

BIOLOGICAL CONTROL OF THE COMPLEX DISEASE OF *Rhizoctonia solani* AND ROOT-KNOT NEMATODE *Meloidogyne javanica* ON CHICKPEA BY *Glomus* spp. AND *Pseudomonas* sp.

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ABSTRACT

Rhizoctonia-Meloidogyne complex disease is a serious problem facing legume production in many countries. The infection of chickpea (*Cicer arietinum*) by pathogens *Rhizoctonia solani* (R) and *Meloidogyne javanica* (M) in a single infection or combination causes severe damage to plant growth. The effect of using *Glomus* spp. (G) and *Pseudomonas* sp. (P) as a biological control agent against Rhizoctonia- Meloidogyne complex disease was tested and assessed by comparing the growth and disease parameters in infected and control plants. Chickpea growth parameters are characterized by measuring shoot and root length, and fresh and dry weight. The results of using (G) and (P) in a single treatment or in combination showed a decrease in the root gall index and in root rot disease severity when compared to the infected and healthy control treatments. The (M+ R+ G) and (M + R + G + P) combination treatment increased polyphenol oxidase (POD) and peroxidase (PPO) enzyme activity value as well as the total phenols content in treated chickpea roots. The combined effects of (G) and (P) on the pathogens' progression and the positive effects on chickpea growth parameters are suggested to be involved in efficient disease control.

Keywords: PPO; POD enzymes; rhizobacteria; rhizoctonia-meloidogyne complex disease; arbuscular mycorrhizal fungi; *cicer arietinum*

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المقاومة الاحيائية للمعقد المرضي للفطر *R. solani* ونيماتودا تعقد الجذور *M. javanica* في نبات الحمص باستخدام

الفطر *Glomus* spp. والبكتريا *Pseudomonas* sp.

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المستخلص

يشكل المعقد المرضي Rhizoctonia-Meloidogyne الناتج عن الإصابة بالفطر *Rhizoctonia solani* ونيماتودا تعقد الجذور *Meloidogyne javanica* مشكلة خطيرة تواجه إنتاج البقوليات في العديد من بلدان العالم. ويعد نبات الحمص *Cicer arietinum* من بين المحاصيل التي تتعرض للإصابة بهذا المعقد المرضي وتسبب له اضرارا جسيمة. استخدم الفطر *Glomus* spp. والبكتريا *Pseudomonas* sp. بشكل منفرد او مع بعضهما كعاملي سيطرة احيائية على هذا المعقد المرضي على نبات الحمص. أظهرت النتائج تأثيرا إيجابيا لهذه المعاملات في خفض مؤشر تعقد الجذور الناجم عن الإصابة بالنيماتودا *M. javanica* وشدّة الإصابة بتعفن الجذور الناجم عن الإصابة بالفطر *R. solani* في النباتات المعاملة مقارنة بالنباتات غير المعاملة فضلا عن زيادات معنوية في بعض معايير النمو لنبات الحمص. كذلك سجلت معاملات السيطرة الاحيائية زيادة معنوية في فعالية إنزيمي بولي فينول اوكسيديز وبيروكسيديز ومحتوى الفينولات الكلي في جذور الحمص المصابة بالفطر والنيماتودا.

الكلمات المفتاحية: البقوليات، الفطريات، إصابة الجذور، معايير النمو.

