolate site	The *Rhizoctonia* solani anastomosis groups	Matching percentage
1	Rs.1	MK841498.1	soil	AG-4 HGIII	KF712285.1 %98.83
2	Rs.2	MK841499.1	soil	AG-4 HGIII	KF712285.1 %98.39
3	Rs.3	MK841500.1	soil	AG-4 HGI	KJ170346.1 %99.71
4	Rs4	MK841501.1	soil	AG-4 HGI	KJ170339.1 %99.57
5	R.5	MK841502.1	soil	BNR AG-A	AB196640.1 %99.48
6	R.6	MK841503.1	soil	BNR AG-A	AB196640.1 %99.65
7	Rs.7	MK968283.1	Potato plant	AG-3 PT	KX650630.1 %98.96
8	Rs.8	MK968284.1	Potato plant	AG-3 PT	MH113810.1 %99.29
9	Rs.9	MK968285.1	Potato plant	AG-3 PT	MH113810.1 %99.58
10	Rs.10	MK968286.1	Potato plant	AG-3 PT	MH113810.1 %99.72
11	Rs.11	MK968287.1	Potato plant	AG-3 PT	MH113810.1 %99.86
12	Rs.12	MK968288.1	Potato plant	AG-3 PT	MH113810.1 %99.86
13	Rs.13	MK968289.1	Potato plant	AG-3 PT	MH113810.1 %99.58
14	Rs.14	MK968290.1	Potato plant	AG-3 PT	MH113810.1 %99.86

Table 3: The infection severity of *Rhizoctonia* solni fungi in the biological sterilization experiment for two-week within the pods.

Treatments	Pods experiments**		% infection severity of biological sterilization in field experiment**
	% infection severity of biological sterilization for 2 weeks	% infection severity of biological sterilization for 4 weeks	
250 bran	6.66	0.010	6.660
500 bran	0.01	0.010	0.010
1000 bran	0.01	0.010	0.010
250 straw	93.33	40.000	46.000
500 straw	93.34	6.660	26.660
1000 straw	60.00	6.660	26.660
250 Cynodon residues	60.00	6.660	6.660
500 Cynodon residues	60.00	6.660	6.660
1000 Cynodon residues	26.66	0.010	6.660
Compared with pathogenic	100.00	100.000	86.000
Compared without pathogenic	0.01	0.010	46.000
L.S.D 0.05	1.028	0.6856	0.7886

* Each number in the table represents three replicates and each replicate represents two plants; ** Each number represents three replicates and each replicate represents 20 plants.

test experiment of the soil that carried out within the pots showed a decreased in the infection severity for all treatments with significant difference from the treatment of the pathogenic fungus (comparison) as shown in table 3. It was also observed that the treatment of wheat bran was superior with a significant difference over all treatments and the infection severity of the pathogenic fungus at the biological sterilization experiment for two weeks was 6.66, 0.01, 0.01% for the concentrations of 250, 500, 1000 g / m^2 soil, respectively. This was followed by the treatment of biological sterilization with the cutoff cynodon dactylon residues at the same concentrations, which its infection severity reached 60, 60 and 26.66%, respectively. While the biological sterilization with wheat straw was ranked the third at the same concentrations, which its infection severity with the pathogenic fungus *R. solani* reached 93.33, 93.34 and 60%, respectively. Furthermore, the infection severity in the treatment of pathogenic fungus (comparison) was 100%. It was also observed when testing the period of biological sterilization for a month within the pots, all treatments was superior with a significant differences in reducing the infection severity of pathogenic fungus *R. solani* as shown in table 3. Moreover, wheat bran treatment was significantly superior in reducing the infection severity of pathogenic fungus at concentrations of 250, 500 and 1000 g/m^2, reaching 0.010% for all concentrations, compared with the comparison treatment (pathogenic fungus) which the infection severity reached 100%. It was followed by the biological sterilization treatment with cutoff cynodon dactylon residues at the same concentrations, which its infection severity reached 6.66, 6.66 and 0.010% respectively. Where the biological sterilization treatment of wheat straw was also ranked third at the same concentrations, with an infection severity of 40, 6.66 and 6.66% respectively.

The biological sterilization efficiency test in the field

Table 4: Effect of biological Sterilization Method on Some Growth Criteria of Potato Plants in the Field.

Treatments*	Number of branch /plant	Branch length (cm)	Average Root wet weight gm/plant	Average Root dry weight gm/plant	Average tubers number / plant	Average tubers weight gm/tuber
250 bran	3.4	13.41	10.60	2.09	7.73	305.4
500 bran	3.5	15.05	11.46	3.27	9.93	323.2
1000 bran	3.6	20.83	11.57	3.76	10.20	346.8
250 straw	2.7	10.52	6.66	1.46	4.93	98.8
500 straw	3.2	10.70	6.84	2.13	5.67	147.9
1000 straw	3.1	12.58	7.88	3.13	7.07	160.1
250 Cynodon residues	3.3	19.61	9.79	1.71	9.93	196.1
500 Cynodon residues	3.1	22.11	10.72	2.55	9.00	233.7
1000 Cynodon residues	3.6	25.72	12.08	3.47	8.93	236.6
Compared with pathogenic	3.5	9.26	2.58	1.68	9.33	217.6
Compared without pathogenic	4	16.70	7.03	1.66	9.67	242.5
L.S.D 0.05	0.8306	3.674	1.962	0.9616	1.572	24.37

• Each number represent three replicates and each replicate includes 20 plants.

