Efficacy of rice husks compost and *Trichoderma harzianum* on *Rhizoctonia solani* and its effect on seeds germination and seedling health

Hisham R. Al-Sharmani¹, Haidar H. Al-Kalabi¹ and Aqeel N. AL-Abedy²

¹Department of Plant Protection, College of Agriculture-University of Kufa, Iraq
²Department of Plant Protection, Agriculture College-University of Kerbala, Iraq
Email: haiderkelabi@uokufa.edu.iq

Abstract. This study was conducted to isolate and identify *Trichoderma harzianum* Rifai and *Rhizoctonia solani* by the polymerase chain reaction (PCR) technique with the presence of the ITS1 and ITS4 primer pair. The study was also carried out to investigate the efficacy of rice husk compost fortified with the bio-control agent, *T. harzianum* for the resistance of wheat seeds to the pathogenic fungus, *R. solani*, and to evaluate the effect of the compost on wheat germination and seedling, and irrigation water usage. The results showed that the highest germination rate which was found to be 100% was achieved with the T.h.+S treatment while the lowest germination was 93.3% with both the T.h.+RHC+S and R.s.+RHC+S treatments compared to germination of 96.6% with the control treatment. The RHC+S treatment resulted in the highest percentage of rotting seeds which was 10% while the lowest percentage of rotting seeds which reached 0% was achieved with the T.h.+S treatment. The results also showed that with R.s.+RHC+S and T.h.+RHC+S treatments, the highest shoot length at 21 post-planting days was achieved which was found to be 1.3 cm and 1.2 cm, respectively compared to other treatments including the control. Regarding fresh and dry weights, treatments with RHC had significantly much higher fresh and dry weights than all other treatments. It was clear from the experimental results that the highest number of branches/plant was recorded in the treatment of T.h.+RHC+S that was 5.3 followed by RHC+S treatment with 4.6 while the lowest number of branches/ plant was recorded in the control treatment which was only 1.8. Amount of water used for irrigation in treatments of T.h.+RHC+S and R.s.+RHC+S was reduced to be 233.33 compared to 600.00 in the control treatments. The highest chlorophyll content was 2.4 and was recorded in the treatment of R.s.+RHC+S with a significant difference from all other treatments. The T.h.+S treatment gave the lowest chlorophyll content. In fact, treatments that incorporated RHC had always higher values of plant total chlorophyll contents. Earliest flowering was achieved with the RHC+S treatment while the R.s.+S treatment resulted in the latest flowering.

1. Introduction
Wheat (*Triticum aestivum* L) is one of the oldest known and most important grain crops cultivated and used as daily food and a major source of energy [1]. Wheat contains high amounts of carbohydrates that are necessary to provide food calories in addition to its good source of protein, vitamins, and minerals [2]. Wheat flour contains 70%-75% starch, 7-17% protein, 2-2.7% fiber, 1%-2.5% minerals,
0.69%-1.62% fat, and low levels of pentosans that make wheat of great economic and nutritional importance [3]. Wheat is prone to infection with many plant pathogens causing seed rot and damping-off diseases at different stages of plant growth [4]. *Rhizoctonia solani* is a widespread soil-borne pathogenic fungus that causes various diseases to many economically important plants including pre-emergence seed rot and post-emergence seedlings death [5; 6]. It can also affect large plants causing root rot and rotting of fruits touching the surface of infested soil [7]. In fact, soil moisture level and organic matter content have a positive impact on plant fortification and reducing the incidence of infection with *R. solani* and other pathogens during and after germination stage and thus reducing the number of plants dwarfed by this fungus in the middle of the growing season [8]. Plant residues in the soil undergo various chemical transformations that change it from insoluble to soluble forms which affect their accessibility and absorption by the roots [9]. Soil microorganisms in addition to other environmental factors play a significant role in increasing the dissolvability of many organic and inorganic plant nutrients and thus affect the nutritional status of the plant and its resistance to diseases [10; 11].

