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Screening of Rhizobacterial Isolates for Biological Control of Fusarium Wilt Disease of Chickpea

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Abstract The use of chemical control causing negative effects non-target environmental impacts and development of pesticide resistance to applied agent, The great interest in eco-friendly and sustainable agriculture, push towards gradually shifting to biological control instead of dependence on chemical. The Fusarium wilt is biotic stress that constraint the production and expansion of chickpea crop in Sudan. The aim of this study was to use rhizobacteria as bio control agent against chickpea Fusarium wilt. Eighteen soil samples taken from chickpea rhizosphere collected from six locations in central and Northern Sudan (three samples from each location). The chickpea rhizospheric bacteria were recovered from the 18 soil samples and their antagonistic activity against the most virulent FOC isolate was evaluated in vitro (using 76 rhizobacterial isolates) and in planta (using the ten most potential rhizobacterial isolates). 31 out of 76 isolates (nominated as SA1, SA2…., SA31) were considered as virulent bacterial isolates, shown clear inhibition zones against the most virulent FOC isolate (FOCS9). The widest inhibition zone diameter (25 mm) was recorded for isolate SA1 (No. 1) and the lowest zones (13.0 and 13.7 mm) were recorded for isolates SA30 (No. 30) and SA31 (no. 31), respectively. Generally, the in planta application of rhizobacterial isolates as biological control agents reduced the disease incidence compared with the controls.

Keywords: *diseases incidence, Fusarium oxysporum, Cicer arietinum, antagonistic bacteria*

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1. Introduction

Fusarium wilt caused by *Fusarium oxysporum* f.sp. ciceris is a major constraint to chickpea (*Cicer arietinum* L.) cultivation throughout the world [1]. Yield losses attribute Fusarium wilt vary from 10-15%, but the disease span completely destroy the crop under unfavorable conditions [2]. The most efficient method for the management of disease is using resistant cultivars [3,4], although new races of the pathogen appear to overcome resistant genes. In addition, chemical control is not satisfactory [5]. Increasing of use of chemical inputs causing several negative effects such as the development of pesticide resistance to applied agent, chemical inputs also have an effect on non-target environmental impacts [6]. The great interest in eco-friendly and sustainable agriculture, push towards gradually shifting to biological control methods instead of dependence on chemical methods [7]. Use of biological control agents, such as plant growth promotion rhizobacteria (PGPR), can be suitable approach in control of disease [8]. PGPR can suppress a broad spectrum of bacterial, fungal and nematode diseases. Also it can provide protection against viral diseases. Some of these rhizobacteria may also be used in integrated pest management programs. Significant control of plant pathogens has been demonstrated by PGPR in laboratory and greenhouse studies, but results in the field have been inconsistent. Progress in understanding of their diversity, colonizing ability and mechanism of action, formulation and application facilitate their development as reliable biocontrol agents against plant pathogens. The major groups of rhizobacteria with potential for biological control include Pseudomonas spp. and Bacillus spp. which are ubiquitous bacteria in agricultural soils, PGPR generally include the strains in the genera Serratia, Pseudomonas, Burkholderia, Agrobacterium, Erwinia, Xanthomonas, Azospirillum, Bacillus, Enterobacter, Rhizobium, Alcaligenes, Arthrobacter, Acetobacter, Acinetobacter, Achromobacter, Aerobacter, Azotobacter, Clostridium, Klebsiella, Micrococcus, Rhodobacter, Rhodospirillum, Flavobacterium, Bradyrhizobium, Frankia, Pseudomonas, Thiobacillus, Paenibacillus, Alicyclobacillus, Aneurinibacillus, Virgibacillus, Solibacillus, Gracilibacillus and others [7].

Different mechanisms have been reported for their performance such as production of antibiotics, siderphore

cyanide hydrogen, competition for nutrition and space, inducing resistance, inactivation of pathogen enzymes and enhancement of root and plant development [9]. Pseudonas and Bacillus strain have great potential in control of Fusarium wilt disease of chickpea [10,11,12].

