Bioefficiency of some indigenous biocontrol agents against Rhizoctonia solani causing cowpea seed rot and pre-emergence damping-off

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Abstract. This study aimed to identify the main causal agent of cowpea seed rot and pre-emergence damping-off in Kerbala province and evaluate some indigenous biocontrol factors in comparison with biological and chemical commercial products in control of the pathogen. The results showed that the main cause of this disease was Rhizoctonia solani where all isolates of this fungus showed a notable pathogenicity. Moreover, all biological agents examined were found to be effective against the pathogen. The maximum inhibition percentages (100 and 89%) were achieved by the commercial products (Beltanol and EM1) while the local biocontrol agents Bacillus cerus and B. subtilis reached 82.66% and 82.22% respectively. However, the third indigenous biocontrol agent Trichoderma harzianum showed inhibition percentage more than 75% and less than 100%. Additionally, all biological control agents demonstrated a high efficiency in protection of cowpea seeds from R. solani infection. This was through decreasing percentage of seeds rot and pre-emergence damping-off in whole treatments compared with control (the pathogen only). The lowest percentage was reached by B. subtilis and Beltanol (0%) followed by B. cerus (5.6%) then T.harzianum (6.7%) and Em1 (21.7%) respectively. However, in control (the pathogen only) was 50%.

1. Introduction

Cowpea (Vigna unguiculata (L.) Walp) is one of major crops of Leguminosae family that is commonly cultivated in tropical and subtropical countries such as Iraq [1]. This crop is of considerable value due to contain a high amount of proteins, starchy and fatty substances as well as metal compounds that are necessary for humans, and their secondary products are of great nutritional importance as feed source of farm animals. It has also an essential role in nitrogen fixation and improving soil texture and fertility due to bacterial nodules on its roots [2]. However, various pathogenic microorganisms affect this crop causing variable diseases including seeds rot and seedling pre-emergence damping-off that affected negatively on the quantity of the yield [3; 4; 5; 6]. This disease is caused by different phytopathogens such as Rhizoctonia solani that is considered one of the most important soil-borne pathogens infecting a wide range of plant hosts including the cowpea crop [7]. Furthermore, several studies such as [8; 9] were found that this fungus is one of devastative fungi that are able to infect the cowpea crop. Additionally, R. solani was found to be the most frequent fungus in samples of infected cowpea seedlings collected from cowpea fields in middle of Iraq [10].
In Iraq, the common management practice used to control plant diseases including the cowpea seeds rot and pre-emergence damping-off is application of pesticides. However, this practice can produce a critical damage to human and animal health in addition to soil microorganisms that are important in sustainability of soil health [11]. Additionally, it encourages the selection of phytopathogenic strains that are increased tolerance to pesticide [12]. Moreover, vast uprising of public opinions are toward obtaining healthy agricultural products free of chemical toxins. As a result of these reasons and others, a considerable attention has been raised to reduce application of these pesticides and find ecofriendly alternative management measures [5]. One of these sustainable measures is application of biological control agents that earns a significant consideration in the new strategies of plant diseases management [13].

Several members of bacterium Bacillus spp. and fungus Trichoderma spp. have been succeeded in combating effectively a wide range of phytopathogens on various agricultural crops in glass houses and fields. This is because of owning diverse control mechanisms such as production range of anti-phytopathogen compounds, nutrient competition, hyperparasitism, and degrading enzymes [14; 15; 16; 17]. Furthermore, there are several other biocontrol factors developed as commercial product such as the Effective Microorganisms (EM1) have been employed to obtain optimal control to soilborne pathogens and to improve plant growth and yields due to consisting of a selection of microorganisms such as Rodopseudomonas spp. that are naturally exist in the environment. These microorganisms produce different substances such as amino acids and carbohydrates that promote plant growth and activate other group of microorganisms that assist in the decomposition of organic matter and increase the fertility of the soil, and produces antibodies against plant pathogens [18]. Thus, as a result of spreading the seed rot and pre-emergence damping-off in most fields of cowpea crop in Kerbala province, this study aimed to isolate and diagnose of the main cause of this disease and assess some local and commercial biocontrol agents in control of the disease.