In eco-friendly farming practices and to reduce the use of chemicals, plant residues are used as organic fertilizers in addition to bio-control factors including *T. harzianum*. Besides being a successful bio-fertilizer, *T. harzianum* is one of the most effective biological control agents against many pathogens [12]. The use of this fungus with organic fertilizers or plant compost increases the possibility of producing healthy plants with high productivity as this fungus is strong antagonistic and is competitive to pathogens on the one hand, and that the organic matter improves soil properties and provides nutrients necessary for plant growth on the other hand [13]. It is obvious that the dissolved plant residues differ in terms of content, composition, and associated species depending on the crop from which they have been dissolved. For instance, rice waste; in particular, rice husk, is one of the most abundant and widely distributed agriculture waste in rice growing areas [14]. Rice waste constitutes about 20% rice husk which is corresponding to more than 100 million ton of annual residues worldwide [15]. Rice husk contains a high percentage of organic matter that is estimated to be about 75%-100% including cellulose, lignin, silica, calcite, and other materials [16]. Therefore, it is highly effective in improving the properties of poor-drainage soils and increasing root-zone exchange activities and thus improving plant growth [17].

In view of the importance of research studies in the field of utilizing plant residues supported by biological agents as organic fertilizers alternative to chemical fertilizers and pesticides and due to the availability of rice husk in most rice growing areas in Iraq, this study aims to investigate the effectiveness of rice husk compost fortified with *T. harzianum* Rifai in improving seed germination and growth of wheat and controlling seeds rot and seedlings death caused by the pathogenic fungus, *R. solani*. A number of plants wastes were used as natural culture media to grow and load *T. harzianum*, including sawdust, barley straw, wheat, millet, rice bran, and wheat [18; 19].

2. Materials and Methods

2.1. Laboratory Experiments

2.1.1. Seed Germination Rate

This was a preliminary experiment to determine seed viability (seed germination ratio) in order to further use in the following experiments. Wheat seeds were put in a Petri dish containing PDA medium and then placed in the incubator for three days. After that, the germination rate was measured as the ratio of the germinated seeds to the total number of seeds in the dish. The germination rate in this experiment was found to be 99%.

2.1.2. Fungal isolates

The fungal isolates of the pathogen *R. solani* Kuhn and the biological agent *T. harzianum* Rifai used in this study were obtained from the Plant Pathology laboratory at the College of Agriculture-University of Kufa. These fungi were identified by PCR amplification and DAN sequencing of rDNA-ITS region. The ITS region of *R. solani* and *T. harzianum* isolates were amplified, using the universal primers
ITS1 (TCCGTTGGTGAACCAGCGG) and ITS4 (TCCTCCGC TTATGATATGC) [20] using Taq DNA polymerase (Roche, Cat. No. 11 146 173 001). The final volume of each PCR reaction mixture (sample) was 20 μl containing; 2 μl 10X PCR buffer, 1 μl of each primer (10 pmol), 2 μl dNTPs (2 mM), 3 μl template DNA (30 ng/μl), 1 unit Taq polymerase, then completed to 20 μl by adding nuclease-free sterile distilled water. PCR amplification was performed using the following conditions: initial denaturation at 94°C for 1 min followed by 35 cycles each consisting of final denaturation at 94°C for 30 sec, annealing temperature at 55°C for 30 sec, initial extension for 1 min, and final extension at 72°C for 5 min [20]. PCR-amplified products were electrophoretically separated on a 1% agarose gel for 140 min at 80 V, 400 mA and visualized with ethidium bromide under UV illumination and images were captured using Vilber Lourmat, Taiwan gel documentation system.

For DNA sequencing, the PCR-amplified products were gel-purified using the FavorPrep PCR Purification Kit (Cat. No. FAGCK 001, Favorgen, Taiwan) and sent along with the primer pair (ITS1 and ITS4) to the Macrogen DNA sequencing service in Korea. PCR products were directly sequenced in both directions using the respective forward and reverse primers. The obtained nucleotide sequences were aligned and compared with the sequences belonged to the R. solani isolates in the NCBI database using the Basic Local Alignment Search Tool (BLAST) [21]. Using the MEGA6 software, multiple alignments of the nucleotide sequences and construction of phylogenetic trees were performed using the neighbor-joining method [22].