INTRODUCTION

Plant diseases are an important factor affecting all types of agricultural production as they reduce quantity and quality of crop productivity due to the vital pressures caused by various pathogens such as bacteria, fungi, and viruses as well as nematodes (4). This impact can be seen in the discrepancy between the production of some crop varieties as compared to what is normally expected. Diseases also limit the cultivation of many crops sensitive to infection, and may even make cultivation impossible in some cases. Legumes are critical economic crops due to their high nutritional value in terms of protein content as well as high consumer demand. Chickpea *Cicer arietinum* is an essential crop that is widely cultivated in Iraq (1). Cultivation of chickpeas also plays a vital role in increasing soil fertility through nitrogen fixation, in breaking the cycles of some diseases in agricultural rotation programs, and in controlling weeds. Chickpea is a crop sensitive to nematode infestation, as it is host to hundred species (25). The wide host range and the geographical distribution make the Root-knot nematode one of the most important diseases that affect this crop in its different geographic cultivation areas (13). The nematode infection reduces the performance of chickpea roots due to damage during nematode feeding and reproduction in the root cells, as well as causing the formation of galls, and nutrient deficiency (21). This leads to the development of nutrient deficiency symptoms such as stunting, yellowing, and decrease in flowers and pods in addition to water stress symptoms, increases crop damage caused by biological pressures as a result of weakening the plant's ability to resist soilborne fungal diseases such as Fusarium wilt and Rhizoctonia root rot (8). According to Ganeshamoorthi and Dubey (10), Rhizoctonia root rot caused by *R. solani* is one of the major diseases threatening chickpea cultivation. The fungus grows in a wide temperature range between 15-36°C and invade the host plants through natural openings or wounds. Study of the fungi and nematodes combined in complex disease dates back to the nineteenth century when Atkinson 1892 referred to the synergistic relationship between pathogenic fungi and the

root-knot nematode *Meloidogyne* sp. on cotton. The pathogen complexes have attracted the attention of researchers all over the world due to the heavy losses they cause. Many researchers refer to the disease "Meloidogyne-Rhizoctonia disease complex" to describe the extent of the relationship between these two pathogens in the development of the disease. Al-Hazmi and Al-Nadary (2) found an increase in the Meloidogyne-Rhizoctonia complex disease index caused by inoculation by both pathogens, especially when inoculation by nematode preceded that by the fungus which led to decrease plant growth and increased root rot severity. Several previous studies have indicated the efficacy of using the chemical pesticides in controlling the nematodes and the fungi each, separately, or when these pathogens collaborated in the formation of complex disease in the plant. However, the environmental and health dangers resulting from the use of these pesticides in agricultural pest control have led to the adoption of new, more effective and safer methods of control. Also supporting this move is an increasing concern over the toxic effect of chemical nematicides on the nontarget microorganisms in the soil works as a nitrogen fixer, which remains the primary source of nitrogen in legumes and the other rhizosphere microorganisms possess a negative effect on some pathogen's developments (15). The use of microorganisms as biological control agents like fungi and bacteria are some of the most important non-traditional or alternative control methods showing remarkable success in suppressing pathogens. The change or manipulation of the rhizosphere microbial content acts as a biological control component against pathogens as these microorganisms are characterized by their high ability to colonize the rhizosphere and competing for the pathogens from invading root cells and work to prevent the nematodes second stage juvenile from invading the roots through antagonism, competition, and parasitism. They also function by inducing systemic resistance in plants and promoting plant growth by increasing roots' ability to use soil nutrients and absorb water (3). The objectives of this study were to determine the effects of using

biological control agents in controlling the Rhizoctonia-Meloidogyne complex disease on chickpea and assess different treatment combinations on chickpea growth and disease parameters.

MATERIAL AND METHODS

Experiment biological materials:

The experiment included two pathogens, *Rhizoctonia solani* and root-knot nematode *Meloidogyne javanica*, and two biological control agents, the bacteria *Pseudomonas* sp., and mixture of three *Glomus* species (*G. aggregatum*, *G. mosseae*, *G. intraradices*), and chickpea *Cicer arietinum* and tomato plants *Lycopersicon esculentum*.

Preparation of nematode inoculum:

Pure culture of identified *M. javanica* maintained under greenhouse conditions on susceptible tomato plants *Lycopersicon esculentum* was used as a source of nematode inoculum. The eggs were extracted from tomato roots using the 0.05% sodium hypochlorite method described by Hussey and Barker (11). Using a nematode counting dish, the suspension of eggs and second stage juvenile (J2) was adjusted to contain 1000 ml⁻¹. Each chickpea plant was inoculated with 2ml of the nematode treatment suspension applied directly in four pencil holes around the stem, followed by watering and covered with soil.