2. Materials and Methods
2.1. Isolation and identification of the causative agent of cowpea seed rot and pre-emergence damping-off in Kerbala province
A filed survey was conducted in five growing sites (Ibrahimeya, Alhindia, Aljadoal algherbi, Al hur and Kerbala Centre) of cowpea crop in Kerbala province during growing season of 2015-2016. Diseased samples of cowpea seeds and pre-emergence seedlings showed dark browning, and decay of all tissues infected were washed with running tap water for 5 minutes to remove the suspended soil. Seedlings were then cut into small pieces (0.5-1 cm long). After that, the symptomatic seeds and pieces of seedlings were sterilized with sodium hypochlorite solution (2%) for 2 minutes, washed with sterile distilled water and dried on sterilized filter paper. Subsequently, they were transferred to petri dishes containing autoclaved Potato Dextrose Agar (PDA) media amended with antibiotic Tetracycline (200 mg / l) then incubated at 25 °C for 3 days. The dishes were examined daily and the fungus associated with diseased samples was purified and diagnosed using the approved taxonomic keys [19; 20].

2.2. Pathogenicity test
Pathogenicity of the fungal isolate was assessed using modified method described previously [21]. Petri dishes (9 cm in diameter) containing 15-20 ml of sterile water agar (WA) media were prepared and inoculated with 0.5 cm in diameter disk taken from the edge of the isolated pure fungal colonies 5 days old individually, and incubated at 25 °C for three days. The inoculated dishes were then planted with cowpea seeds that were surface sterilized with sodium hypochlorite solution 2%. The sterilized seeds were placed around of the edge of the fungal growth colonies as 15 seeds per dish. In addition to this, sterilized seeds were placed in WA dished without fungal inoculation to be as a control. Subsequently, all dishes were incubated at a temperature of 25°C. The percentage of seed germination was measured after 7 days of planting the seeds in control dishes using the following equation:
2.3. The biocontrol fungus Trichoderma harzianum

The biocontrol efficacy of T. harzianum, previously isolated from fields of Agriculture College, University of Kerbala and identified in its laboratory of plant pathology, was examined using the dual culture technique [22]. The PDA media was prepared, sterilized in the autoclave, and distributed in 9 cm diameter Petri dishes. These dishes were then inoculated with a 0.5 cm disc obtained from the edges of 7-days old pure colonies of R. solani and T. harzianum each placed in the centre of the half of the Petri dish that were then placed in the incubator at 25° C for 7 days. The antagonistic ability of T. harzianum was estimated according to the five-grade scale described formerly [23] as following:

Grade 1 - The growth of the bio-control fungus covers the whole of the dish without allowing of the pathogenic fungus to grow and this equals the inhibition percentage 100%. Grade 2 - the growth of the bio-control fungus covers three-quarters of the dish while the growth of pathogenic fungus, covering the remaining quarter and this equals the inhibition percentage 75%. Grade 3 - the growth of the bio-control fungus covers half of the dish, whereas the growth of the pathogenic fungus covers the other half, and this equals the inhibition percentage 50%. Grade 4 - The growth of the bio-control fungus covers a quarter of the dish. In contrast the growth of pathogenic fungus covers the remaining three quarters, and this equals the inhibition percentage 25%. Grade 5 - Non-growth of the bio-control fungus exists while growth of the pathogenic fungus covers the full of the dish, and this equals the inhibition percentage 0%. The bio-control fungus is considered effective when the antagonistic grade is 1 or 2.