2.1.3. Preparation of fungal inoculums
The isolates were cultured on millet seeds. First, the millet seeds were macerated with water for six hours and then were taken out and exposed to air for 10 minutes. Thereafter, they were placed in tightly-capped bottles. The bottles were kept in a sterilizer at a temperature of 121°C and pressure of 15 lb/in² for an hour on the first day. The same sterilizing procedure was repeated on the second day. After 3 days, the fungal isolates were added to the millet seeds in the bottles. Finally, the bottles were incubated for seven days for fungal growth.

2.2. Field (Pot) Experiment
Soil (S) was prepared and mixed with the rice husk compost (RHC) at a rate of 1: 3 (V/V), respectively. The potting soil was combined with the experimental treatments T. harzianum (T.h.), R. solani (R.s.), RHC and all their possible combinations of treatments as follows:

1- T.h.+S
2- RHC+S
3- T.h.+RHC+S
4- R.s.+S
5- R.s.+RHC+S
6- T.h.+R.s.+RHC+S
7- S, soil only treatment served as a control for comparison.

The fungi cultured on millet seeds were added to the soil or soil mix at a rate of 5 g/pot according to the abovementioned treatments. Pots were slightly watered and left for 2 days for fungus to grow and penetrate the soil before planting. After that, pots of the size of 20x20 were sewed on 14/11/2012 and treated with each of the treatments listed above. At 10 days post-planting (DPP), seeds germination rates and the number of healthy and infected plants in each pot were recorded. At 25 DPP, pots were fertilized with 5 g of nitrogen (diammonium phosphate (DAP)). Plant growth parameters were measured including shoot and root length, shoot and root fresh and dry weight, number of branches/plant and total chlorophyll content. After that, the growth parameters were tabulated and compared to all investigated treatments.
3. Results and Discussion

3.1. Fungal isolates

PCR amplification of DNAs extracted from these isolates showed the possibility of amplifying PCR products of approximately 500 bp using the ITS1-ITS4 primers (Figure 1).

Figure 1. DNA products amplified by polymerase chain reaction (PCR) from T. harzianum (1) and R. solani (2) (obtained from Plant Pathology graduate laboratory, Dept. of Plant Protection, College of Agriculture-Univ. of Kufa), NC: negative control (no template DNA added). M, 1Kbp DNA ladder marker (Promega, Madison, USA)

The PCR-amplified ITS region (ITS1, 5.8S rDNA, and ITS4) of each T. harzianum and R. solani isolates were sequenced and the generated nucleotide sequences were subjected to a BLAST search. The comparison of the whole ITS region (ITS1, 5.8S rDNA and ITS4) of the T. harzianum isolate with those previously deposited in the GenBank revealed that the nearest genetic similarity (100%) of the generated ITS sequence was with T. harzianum from India (JX518919) (Fig. 2 and Fig. 3). Comparison of the sequence obtained from R. solani with the other R. solani isolates deposited in GenBank showed that the highest genetic similarity was 100% with the R. solani isolate previously identified in Iraq (KY055374) ((Fig. 4 and Fig. 5). Minimum nucleotide sequence similarity (88%) for this R. solani isolate was observed with R. solani isolates identified in USA, China, and India (FJ746916, HQ270173 and JF701745.1, and JF701771, respectively). Polymerase chain reaction (PCR) technology was used in this study to diagnose the isolates of F. solani and R. solani. Due to its high accuracy in the diagnosis of many organisms, including pathogenic and non-pathogenic fungi such as F. solani, R. solani, Alternaria alternata and Aspergillus spp. [23].
**Figure 2.** Similarities and differences in some regions of the nucleotide sequence alignments produced by PCR product amplified from *T. harzianum* isolate.
Figure 3. A phylogenetic tree, generated using the neighbor-joining method showing the genetic relationship among the Iraqi *T. harzianum* isolate, with those of other *T. harzianum* isolates available in GenBank (NCBI).
Figure 4. Similarities and differences in some regions of the nucleotide sequence alignments produced by PCR product amplified from *R. solani* isolate.
Figure 5. A phylogenetic tree, generated using the neighbor-joining method showing the genetic relationship among the Iraqi R. solani isolate, with those of other R. solani isolates available in GenBank (NCBI).

