ZANCO Journal of Pure and Applied Sciences
The official scientific journal of Salahaddin University-Erbil
https://zancojournals.su.edu.krd/index.php/JPAS

ISSN (print):2218-0230**, ISSN (online):** 2412-3986**, DOI: http://dx.doi.org/10.21271/zjpas**

RESEARCH PAPER

Evaluation of Pseudomonas and Bacillus Strains as Potential Biocontrol Agents against Fusarium Wilt of Chickpea

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A B S T R A C T:

Chickpea (*Cicer arietinum* L.) production is severely reduced by Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris (Foc)* in most chickpea growing areas worldwide.

The effect of treating chickpea seeds with three bacterial strains of *Bacillus* and two strains of *Pseudomonas* to control wilt caused by this pathogen was carried out in plots under field conditions. The *Bacillus* strains had much better effect than *Pseudomonas* strains on plants health. *Bacillus* strain (K3) significantly increased seed germination, plant weight, number of pods and the yield. These were increased by (12, 21.2, 39.8 and 14.2%) respectively. Also, the strain *B. amyloliquefaciens* (5113) showed significantly increases of plant weight, number of pods and the yield (19.6, 19.9 and 10.1%) respectively. It can be concluded that bacterial seed coating combined with other control strategies as integrated pest management could be used successfully to control or at least decrease the effect of Fusarium wilt on chickpea production.

KEY WORDS: *Bacillus subtilis, Fusarium oxysporum* f. sp. *ciceris*, Non-chemical control, *Pseudomonas fluorescens*, Seedborne pathogens.
DOI: http://dx.doi.org/10.21271/ZJPAS.34.2.6
ZJPAS (2022) , 34(2);53-61 .

1.INTRODUCTION :

Chickpea (*Cicer arietinum* L) contributes 18% of the global production of grain legume (Murali Sankar et al., 2018) and it is a major source of human food and animal feed because of their high content of quality protein (Jukanti et al., 2012; Hossain et al., 2010). In addition, chickpea cultivation plays a significant role in farming systems as a substitute for fallow in cereal rotations and it also helps in the management of soil fertility, particularly in dry lands. Those features make chickpea cultivation of particular importance to food security in the developing countries (Rafael et al., 2015; Stagnari et al., 2017).

Chickpea is affected by several diseases. Wilt caused by *Fusarium oxysporum* Schlecht and Emnd Snyd. & Hans. f.*sp. ciceri* (Padwick) Snyd. & Hans (Foc) is the most serious disease and causes heavy losses (Dubey et al., 2007). The disease was first reported from India by Butler (1918). It is a destructive disease and has become a major factor limiting chickpea production worldwide (Rafael et al., 2015; Dubey et al., 2007). Annual chickpea yield losses vary from 10 to 15%, but can result in total loss of the crop depending on fungal inoculum and environmental condition (Golakiya et al., 2018; Halila and Strange, 1996; Navas-Cortés et al., 2000). In particular, disease attacks are devastating if they occur when the crop is under heat and water stresses during the reproductive and seed filling phases (Landa et al., 2004a).

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Article History:
Received: 06/05/2020
Accepted: 10/07/2020
Published: 13/04 /2022

Disease management is difficult to achieve. Although many strategies have been investigated to control the disease in the field but no single control measure is fully effective. Resistant cultivars, sowing date, crop rotations, fungicide seed treatment and biocontrol agents have been used (Meki, et al., 2009; Merkuz et al., 2011; Haware et al., 1996). Highly resistant varieties are neither available nor can be effective against different races of the pathogen (Meki, et al., 2009; Merkuz et al., 2011; Merkuz, 2012) and significant pathogenic variability and new races of the pathogen have undermined their importance (Haware and Nene, 1982). Functional genomics studies could play a significant role to help better understanding of pathogen plant interaction and play an important role in resistance development against legume wilt (Hashem *et al.,* 2020). Sowing date, as the effect of temperature, affect slightly disease development. Because of the long inoculum survival in the soil, the crop rotation strategy in not so sufficient. Neither the use of fungicides is usually effective as it is used mainly for the seed borne inoculum and the effect is short lived. Biological control using microorganisms provides an alternative to the use of synthetic fungicides with the advantages of reduced environmental impact, greater public acceptance and becoming a critically needed component of plant disease management, particularly in reducing root diseases (Meki et al., 2009; Anjajah et al., 2003; Landa et al., 2004b). These antagonists besides of helping to cope with plant diseases, they also provide batter nourishment to host plants (Glick, 1995; Burr et al., 1998).