2.4. The biocontrol bacteria Bacillus subtilis and Bacillus cereus

Two bacterial biocontrol agents (B. subtilis and B. cereus), previously isolated from agricultural fields and identified in laboratory of plant pathology belonging to Agriculture college/University of Kerbala were grown on the sterilized Nutrient agar medium for 48 hours. One ml of the bacterial dilution 7x10^6 was added to each dish containing 20 ml of sterilized PDA media. These dishes were then shook circularly to homogenize the bacterial growth with the medium. A 0.5 cm disc of R. solani (7-days old) was placed in the centre of each dish. Each biocontrol agent was replicated four times in addition to control treatment that was the R. solani only. All dishes were subsequently incubated at a temperature 27°C for 7 days. The inhibition percentage was determined by measuring the mean diameter of the R. solani growth in all treatments, and the following equation was applied:

\[
\text{Inhibition} \% = \left( \frac{C - T}{C} \right) \times 100
\]

C: mean growth of the fungus in control
T: mean growth of the fungus in treatment

2.5. The Commercial products

Three concentrations (3, 6, and 10%) of the biological product EMI were assessed against R. solani. These concentrations were added separately to an autoclaved PDA medium. This medium was then mixed well until homogenized and poured in sterilized 9 cm Petri dishes as a first layer that was left to the second day, and then applied the a second layer of the PDA medium only, which was free of any additives [24]. The centre of each dish was inoculated with 0.5 cm disc obtained from pure 7 days old colony of R. solani. As well as, dishes of PDA free additives were inoculated with R. solani only as a control. Additionally, the fungicide Beltanol (8-Hidroxiquinolene 37.5% (Sulfate) w/v) was used (1ml/l) as a control beside control treatment that was represented the pathogenic fungus.
only without any additions. All dishes were then incubated at 25 °C. The efficacy of the bio and chemo products against the pathogenic fungus was revealed by determining the mean of two diagonal radius of each growing colony in treatments and control dishes, and the percentage of inhibition was calculated on the basis of the following the same equation mentioned in section 2.4.

2.6. Evaluation efficiency of the local biocontrol factors T. harzianum, B. subtilis, and B. cerus, and the commercial products in protection of cowpea seeds and seedlings from R. solani

This experiment was carried out in a plastic house located at Agriculture College / University of Kerbala to identify the in vivo efficiency of the local biocontrol agents in comparison with the two products. The following treatments were implemented:

- Treatment of negative control (the pathogen R. solani only).
- Treatment of positive control (without the pathogen R. solani).
- Treatment of B. subtilis and the pathogen R. solani.
- Treatment of B. cerus and the pathogen R. solani.
- Treatment of T. harzianum and the pathogen R. solani.
- Treatment of the bio-product EM1 and the pathogen R. solani.
- Treatment of the fungicide Beltanol and the pathogen R. solani.

The pathogenic R. solani and biocontrol T. harzianum inoculums were made by growing them separately on locally millet seeds that were autoclaved at 121°C for 60 minutes two times, once in each day. The inoculated seeds were monitored and shaken every day for two weeks. A 10 g of each inoculum was subsequently added in each pot. In contrast, a 20 ml of B. subtilis and B. cerus inoculum at concentration 8 x10^7 was added separately in each pot. The bacterial and fungal inoculum were mixed thoroughly with the autoclaved soil and irrigated with water, covered with punctured polyethylene bags and left for 3 days [25]. On the other hand, the fungicide Beltanol (1ml/l) was added to the soil after adding the inoculum of R. solani. Each pot was planted with six seeds of a local cowpea variety. Subsequently, all pots were placed in the plastic house and monitored daily. After one month of planting the cowpea seeds, number of germinated seeds and the percentage of seed decay and pre-emergence damping-off were calculated in each pot as a following:

\[
\text{seed decay and pre-emergence damping-off \%}= \frac{\text{Number of non-germinated seeds and pre-emergence damping-off seedlings}}{\text{Total number of seeds}} \times 100
\]

2.7. Statistical analysis

All data were analysed by operating one-way ANOVA (SPSS version 13.0) at a significance level of p < 0.01.