3.2. The effect of treatments on seeds germination and water usage

The results of the experiments with different treatments are listed in Table 1. It is apparent from the table that seeds vitality and germination rate were affected by the applied treatments. Interestingly, the highest germination rate of wheat seeds was achieved with the treatment of T.h.+S which was found to be 100% while the germination rate was 96.67% with control only. The rest of the treatments had no effect on the germination rate. The results also showed that all treatments resulted in a reduction in the percentage of rotting seeds. This improvement in seeds germination and reduction in seeds rot can be attributed to two factors. The first factor is the chemical effect resulting from the decomposition of organic matter that improves the soil properties and processes. The second factor is related to change in the biological composition of the soil as the number of fungi and bacteria that affect the pathogens through competition, antagonism or other mechanisms increases [24].

Regarding the amounts of water used for irrigation, the results indicated that water amounts in the treatments of T.h.+RHC+S and R.s.+RHC+S were reduced to be 233.33 each compared to 600.00 in the control treatment. This can be attributed to the contribution of these treatments to improving the physical properties of the soil that improved the soil water holding capacity.

3.3. Effect of treatments on plant shoot and root length and chlorophyll content at 21 dpp

The experimental results of the effects of the treatments on plant growth are presented in Table 2. The results revealed that the T.h.+RHC+S treatment led to a significant increase in the shoot length, which amounted to 24.97 cm compared to 16.57 cm in the case of control treatment. This is connected to the organic substances added to the soil which helps in releasing large amounts of carbon dioxide and then the formation of carbonic acid, which increases the efficiency of photosynthesis, and thus increases the plant vegetative and root growth [25]. Chlorophyll content in treated plants especially with RHC was increased regardless of the presence or absence of the pathogenic or bio-agent fungi.
Table 1. The effects of different treatments of *T. harzianum* (T.h.), *R. solani* (R.s.) and rice husk compost RHC on seeds germination and water use.

| Treatment       | Germination (%) | Seed Rot (%) | Water Amount |
|-----------------|-----------------|--------------|--------------|
| S               | 96.67           | 3.33         | 600.00       |
| T.h.+S          | 100.00          | 0.00         | 416.67       |
| RHC+S           | 90.00           | 10.00        | 316.67       |
| T.h.+RHC+S      | 93.33           | 6.67         | 233.33       |
| R.s.+S          | 96.67           | 3.33         | 383.33       |
| R.s.+RHC+S      | 93.33           | 6.67         | 233.33       |
| T.h.+R.s.+RHC+S | 97.67           | 3.33         | 250.00       |
| **L.S.D.**<sub>0.05</sub> | **2.691** | **2.597** | **10.697** |

*Treatments are Potting soil (S) treated with rice husk compost (RHC), *T. harzianum* (T.h.), *R. solani* (R.s.) or any of their combinations.

This increase can be due to increased photosynthetic activity in plants as a result of increased plant height and leaf area as well as an increase in root functional activities and thus improving total cellular activities. Such high activity levels in plants led to an increase in photosynthetic products and eventually increase general plant health as indicated by the high levels of total chlorophyll content [26].

Table 2. The effects of different treatments of *T. harzianum* (T.h.), *R. solani* (R.s.) and rice husk compost RHC on plant shoot and root length and chlorophyll content.

| Treatment       | Shoot (cm) | Root (cm) | Chlorophyll content (Total) |
|-----------------|------------|-----------|----------------------------|
| S               | 16.57      | 5.07      | 0.96                       |
| T.h.+S          | 14.63      | 6.00      | 0.90                       |
| RHC+S           | 20.93      | 3.53      | 2.19                       |
| T.h.+RHC+S      | 17.57      | 4.03      | 2.10                       |
| R.s.+S          | 15.30      | 6.53      | 2.08                       |
| R.s.+RHC+S      | 24.97      | 4.30      | 2.45                       |
| T.h.+R.s.+RHC+S | 21.90      | 3.63      | 2.16                       |
| **L.S.D.**<sub>0.05</sub> | **1.529** | **1.069** | **0.159**                  |