Plant growth promoting rhizobacteria (PGPR), such as *Bacillus* and *Pseudomo*nas strains, are the major root colonizers (Manikanda et al., 2010; Joseph et al., 2007), and can elicit plant defences (Kloepper et al., 2004). They have great potential in control of fusarium wilt disease of chickpea (Hervas et al., 1997; Zain et al., 2013: Chen et al., 2010: Karimi et al., 2012: Subhani et al., 2013).

The aim of this study was to evaluate the efficacy of three bacterial strains of the genus Bacillus and two strains of Pseudomonas genus that were previously identified as having potential biocontrol activity against different plant pathogens (Koch et al., 2010), (Amein et al., 2011), (Schmitt et al., 2009), (Amein & Weber, 2002) and (Amein et al., 2008) on control of chickpea Fusarium wilt disease caused by (Foc) in the field conditions.

2. MATERIALS AND METHODS:

2. 1. MATERIALS

2. 1. 1. Plant, fungal and bacterial source

Kabuli chickpea cultivar Flip 06 – 15 was obtained from *Agricultural Research Centre / Erbil.*

An isolate of Foc which was isolated from chickpea plant roots and maintained at Plant Protection Dept., laboratory was used in all experiments.

Five strains were selected from a group of bacterial collection, isolated from roots and seeds of different cereals, vegetable and oilseed rape, showing good results in earlier experiments against different pathogens in different crops were tested for their efficacy against the fungal pathogen *Foc* on chickpea.

These strains were three *Bacillus* and two *Pseudomonas*;

Bacillus subtilis strain (K3) was isolated from seed of oilseed rape in Sweden.

Bacillus amyloliquefaciens strain (5113) from collection of Dept. Plant Biology/ Swedish university of Agricultural Science.

Bacillus sp. strain (M1) was isolated from carrot seeds in Sweden.

*Pseudomo*nas *fluorescens* strain (L18). This bacteria was originally isolated from roots of golf grass at a golf course in Sweden.

*Pseudomo*nas sp. strain (53) was isolated from wheat plant root in Poland.

2. 2. Preparation of pathogen inoculum

To induce conidia formation, (Foc) was grown on potato dextrose agar plates for 2 weeks. Mycelium and conidia were collected from lawn cultures first by saturating the cultures with 2 ml sterile distilled water then by scrapping the mycelium with spores from the agar surface with the aid of a sterile glass spreader. The solution with spores then filtered through two layers of cheesecloth into 250 ml Erlenmeyer flasks. The culture filtrate were drained briefly for a minute, then washed the filtered mycelia while still in the filter twice with sterile distilled water.

The number of spores were counted using a haemocytometer, diluted to 1x 10^6 spores/mL as a stock spore solution, and kept at 4 °C until use.

2.3. Screening of antagonistic bacteria in in vitro and in greenhouse:

Before testing in the field, a screening of the antagonists in the Lab., and in greenhouse were done. The assay for antagonism was performed on PDA plates by the dual culture method. A mycelial plug (5 mm diameter) of the pathogen *Foc* culture, 5 days old was placed on the one side of a Petri dish and a loopful of bacteria was then streaked 2 cm away from the disc of *Fusarium* isolate on the same dish. Paired cultures were incubated at 25ºC. Plates inoculated only with test pathogen served as controls. The experiment had three replications of each treatment. The inhibition zone was recorded as the distance between the fungal pathogen and the area of antagonist growth after 24 to 72 h.

Greenhouse experiment was prepared by sowing infested seeds treated with individual bacterial suspension in pots (the method used was the same as described below for field experiments). Pots (10 x15 cm) field with sterilized soil were sown with 1 seed in each with 5 replicate for each treatment and were placed on greenhouse bench. After one month the plants were scored for disease development.

2. 4. Seed treatment for field experiments

Bacterial strains were pre- cultured for 24 h on Nutrient agar (NA) medium in Petri dishes at 26 - 28 °C, and were then further cultured by using single colony in Luria - Bertani (LB) broth medium on a rotary shaker for 48 h at 24 - 26 °C.