3. Results and Discussions

3.1. Isolation and identification of the causative agent of cowpea seed rot and pre-emergence damping-off in Kerbala province

The results of the cowpea field survey revealed that the disease of seed rot and pre-emergence damping-off was present in all fields examined. Furthermore, the most common fungus associated with these diseased seeds and seedlings samples was found to be R. solani. This fungus was isolated from all fields of five areas surveyed in Kerbala province. The occurrence of this disease in all surveyed fields of cowpea may belong to the continuous cultivation of the susceptible cowpea cultivars and present of the favorable environmental conditions for diseased development, which lead to accumulation of R. solani inoculum. Additionally, control strategies followed against this pathogen such as crop rotation is not fully succeeded because of pathogen ability producing sclerotia that are resistant to unfavorable soil conditions as well as this pathogen has a wide range of plant hosts [26]. Additionally, no such a resistant cultivar or sufficed fungicides are existing to
control the pathogen in Iraq. This result is also in consistent with several researchers’ findings [6; 27].

3.2. Pathogenicity test
All *R. solani* isolates examined showed a noteworthy pathogenicity through reduction of the percentage of cowpea seeds germination between 47-67%. The pathogenicity of *R. solani* is due to the secretion of some of phosphatase, ligninperoxidase, pectinase, and cellulase that facilitate the infection [5]. In addition, several phytotoxin compounds such as Phynel acetic acid and hydroxin para hydroxy derivatives can be produced and affect directly on seed embryos and cause death. However, the differences among *R. solani* isolates in their pathogenicity are possibly due to their genetic variation which influence by common environment conditions, type of soil and cultivars planted. This result is in consistent with many studies conducted on different plants that showed pathogenic ability of *R. solani* causing different disease including seed rot and pre-emergence damping-off [6].

3.3. The biocontrol fungus *T. harzianum*
*T. harzianum* showed a significant antagonistic capability against the pathogenic that was more than 75% (Fig. 1). This ability is due to several reasons such as rapid growth and lack of need special nutritional requirements. It includes also direct *Trichoderma* intrusion on the mycelia of pathogenic fungus, and analyse the cell walls via numerous of enzymes produced such as chitinase and B-1-3 gluconase. Moreover, *T. harzianum* has competitive advantage over the place and food and able to grow in all types of from light sandy to heavy clay with different moisture and organic content and pH. It is capable of survival and reproduction in varying temperatures, as well as the production of antibiotics such as *Trichodermine*, *Demadine* and *Viridene* [25].

![Figure 1. Test of the antagonistic potential of *T. harzianum* (Green growth) against the pathogenic isolate of *R. solani* (Brown growth).](image)

3.4. The biocontrol bacteria *B. subtilis* and *B. cerus*
The two local biocontrol factors, *B. subtilis* and *B. cerus* showed a high antagonism efficacy against the pathogenic fungus *R. solani* (Fig. 2). The *B. subtilis* achieved an inhibition ratio of 82.22% while the *B. cerus* reached 82.66%. The antagonistic properties of both bacteria can be attributed to their rapid growth that leads to prevent the growth of the fungus. Furthermore, they produce many antifungal compounds such as subtilin, Bacteriacin, Bacillomycin, Zwittermicin and Kanosamicin. They also produce numerous of enzymes such as Chitinase and B-1-3 gluconase, Endo gluconase,
Gluconase and Protenase, which lead to decomposition of the cell walls of fungal pathogens [28]. This result is in agreement with findings of previous study [15] that was identified a high efficiency of both biocontrol agents in control of *R. solani* that caused root rot disease on legume crop.