*Treatments are Potting soil (S) treated with rice husk compost (RHC), *T. harzianum* (T.h.), *R. solani* (R.s.) or any of their combinations.
3.4. Effect of treatments on fresh and dry weight and the number of branches/plant at 21 dpp

The observations regarding fresh and dry weight and the number of branches/plant are recorded in Table 3. It can be noticed that the treatment R.s.+RHC+S resulted in a considerable increase in the plant’s fresh and dry weight which reached 1.35 g and 0.40 g, respectively compared to 0.71 g and 0.07 g, respectively for the control treatment. The two treatments T.h.+S and R.s.+S have reduced the dry and fresh weight of the plant which reached 0.62 g and 0.09 g, respectively. This improvement might be attributed to the ability of the *T. harzianum* fungus to decompose the nutrients in organic compost and turn it into highly absorbable materials by plants or due to the biological factor produced by metabolites that improve plant growth [10]. The number of tillers increased with the T.h.+RHC+S treatment which was 5.33 branch/plant compared to other treatments including the control which was 1.1 branch/plant. The number of tillers dropped to 0.77 branch/plant with the T.h.+S treatment.

Table 3. The effects of different treatments of *T. harzianum* (T.h.), *R. solani* (R.s.) and rice husks compost RHC on fresh and dry weight and number of branches/plant.

| Treatment            | Fresh Weight (g) | Dry Weight (g) | No. of branches/plant |
|----------------------|------------------|----------------|-----------------------|
| S                    | 0.71             | 0.07           | 1.10                  |
| T.h.+S               | 0.62             | 0.09           | 0.77                  |
| RHC+S                | 1.16             | 0.10           | 4.67                  |
| T.h.+RHC+S           | 1.03             | 0.11           | 5.33                  |
| R.s.+S               | 0.62             | 0.09           | 1.33                  |
| R.s.+RHC+S           | 1.35             | 0.40           | 3.83                  |
| T.h.+R.s.+RHC+S      | 1.28             | 0.14           | 3.83                  |
| **L.S.D**<sub>0.05</sub> | **0.267** | **0.021** | **1.029** |

*Treatments are Potting soil (S) treated with rice husk compost (RHC); *T. harzianum* (T.h.), *R. solani* (R.s.) or any of their combinations.

4. Conclusion

It can be concluded from this study that the addition of organic fertilizer, fortified with the biological agent *T. harzianum* increased the germination rates of wheat seeds and protected them from rotting. Such treatment also encouraged the vegetative growth of the treated plants and all the studied indicators including plant content of total chlorophyll, plant height, number of branches per plant and root length. Organic fertilizer in the presence of bio-control agent also helped induce plant resistance against the seed rotting causal *R. solani*. The presence of organic fertilizer increased the water use efficiency by increasing plant water absorption and reduced the amount of water used for watering.

5. Acknowledgment

The authors are grateful to Dr. Sabah L. Alwan, Dept. of Plant Protection, Faculty of Agriculture, for advising throughout the study and to Dr. Basil H. Kandouh, Dept. of Plant Protection, Faculty of Agriculture, for being extremely helpful in editing this paper.
References

[1] Pontonio E and Rizzello C G 2019 Minor and Ancient Cereals: Exploitation of the Nutritional Potential Through the Use of Selected Starters and Sourdough Fermentation. In Flour and Breads and their Fortification in Health and Disease Prevention (Florida, USA: Academic Press).

[2] Lamothe L M et al 2019 The scientific basis for healthful carbohydrate profile Crit. Rev. Food Sci. Nutri. 59(7) 1058-1070.

[3] Al-Moussawi M N 2010 Wheat is the world’s first strategic crop (Kufa, Iraq: Books and Documents - University of Kufa).

[4] Ishtiaq M et al 2019 Management of root rot diseases of eight wheat varieties using resistance and biological control agents techniques Pakistan J. Bot. 51(1) 327-339.