Preparation of *R. solani* inoculum:

R. solani was isolated from infected chickpea plants showing root rot symptoms. The surface of the roots was sterilized with 3% sodium hypochlorite and rinsed thoroughly with distilled water. The root pieces were dried out by filter papers, moved to Petri dishes containing potato dextrose agar (PDA) enhanced with streptomycin 100 ppm, incubated at 25±2°C. The *R. solani* soil inoculation culture was prepared on Proso millet seed *Panicum miliaceum* according to El-Tarabily (9) method. The soil was inoculated at the rate of 10g/ soil before sowing of seeds.

Preparation of *Glomus* spp. inoculum:

ERS is a commercial arbuscular mycorrhizal fungi (AMF) inoculum product from Bioglobal, Turkey was used as a mycorrhizal inoculum. It comprises three mixed *Glomus* spp. (*G. aggregatum*, *G. mosseae*, and *G.*

intraradices) consisting of 1 x10⁻⁴ inoculum potential units/g. The AMF inoculation was done during the sowing stage by adding 10 mL of inoculum (about 10000 inoculum potential units) to each pot (22).

Preparation of *Pseudomonas* sp. inoculum:

A commercial product of Bactogen company, Turkey was used. Each ml contains 1 x 10⁸ CFU of *Pseudomonas* sp. bacteria. Each pot received 20 ml of diluted suspension 4 x 10⁶ (CFU ml⁻¹) during the sowing stage.

Experimental design:

This experiment was carried out in the College of Agriculture and Forestry- University of Mosul by using a completely randomized blocked design RCBD with the following 14 Treatments: control (C), *R. solani* (R), *M. javanica* (M), *Glomus* spp. (G), *Pseudomonas* sp. (P), (R+G), (R+P), (R+G+P), (M+G), (M+P), (M+G+Ps), (R+M+G), (R+M+Ps), (R+M+G+Ps). Each treatment was replicated three times. Four chickpea seeds were sown in each pot containing a previously sterilized soil mixture (Soil, river sand, and peat moss in a ratio of 3:1:1 v/v). Pots were placed in a greenhouse and watered as needed.

Parameters analysis of the disease and plant growth:

After 60 days all plants were uprooted, disease and growth data recorded of the following parameters; disease severity, root gall index, Peroxidase POD, Polyphenol oxidase PPO, Total Phenolic compounds, root and shoot length (cm), root and shoot fresh weight (g), root and shoot dry weight (g).

Assessment of root galls:

0-5 scale root gall index were calculated according to Baker (7) as: 0 = 0-10%, 1 = 11-20%, 2 = 21-50%, 3 = 51-80%, 4 = 81-90%, and 5 = 91-100%.

Assessment of disease index severity:

Disease index severity of root rot was also calculated according to Aoyagi et al. (5) as following: 0 = healthy, 1 = 1 - 25% diseased roots (D.R.), 2 = 26 - 50% (D.R.), 3 = 51- 75% (D.R.), and 4 = 76 - 100% (D.R.).

Extraction of enzymes:

0.5 g of fresh chickpea root was ground in a prechilled mortar with 10 ml of 0.1M ice-cold phosphate buffer (pH 7) and centrifuged at 2000 rpm for 10 min. The supernatant was

made up to 10 ml by adding distilled water and used for enzyme assay (16).

Estimation of polyphenol oxidase activity (PPO):

The reaction mixture, containing 0.5 ml of enzyme extract and 2.3 mL of a 0.1 M phosphate buffer (pH 6), was mixed in a spectrophotometer cuvette and adjusted to zero absorbance. 200 μ l of 0.1 M of fresh catechol solution was added to the mixture and mixed quickly. Enzyme activity was measured as the change in absorbance at 420 μ M was recorded at an interval of 60 sec for a total of 3 minutes (6).

Estimation of peroxidase activity (POD):

Enzyme activity was estimated spectrophotometrically according to War et. al. (20) method. The reaction mixture contained 3 ml of 0.05 M guaiacol in 0.1 M phosphate buffer (pH 7), 0.02 M H₂O₂, and 0.2 ml of enzyme extract. Absorbance was recorded at 420 nm at an interval of 60 sec for a total of 3 minutes. Activity of both assays is defined as change in absorbance min⁻¹ g⁻¹ fresh weight. One unit of enzymatic activity (U) is defined as absorbance increase of 0.001 per minute under the assay conditions.