**Figure 2.** Test of the antagonistic potential of *B. subtilis* (A) and *B. cereus* (B) against the pathogenic isolate of *R. solani*

3.5. **The Commercial products**

The different concentrations of the bio-product EM1 showed a high efficiency in inhibition of *R. solani* growth (Fig. 3). The inhibition percentages increased gradually related to rising of the concentrations. Concentration 10% achieved the highest inhibition percentage (89%), while concentrations 6% and 3% reached the inhibition rates of 67.56 and 62.50, respectively. On the other hand, the chemo-product Beltanol showed a 100% inhibition percentage against the *R. solani*

This gradually increasing of inhibition percentages achieved with EM1 product is in consistent with several previous studies [24] that was found the concentrations 0.2, 0.5 and 1% of EM1 causing a steady increase in growth inhibition of several phytopathogenes. The mechanism of this inhibition may be due to the toxic compounds produced by the component microorganisms *Lactobacillus* spp. that secretes lactic acid and reuterin. These substances can inhibit growth of several fungi and possess an ability to reduce the pathogenic toxins [24]. Regarding to the efficiency of Beltanol, the results obtained in this study agreed with previous studies such as [10].

**Figure 3.** Inhibition effect the commercial products EM1 and Beltanol against the pathogenic fungus *R. solani*
3.6. Evaluation efficiency of the local biocontrol factors *T. harzianum*, *B. subtilis*, and *B. cerus*, and the commercial products in protection of cowpea seeds and seedlings from *R. solani*

The results of this experiment showed that all the indigenous biological control agents were highly efficient in protection of seeds and seedlings of cowpea from infection by *R. solani* (Fig. 4). This was discovered by rising of the percentage of seed germination and seedlings growing between 83 to 100% compared to the treatment of the pathogen only (Control -) that was 50.00%. The highest biocontrol factor was *B. subtilis* achieved 100% that did not differ with the treatment of the chemo-product Beltanol, followed by the treatment of bacteria *B. cerus* that was 94.4%. However, *T. harzianum* increased the percentage to 83.3% while the bio-product was 78.3%.

The effectiveness of both biocontrol agents *B. subtilis* and *B. cerus* can be attributed to their rapid growth and spreading in the dynamic space leading to compete with the pathogen. They also produce many of antifungal substances such as Kanosamicin and Zwittermicin and enzymes B-1-3 gluconase and chitinase, Endo gluconase, protonase, glucnase and chitinase, which destroy fungal cell components [25].

The effect of the chemical pesticide Beltanol on *R. solani* is possibly due to its active ingredient substances Hydroxoy quinolone and sulfate, which combine with some minerals leading to form a complex compound. These minerals such as iron and sulfur are very important in some metabolic processes of the pathogens. For instance, they affect the respiratory chain of pathogen leading to kill of the pathogen [10].

Effect of *T. harzianum* can attributed to the direct parasitism on the pathogenic fungi and secretion a number of enzymes such as B-1-3, chitinase, and gluconase that affect negatively on plant pathogens such as *R. solani*. It also has the ability to induce the resistance in the plant [16]. Additionally, this biocontrol agent has positive effect on plant by improving various growth parameters due to production of hormones such as oxins and ethylene, which stimulates germination of seeds [29].

The influence of EM1 was recognized as a result of containing microorganisms that produce hormones such as gibberellin, auxin, cytokinin, enzymes, vitamins and antibiotics that cause encouraging results in control of numerous soil-borne fungal pathogens [24].

![Figure 4. Evaluation of the efficiency of some local biocontrol agents *B. subtilis* (Bs.) and *B. cerus* (Bc.) *T. harzianum* (Tr.) and commercial products Beltanol (Bel.) and Em1 in control of *R. solani*](image-url)
4. Conclusions
The present assessment obviously indicated that all native biological control agents (T. harzianum, B. subtilis and B. cerus) examined showed robust antagonistic activity against R. solani infection on cowpea crop. Consequently, they could be used as alternative methods of fungicide application for management of the diseases caused by R. solani on cowpea crop or others crops.

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