Before treating with biocontrol agents, seeds were first surface disinfested in 75% ethanol for 30 sec, washed twice in sterilized distilled water, and dried on sterilized filter paper. Seeds were then inoculated with *Fusarium* suspension (1 x 10^6 spore/ml.) in conical flasks. After five min. of flasks shaking by hands, seeds were dried on filter paper and after 2h were coated in new flasks with a suspension of an individual strain of bacteria (20 mL of bacterial biocontrol agent suspension / 100 g of chickpea seeds) and shaken for 5 min, then excess liquid was drained and the seeds were dried overnight on a laboratory bench at room temperature before sowing. The concentration of bacteria were between $10^4 – 10^6$ cfu/seed.

2. 5. Field experiments

Field experiments were conducted at the Experimental Farm of Gerdarash (Agriculture College Experiment Station) for two consecutive growing seasons. The experimental layout was randomized block (2m x 2m) with three replicates and 0.5 m in between. Treatments within blocks consisted of five rows with (15 seeds/ row). Chickpea seeds cv. Flip 06 - 15 artificially infested with *Fusarium* spore suspension and individual bacterial suspension were sown in the beginning of March.

Seeds treated with *Fusarium* alone were sown as control. Plots were watered after one week and then as needed. Number of germinated seeds were counted after two weeks of sowing date and results of other parameters (plant weight, plant length, seed weight, number of pods and disease severity index) were evaluated at harvesting in late June.

The disease severity index of chickpea symptoms was scored on a 0 – 5 scale, were 0 = healthy (no symptoms); 1 = 1 – 25% yellowing or wilting of the plant; 2 = 26 – 50% yellowing or wilting; 3 = 51 – 75% yellowing or wilting; 4 = 75 – 100% yellowing or wilting and 5 = plant dead.

A disease severity index (DSI) was calculated using the following formula:

$$DSI\ (\%) = \frac{\sum (\text{class} \times \text{no. of plants in class})}{\text{Total no. of plants} \times (\text{no. of classes} - 1)} \times 100$$

2. 6. Statistical analysis

For interpretation of data, analysis of variance (ANOVA) was used, with sources and amounts of variation compered using an F ratio test. To compare treatment means Duncan's Significance Level test ($P < 0.05$) was used.

3. RESULTS:

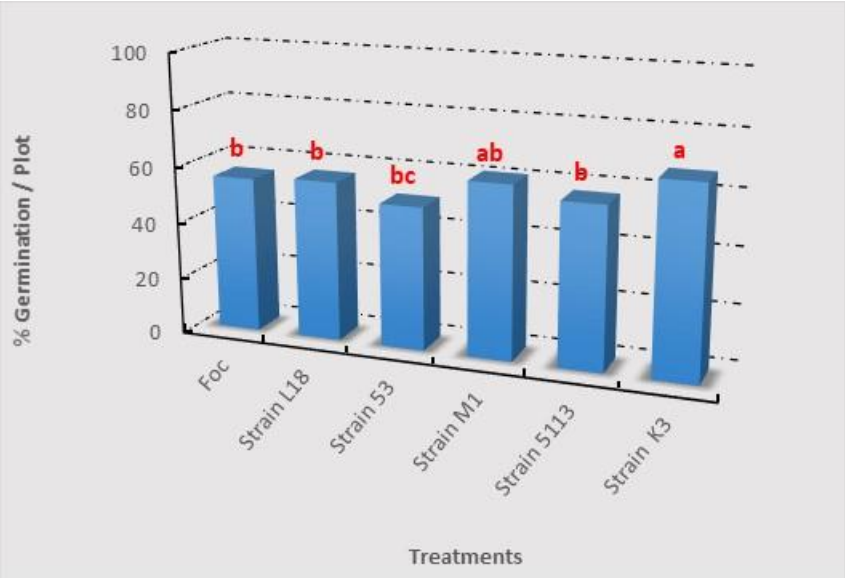
3. 1. Dual culture and greenhouse screening

In general all tested bacteria inhibited the growth of Fasarium mycelium in Petri dish plates, but there were differences in inhibition zones among different strains. Also in greenhouse screening most strains had positive effect in germination and plant health. (data not shown) because these were single experiments.

3. 2. Field Trials

3. 2. 1. Bacterial Effect on Seed Germination

Only Bacillus strain (K3) significantly increased the number of germinated seedlings. This was increased by (12%). The two other Bacillus strains (M1 and 5113) increased these numbers by (6 and 2% respectively). Also Pseudomonas strain (L18) slightly increased germination (1.4%), while Pseudomonas strain (53) decreased the germination by (4.3%) compared with the control (infested, untreated) (Fig. 1).