[5] Schwartz H F and Gent D H 2007 Eggplant, pepper, and tomato-damping-off and seedling blight Plant Prot. Sci. 40 110-114.

[6] Benbow H R et al 2019 Serpins: genome-wide characterization and expression analysis of the serine protease inhibitor family in Triticum aestivum Genetics 3 400444.

[7] Mahmoud Y M et al 2007 Genetic Diversity among Nile delta isolates of Rhizoctonia solani Kuhn based on Pathogenicity, Compatibility, Isozyme Analysis and total protein pattern J.Bot. 31 19-29.

[8] Paulitz T C et al 2002 Insights into the prevalence and management of soil borne cereal pathogens under direct seeding in the Pacific Northwest, USA. Can. J. Plant Pathol. 24 416-428.

[9] He Y et al 2019 Bacterial community and phosphorus species changes in pepper rhizosphere soils after Pseudomonas putida Rs-198 inoculation Rhizosphere 100164.

[10] Harman G E 2000 Myths and dogmas of biocontrol change in perceptions derived from research on Trichoderma harzianum T22. Plant Dis. Rep. 84 (4) 377-393.

[11] Altimore C et al 1999 Solubilization of phosphates and micronutrients by the Plant growth promoting and biocontrol fungus Trichoderma harzianum 22-129 Appl. Environ. Microbiol 65 2926-2933.

[12] Ranasingh N et al 2006 Use of Trichoderma in disease management Ori. Rev. 68-70.

[13] Quideau S A 2002 Organic matter accumulation In: Encyclopedia of soil science (New York, USA: Marcel Dekker Inc.).

[14] Fu Y et al 2019 Activated bio-chars derived from rice husk via one-and two-step KOH-catalyzed pyrolysis for phenol adsorption Sci. Tot. Envir. 646 1567-1577.

[15] Tun M M et al 2019 Biomass energy: An overview of biomass sources, energy potential, and management in Southeast Asian countries Resources 8(2) 81.

[16] Terzioğlu P et al 2019 Review on a novel biosilica source for production of advanced silica-based materials: Wheat husk Asia-Pacific J. Chem. Engin. 14(1) 2262.

[17] Badar R and Qureshi S A 2014 Composted Rice Husk Improves the Growth and Biochemical Parameters of Sunflower Plants J. Bot. Arti. 427 64-86.

[18] Paningbatan R A 1997 Trichoderma species for the biocontrol of sweet pepper stem rot (Sclerotinia rolfsii Sacc.) in Philippine Phytopathol.30 (1) 16-25.

[19] Alwan S L 2005 Manufacture of a new herbicide Trichoderma harzianum Rafai to control seed rot and seedling death caused by Rhizoctonia solani and Pythium aphanidermatum (Kufa, Iraq: PhD thesis, University of Kufa).

[20] White T J et al 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic In: PCR protocols: a guide to methods and applications (New York, USA: Academic Press Inc.).

[21] Zhang S et al 2012 Molecular detection of Fusarium oxysporum in the infected cucumber plants and soil Pak. J. Bot. 44(4) 1445–1451.

[22] Tamura K et al 2013 MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30 2725–2729.
[23] Al-Abedy A N et al 2018 Genetic variability of different isolates of Rhizoctonia solani Kuhn isolated from Iranian imported potato tubers (Solanum tuberosum L.) Int. J. Agricult. Stat. Sci. 14(2) 587-598.

[24] Al-Haidari A J 2003 Detect and diagnose some fungi that cause seeds rot and seedlings death of plant plants and their resistance with different techniques of fungi Trichoderma harzianum Rafai (Kufa, Iraq: MSc. thesis, University of Kufa).

[25] Al-Hadeethi B A 2002 Trichoderma harzianum in the soil and growth of tomato plant (Baghdad, Iraq: University of Baghdad).

[26] Maroof A K 2007 Effect of magnetization of saline irrigation water in some soil characteristics and growth and yield of tomato crop in Zubair and Safwan regions (Baghdad, Iraq: PhD thesis University of Baghdad).