Estimation of total phenols:

For total phenol estimation, 0.5 g of fresh root samples were homogenized in 10 ml 80% methanol, kept at 70°C and agitated for 15min (24) 200 μ l of this methanolic extract was

added to 2ml of distilled water plus 50 μ l of Folin-Ciocalteu reagent. The solution was kept at 25°C. The resulting blue color was measured using a UV-Visible spectrophotometer at 750 nm. Gallic acid was used as the standard. The number of phenolics was expressed as mg⁻¹ Gallic acid equivalent (GAE).

Statistical analysis: Data were subject to factorial analysis of variance (ANOVA). using the procedure GLM of the SAS system. Duncan multiple range tests were used for comparisons of means at (p < 0.05).

RESULTS AND DISCUSSION

Effect on plant growth parameters:

Results presented in Table 1 show the effect of using the biological agents in controlling *M. javanica* and *R. solani* and the interaction of the two on the chickpea growth parameters. Shoot and root length and fresh and dry weight were increased when the chickpea plants infected with *R. solani* were treated with *Glomus* spp. and *Pseudomonas* sp. in a single treatment or in combination with the two biocontrol agents (G) and (P) compared to the control treatments (C) and (R). Chickpea infected with *M. javanica* (M) also showed a significant increase in growth parameters when treated with the same fungal and bacterial biocontrol agents (G) and (P) alone or in combination, as compared to the infected and control treatment (C) and (M).

Table 1. The effects of using biological control agents *Glomus* spp. and *Pseudomonas* sp. against *M. javanica* and *R. solani* on the chickpea growth parameters after 60 days of inoculation.

Treatments	Shoot length (cm)	Root length (cm)	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Root dry weight (gm)
Control	35.65 a-d	29.0 b-e	2.46 b	0.78 b	5.55 b-c	1.85 b
M	30.67 b d	25.22 b-e	1.57 d e	0.50 c e	3.55d-g	1.27 b d
M+G	37.14 a-c	32.87 ac	2.12 c	0.63 b d	4.49 b-d	1.53 b
M+P	39.22 a-d	28.19 b c	2.00c d	0.61 b d	4.53b-d	1.37 b c
M+G+P	35.64 a-d	29.3 a-e	1.66d e	0.53 c e	2.47 g	0.74 e
R	21.84 d	18.16 e	1.27 f g	0.46 d e	2.39 g	0.84 d e
R+G	44.58 a b	36.63 ab	2.03 c d	0.63 c d	4.53 b-d	1.32 b c
R+P	38.4 a-c	33.87 a b	2.50 ab	0.8 a b	5.47 a-c	1.75 b
R+G+P	33.73 a-d	28.0 b e	2.48 b	0.77 b	5.52 b c	1.6 b
M+R	25.30 c d	20.47 c e	1.26 f g	0.39 e f	2.55f g	0.79 e
M+R+G	36.12 a-d	31.77 a-d	2.23 b c	0.73b	5.03 b c	1.39 b c
M+R+P	29.82 b-d	22.04 c d	2.20 b c	0.68 c	3.80d e	1.30 b c
M+R+G+P	45.7 a	40.94 a	2.16 c	0.87 a	5.15 a c	1.68 b
G	43.1 a b	36.11 a b	2.8a	0.90 a	6.19a	2.13 a
P	33.85 a-d	26.59 b e	2.26 c	0.52 c d	4.33c-e	0.91 c e
G+P	38.40 a-c	34.61 a b	2.13 c	0.67 c	5.36 a c	1.39 b c

Values are means represent of three replicates. Means in each column followed by the same letter do not differ significantly according to Duncan's multiple range tests (P<0.05).

Effect on Root gall index: The nematode root gall index data in Table 2 indicates the effect of using both *Glomus* spp. (3.66) and *Pseudomonas* sp. (2.66) with *M. javanica* individually or in combination in (2.66) reducing the root-knot gall index. The infected root with both *R. solani* and *M. javanica* decreases the root gall index compare to the infected plant with *M. javanica* alone (4.66). The best results are when using both biocontrol agents in combination (M+R+G+P) to reduce the root-knot gall index (1.00).