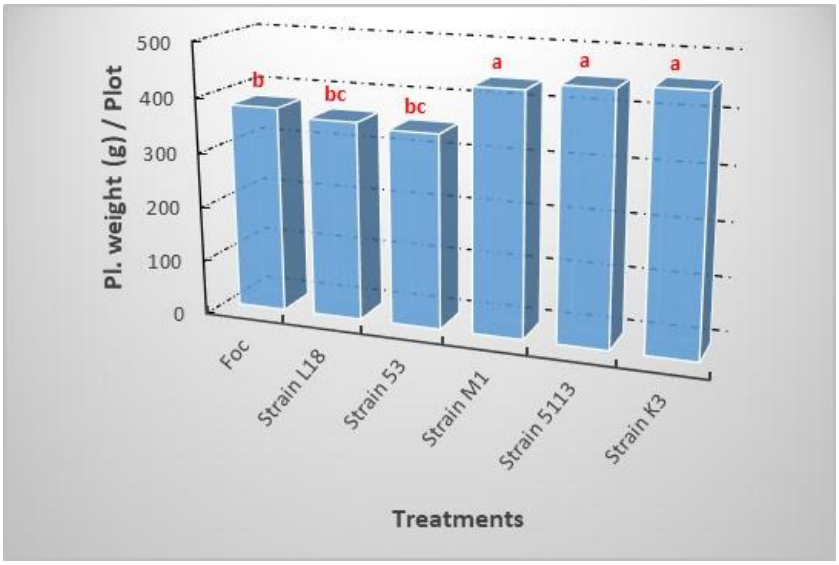


Different letters within columns indicate statistically significant differences, according to Duncan's Significance Level test ($P < 0.05$).

Figure 1: Effect of selected bacterial seed treatments on chickpea cv. Flip 06 – 15 plant germination, artificially infested with *Foc*. Means are for two field trials (each with three replicates, seventy five plants/replicate).

3. 2. 2. Bacterial Effect on Plant Fresh Weight

The result of bacterial effect on plant fresh weight is shown in (Fig.2). All three Bacillus strains (K3, 5113, and M 1) significantly increased plant weight (21.2, 19.6 and 16.4% respectively) whereas the two Pseudomonas strains (L18 and 53) both decreased plant weight (4 and 6.3% respectively).

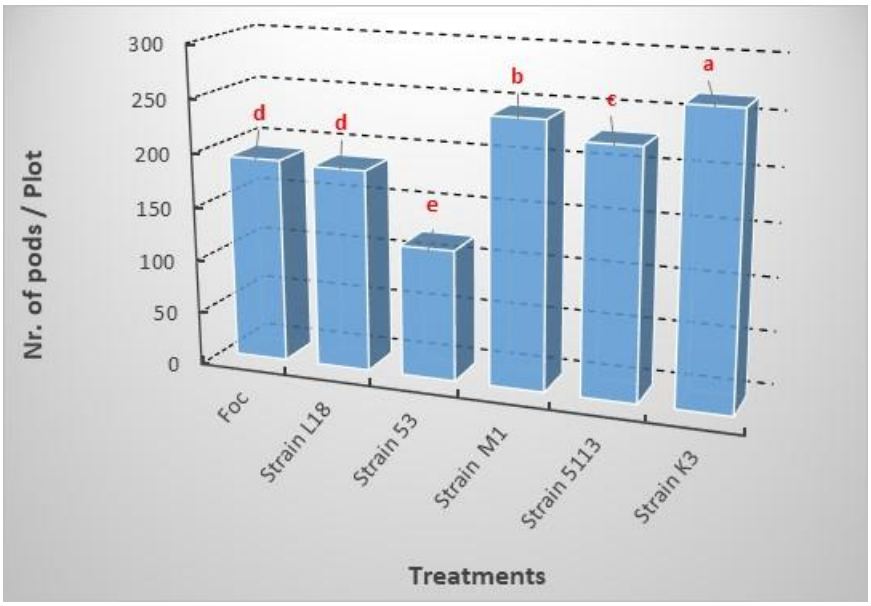


Different letters within columns indicate statistically significant differences,according to Duncan's Significance Level test ($P < 0.05$).

Figure 2: Effect of selected bacterial seed treatments on chickpea plant fresh weight, cv. Flip 06 – 15 artificially infested with *Foc.* Means are for two field trials (each with three replica, seventy five plants/replicate).