Efficiency of biological sterilization of soil in the field experiment showed that the infection severity in all treatments was significantly reduced as shown in table 3. The wheat bran treatment was significantly superior to that of the pathogenic fungus (comparison) (86%), which led to reduce the infection severity of the pathogenic fungus that reached 6.66, 0.01 and 0.01% for concentrations of 250, 500 and 1000 g/m^2 soil respectively. Followed by the biological sterilization treatment with cutoff cynodon dactylon residues at the same concentrations with a significant difference from the pathogenic fungus treatment (comparison), which had the infection severity 6.66%. While the biological sterilization treatment of wheat straw at the same concentrations was ranked the third, which was also significantly superior over the pathogenic fungus treatment (comparison) (86%) and the infection severity with the pathogenic fungus *R. solani* reduced to 46, 26.66 and 26.66, respectively. The results of some growth parameters showed significant differences between the biological sterilization experimental treatments in the field as shown in table 4. There was a significant superiority was observed in the wheat bran treatment in three concentrations of 250, 500 and 1000 g/m^2 over the other treatments, in branch length trait, average wet weight, average dry weight and average tuber weight, which differed significantly from the pathogenic fungus *R. solani*, treatment and in the branche's length, which reached 13.41, 15.05 and 20.83 g/plant respectively, compared with the pathogenic fungus 9.26 g/plant, The average wet weight was 10.60, 11.46 and 11.57 g/plant respectively, compared with the pathogenic fungus treatment 2.58 g/plant. The average dry weight was 2.09, 3.27 and 3.76 g/plant, respectively, compared with the pathogenic fungus 1.68 g/plant. Average tuber weight was also significantly superior at 305.4, 323.2 and 346.8 g/plant, respectively, compared with the pathogenic fungus 217.6 g/plant, whereas did not significantly differences in the number of branches which reached 3.4, 3.5 and 3.6 g/plant respectively, with pathogenic fungus 3.5 g/plant.

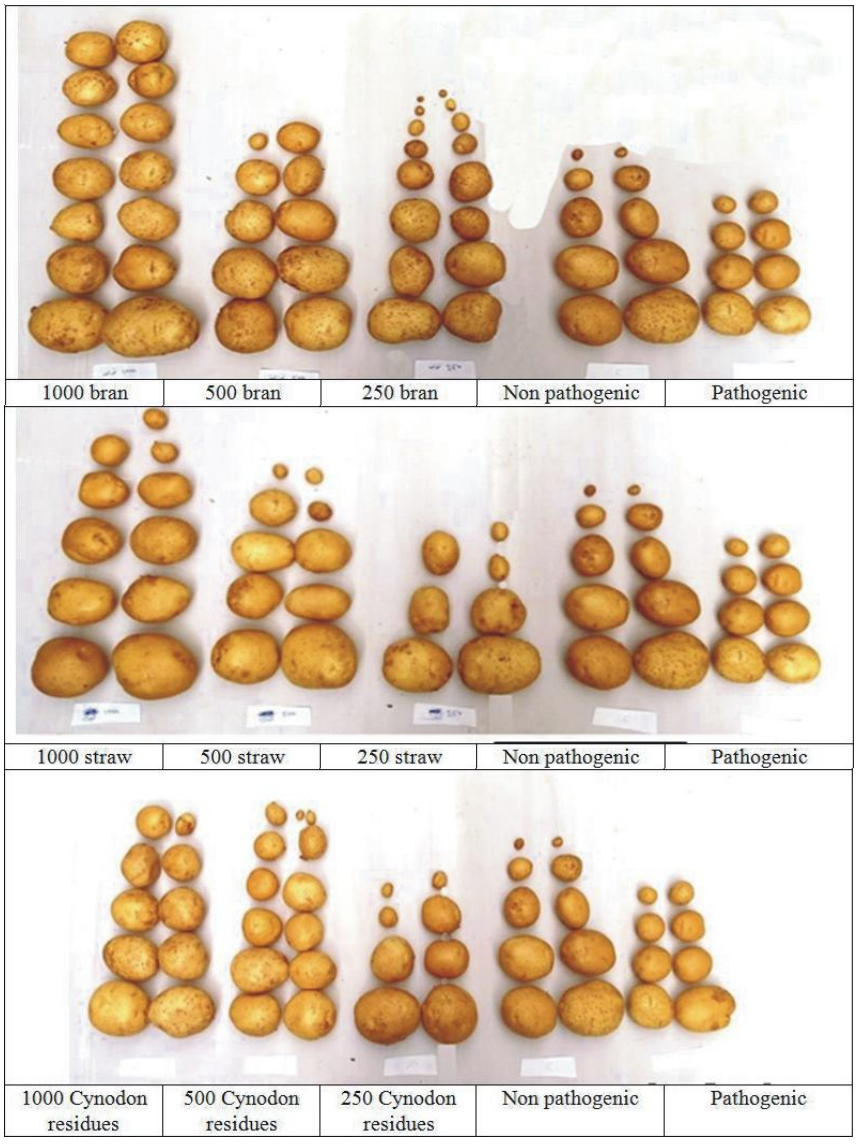

Fig. 2: Potato tubers of biological sterilization experiment in the field against the pathogenic fungus *Rhizoctonia* spp.

Also, there are a significant differences were observed in the average tuber number as shown in fig. 2, which reached 7.73, 9.93 and 10.20 respectively, compared with the pathogenic fungus 9.33 g/plant. While the cutoff cynodon dactylon residues with a concentration of 1000 ranked secondly, which superior in some growth parameters. It was significantly superiority in branch length, average wet weight and average dry weight rate, which amounted 25.72, 12.08 and 3.47 g/, respectively, compared with the pathogenic fungus treatment (comparison), which amounted to 9.26, 2.58 and 1.68 g/plant respectively. Moreover, it did not differ significantly in the branch number and the average tuber number and the average tuber weight, which amounted to 3.6, 8.93 and 236.6 g/plant respectively, compared with the pathogenic fungus treatment, which was 3.5, 9.33, 217.6. g /plant respectively.

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