Effect on disease severity:

Disease severity reflects the role of *R. solani* in the root rot development: the higher severity occurred when chickpea plants infected with *R. Solani*, whether alone (0.76) or with *M. javanica* (0.73). Meloidogyne-Rhizoctonia disease severity was decreased when using *Gloms* sp. and *Pseudomonas* sp. in combination to control the infected chickpea plants.

Effect of *Glomus* spp. and *Pseudomonas* sp. on activities of PPO, POD enzymes, and total phenol content in chickpea plants:

Results presented in Table 2 show the effect of *M. javanica* and *R. solani* and their interaction on POD and PPO enzyme activity and on total

phenolic content. The significant increase in POD enzyme activity showed the influence of *Pseudomonas* sp. treatment in the presence of *R. solani* 2.76 unit.min⁻¹.gfw⁻¹, and *Glomus* spp. treatment in presence of both *M. javanica* and *R. solani* 2.8 unit.min⁻¹.gfw⁻¹ as compared to the single infection of *R. solani* 1.66 unit.min⁻¹.gfw⁻¹ or *M. javanica* 1.57 unit.min⁻¹.gfw⁻¹. The POD in control treatment was 1.27 unit.min⁻¹.gfw⁻¹. PPO enzyme activity indicating a significant superiority of the *Pseudomonas* sp. treatment in the presence of *R. solani* 6.19 unit.min⁻¹.gfw⁻¹ as compared to treatment with *R. solani* alone- 2.47 unit.min⁻¹.gfw⁻¹. The result of treatment with *M. javanica* 3.55 unit.min⁻¹.gfw⁻¹ differed significantly from that of the control treatment 2.39 unit.min⁻¹.gfw⁻¹. Significant differences were also recorded in the total phenolate content. content being significantly higher in *Glomus* spp. and *Pseudomonas* sp. in the presence of *R. solani* and nematodes treatment 4.50 ug⁻¹.gfw⁻¹ as compared with outcome of treatment with *R. solani* fungus alone 1.83 ug⁻¹.gfw⁻¹. Results of treatment with *M. javanica* 2.20 ug⁻¹.gfw⁻¹ were also significantly different from the results of the control treatment 1.40 ug⁻¹.gfw⁻¹.

Table 2. The effects of using biological control agents *Glomus* spp. and *Pseudomonas* sp. against *M. javanica* and *R. solani* on the chickpea root gall index, disease severity, POD, PPO, and total phenolic content of fresh weight (FW).

Treatments	Nematode Root gall index	Disease severity	POD unit.min-1.g fw-1	POD unit.min-1.g fw-1	Total phenol mg ⁻¹ fw
Control	-	-	1.27 f g	2.39 g	1.4 g
M	4.66 a	-	1.57 d e	3.55 d-g	2.20 e f
M+G	3.66 b	-	2.12 c	4.49 b-d	3.36 c d
M+P	2.66 c	-	2.00 c d	4.53 b-d	3.31 c d
M+G+P	2.66 c	-	2.46 b	4.48 b-d	4.00 a-c
R	-	0.76 a	1.66 d e	2.47 g	1.83 f g
R+G	-	0.33 b	2.50 ab	5.47 a-c	2.53 e
R+P	-	0.26 b c	2.76 a	6.19 a	3.50 c d
R+G+P	-	0.26 b c	2.48 ab	5.52 a b	3.15 d
M+R	1.33 d	0.73 a	1.26 f g	2.55 f g	2.43 e
M+ R+G	1.33 d	0.26 b c	2.8 a	5.03 b c	3.56 b-d
M +R+P	1.00 d	0.23 c	2.20 b c	3.80 d e	4.16 a b
M+R+G+P	1.00 d	0.16 d	2.23 b c	5.15 a-c	4.50 a
G	-	-	2.03 c d	4.53 b-d	3.46 c d
P	-	-	1.76 d e	4.33 c-e	3.20 d
G + P	-	-	2.13 c	3.36 e-g	3.66 b-d

Values are means represent of three replicates. Means in each column followed by the same letter do not differ significantly according to Duncan's multiple range tests (P<0.05).