3. 2. 3. Bacterial Effect on Number of Pods

The Bacillus strains (K3, M 1 and 5113) increased the number of pods significantly (39.8, 28.8 and 19.9% respectively). Both Pseudomonas strains (L18 and 53) decreased the number of pods by (1.6 and 36.1% respectively), compared with the control (infested, untreated) (Fig. 3).

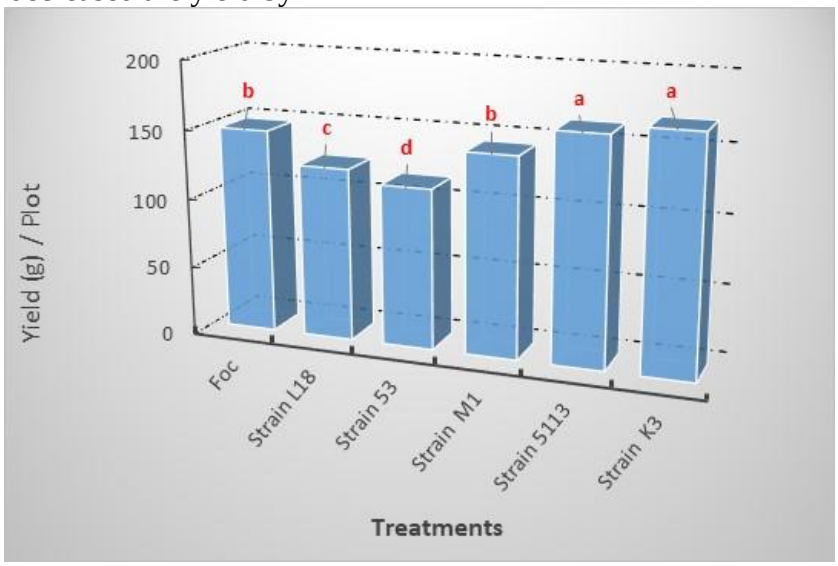


Different letters within columns indicate statistically significant differences, according to Duncan's Significance Level test ($P < 0.05$).

Figure 3: Effect of selected bacterial seed treatments on chickpea pods number, cv. Flip 06 – 15 artificially infested with *Foc*. Means are for two field trials (each with three replicates, seventy five plants/replicate).

3. 2. 4. Bacterial Effect on Yield

Two Bacillus strains (K3 and 5113) significantly increased the yield (14.2, and 10.1% respectively) while the strain (M 1) decreased the yield by (2.7%). Also the two Pseudomonas strains (L18 and 53) decreased the yield by (15.5 and 21.6% respectively) (Fig.4).

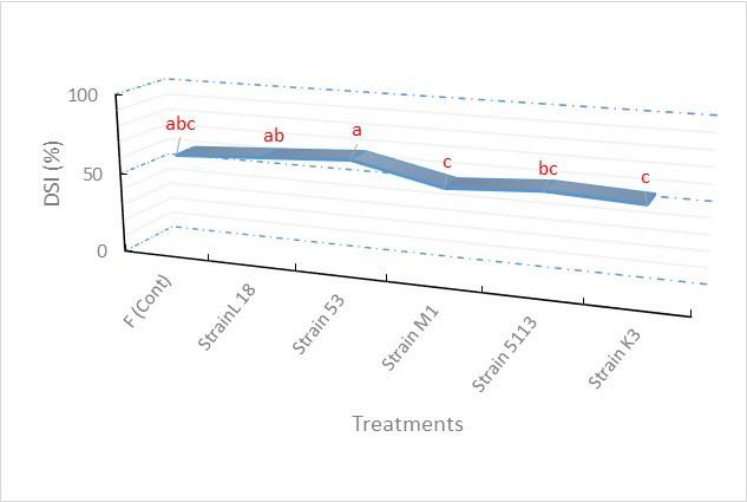


Different letters within columns indicate statistically significant differences,according to Duncan's Significance Level test ($P < 0.05$).

Figure 4: Effect of selected bacterial seed treatments on chickpea yield cv. Flip 06 – 15, artificially infested with *Foc*. Means are of two field trials (each with three replicates, seventy five plants/replicate).