Discussion

Bioagents are a powerful tool that can be used to protect crops against various biological stresses (14). *Glomus* and *Pseudomonas*

species have been included in many biological control programs and in many commercial products used for this purpose. In our experiment results, the inoculation of chickpea

with *Glomus* spp. or *Pseudomonas* sp. in single treatments or in combination improved plant growth parameters and suppression of disease significantly when compared to the untreated control plants or the infected treatments.

Effect on plant growth parameters:

Chickpea inoculation results with one or both pathogens suppressed plant growth, these results agree with several similar studies described the positive effects of *M. incognita* infection in a synergistic interaction with *R. solani* in disease severity, leading to greater plant root damage (2). The biological control treatment decreased the negative effects of parasitism and work together to inhibit the disease and enhance the plant's nutrient supply of phosphorous and nitrogen (15).

Effects on *M. javanica* and root gall index:

Our results support findings of several studies that have used bacteria and fungi in biological control of Meloidogyne-Rhizoctonia complex disease or in a single infection. Khan et al., (14) found the application of *P. fluorescens* suppressed *M. incognita* egg hatching and induced mortality of the J2 and reduced nematode reproduction and galls formation. As well as treated soil with *Glomus* spp. as a biocontrol agent decreased nematode population less than half compared with infection control with nematodes only by producing hydrogen cyanide, ammonia, siderophore, indole acetic acid, and solubilized phosphorus. including producing antibiotics, enzymes, and toxins (19). The roles of nematode in developing complex disease described by Al-Hazmi and Al-Nadary (2), nematode is preparing plant roots physiologically for root rot fungi infection through the accumulation of amino acids resulting from the nematode parasitic activity.

Effect on *R. solani* on the disease severity:

There was a notable increase in root rot severity caused by *R. solani* in the presence of root-knot nematode *M. Javanica*. This increase reflects the synergistic interaction between the two pathogens in complex disease. This study found a significant positive effects of inoculated infected chickpea plants with *Pseudomonas* sp. and *Glomus* spp. individually or in combination and confirmed the earlier studies results of the ability of these

agents to secrete antifungal metabolites and enzymes and induce plant immunity and cell defense mechanisms through enhance the concentration of phenolic compounds and chitinolytic enzymes (3). In the present study, inoculated plants with mycorrhizal fungi reduced significantly the disease severity of *R. solani* pathogen, which may be attributed to increased nutrients status in the rhizosphere, reduced direct competition for root space and resources with pathogen, induce plant immunity to involve certain systemic mechanisms such as systemic acquired resistance (SAR) and cell wall defenses, and enhance production of defense-compounds such as phenolics, -1,3- glucanase and chitinolytic enzymes (12). Additionally, inoculated plants with mycorrhizal fungus *G. mosseae* showed a lower disease severity than the other species *G. clarum*, which may lead to a potential active control tool. Furthermore, the inoculation with mycorrhizal fungi increases both root dry weight and shoot dry weight, which may work as extra fertilizer for fields that have nutrition deficiency.

Effect on POD and PPO Enzyme activity and total phenolic content:

Activation of systemic resistance is one of the most important biological control methods. Rhizoctonia- Meloidogyne complex disease is one cause of increased concentration of plant defense enzymes (POD and PPO) activity due to the activation of systemic defense mechanism under biological stress. Soil microorganisms play an important role in this area. *Pseudomonas* sp. and *Glomus* spp. have been reported in many studies as biocontrol promoting factors that can activate plant defense mechanism against pathogens and increase total phenolic compounds (18). Our experiment results support these studies.

Conclusion

The present results support findings of many other similar studies that described the synergistic effect of *R. solani* and root-knot nematode *M. javanica* on the root gall index, disease severity and, the negative effects on plant growth parameters caused by (or, as a result of) the complex disease (Rhizoctonia-Meloidogyne) of chickpea. Significant positive effects of using both *Glomus* and *Pseudomonas* species as a biological control

against the complex disease in single or in combination treatments were observed.

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