3. 2. 5. Bacterial Effect on Disease Severity Index (DSI)

None of the tested bacterial strains decreased the disease severity index significantly compared with the control. All *Bacillus* strains reduced the disease severity. This was reduced by (9.2, 7.5 and 3.3) for strains M1, K3 and 5113 respectively. Both *Pseudomonas* strain L18 and 53 increased the disease severity by 5.3 and 10.7% respectively (Fig. 5).



Different letters within columns indicate statistically significant differences, according to Duncan's Significance Level test ($P < 0.05$).
Figure 5: Effect of selected bacterial seed treatments on chickpea disease severity, cv. Flip 06 – 15 artificially infested with *Foc*. Means are of two field trials (each with three replicates, seventy five plants/replicate).

4. DISCUSSION:

Chickpea production is severely reduced by Fusarium wilt caused by *Foc* in most chickpea growing areas of the world.

Field results indicate clearly the positive effect of all three *Bacillus* strains on chickpea seed infested with *Foc.* Of all strains tested, *Bacillus* strains K 3 had the best effect. It had significant effect on seed germination, plant fresh weight, number of pods and the yield. In previous experiments this strain has shown fabulous results in controlling some important plant pathogenic fungi f. ex. carrot seed infected by *Alternaria dauci* and *A. radicina* (Koch et al., 2010), cabbage seeds infected by *A. brassicola* (Amein et al., 2011) and also good effect against lamb's lettuce seeds infection by *Phoma valerianellae* (Schmitt et al., 2009).

Despite the high inhibition zone of Foc mycelium in dual culture experiment and positive effect in greenhouse screening and also shown good effect in controlling some important crop pathogens such as *Gaeumannomyces graminis* the cause of take- all disease of wheat (Amein & Weber, 2002) *Microdochium nivale*, the causal agent of snow mould and seedling blight of winter wheat (Amein et al., 2008), also good effect on carrot seed infection by *Alternaria dauci* and *A. radicina* (Koch et al., 2010) and *A. brassicola* on cabbage seeds (Amein et al., 2011), the *Pseudomo*nas *fluorescens* strain L18 reduced chickpea seed germination and showed negative effect on all other parameters tested in field trails. Reduction of seed germination and the negative effect it had on other parameter could be due to high bacterial population on seeds. This strain has shown toxic effect on wheat seeds and reduced germination when treated with high suspension (data not published). Therefore an optimization of bacterial number (seed testes in greenhouse) are needed before performing any field trails. The results of the other Pseudomonas strain (53) do not differ much from results of this strain and the reason could be the same

Both *Bacillus* and *Pseudomonas* strains have been reported for their positive effect in controlling many plant pathogenic fungi. Several reports have described *Bacillus* strains worthy to

be used as biocontrol agents for plant diseases. In many studies *B. subtillis* have shown to be one of the most effective agents in controlling *Fusarium oxysporum* of chickpea ((Belabid et al., 2018; Baysal et al., 2008; Gajbhiye et al., 2010; Chen et al., 2010). Zaim et al (2013) reported the positive effect of five *Bacillus* strains in reducing the disease severity of chickpea *Fusarium* wilt in pot assays and field trials. Also Moradi et al., (2012) reported significant reduction of disease severity by a strain of *B. subtillis* when this was used as seed treatment and as suspension added to the soil.

Also *Pseudomonas* strains have been reported for positive effect against the *Fusarium* wilt disease in chickpea (Karimi et al., 2012: Subhani et al. 2013; Murali Sankar *et al*., 2019). Karimi et al., (2012) reported that growth parameters (plant height, fresh and dry weight of plants) were significantly increased by *Bacillus* and *Pseudomonas* strains besides to good disease control. In greenhouse experiment a strain of *P. fluorescens* showed highly disease reduction percentage (76.78%) over uninoculated control (Mahmood et al., 2015). Also (Khan et al., 2004) reported yield increased by 39% when this was examined in chickpea plants growing in microplots under field conditions.

Many factors affect disease suppression by microbial agents, f. ex. the inoculum density and the race of the pathogen, the environmental conditions prevailing when biocontrol activity should operate and the chickpea genotype (Hervas et al., 1997; Landa et al., 2001).

5. CONCLUSION

In conclusion, seed coating with *Bacillus* and *Pseudomonas* bacterial strains in combination with other control strategies could be used successfully for control or at least decrease the effect of *Fusarium* wilt on chickpea production, but more experiments are needed to optimize the preparation of the most interesting bacterial strains and also testing their effects on other chickpea cultivars.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest (1)

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