PGPR have been reported as biocontrol agents of soil borne plant pathogen, production of siderphores that chelate iron, making it unavailable to phytopathogens, antagonism by synthesis of volatile and diffusible antifungal metabolites such as phenazine and hydrogen cyanid, the ability to successfully compete with pathogens for nutrients and niches on the root to induce systemic resistance [13,14].

The objective of this study is to screen Rhizobacteria isolates from chickpea rhizosphere with antagonistic activity against *Fusarium oxysporum* f.sp.ciceris invitro and inplanta.

2. Materials and Methods

2.1. Source of Potentially Antagonistic Bacteria

Eighteen soil samples as a good source of antimicrobially-active rhizobacteria were taken from chickpea rhizosphere collected from six site in centeral and Northern Sudan (El-madina Arab, Ganeb, Abugota, El- moaileg, Agricultural Research Corporation-Madani and Hudeiba Research Station) during season (2018/2019). Three samples from each location were taken). Samples were cooled to 4°C so that any change in the original microflora would be prevented. The weight of each sample was 100 g.

2.2. Isolation of Antagonistic Bacteria

Serial dilution technique described by Abdalla *et al*. [15] was adopted for isolation of rhizobacteria from the 18 soil samples.

In vitro screening of rhizobacteria for their antagonistic activity against F. oxysporum

Antagonistic activity of rhizobacteria isolates on *Fusarium oxysporum* f.sp ciceris was examined following the agar diffusion method as described by Pajand and Paul [16]. The fungus was tested as a plug of mycelium at the center of a Petri dish (9 cm) of half-strength PDA using sterile cork borer with a diameter of 0.78 mm. Sterile toothpicks were used to transfer each of rhizobacterial isolate from 2-days old cultures and spot it onto the agar surface near the outer edge of the dish. Plates were incubated at 27°C and inhibition zones diameters were measured after seven days. All bacterium-fungus combinations were replicated in three plates. Only those isolates that produced a clear inhibition zone against the fungus growth were considered to have an antagonistic activity [17].

In planta screening of antagonistic bacteria

The effect of the potential rhizobacterial isolates on controlling Fusarium wilt disease was tested on the most resistant and most susceptible chickpea genotypes. Bacterial isolates were grown, separately, in 250 ml Erlenmeyer flasks each containing 100 ml of nutrient broth medium and shook for 24 hrs onto a rotary shaker. The growth was diluted with an adequate amount of non-inoculated nutrient broth to obtain a bacterial suspension of 108 cfu/ml using a spectrophotometer (660 nm).

Chickpea seeds were surface sterilized with 70% ethanol, then immersed for 2 minutes in 2% sodium hypochlorite and washed four times with sterilized distilled water and left to dry. A total of 20 seeds were impressed in a Petri-dish containing bacterial suspension, for 24 hrs.' then placed on moistened sterile filter paper in Petri plates (four plates with 20 seeds/plate) and left to germinate at room temperature for five-days. Control plates were arranged in a similar way, except that they were treated with bacterial-free nutrient broth.

To evaluate the incidence and severity of Fusarium wilt disease, the germinated seeds of the two chickpea cultivars were treated with rhizobacterial isolates and transferred to 30×40inch plastic sacks containing a sand: clay soil mixture (1:1 w/w). In addition, a control set of germinated seeds treated with non-inoculated nutrient broth was also included. A factorial experimentwas arranged in a Completely Randomized Design (CRD) with four replicates each consisted of three plants per sack. Data generated from factorial experiment in CRD was analyzed using STATISTIX 8.0 analytical software.

2.3. Assessment of Biological Control of Wilt Disease

Disease reactions were assessed by the incidence and severity of symptoms according to Abdalla et al. [15] (2014).

3. Results and Discussion

3.1. In Vitro Screening of Rhizobacterial Isolates against FOC

Seventy-six rhizobacterial isolates were recovered from the 18 rhizospheric soil samples. The isolates were all tested for their efficacy in inhibiting the growth of *Fusarium oxysporum* f. sp. ciceris. A total of 31 isolates were considered as virulent isolates since they have shown clear inhibition zones against FOCS9 isolate. Analysis of variance for inhibition zones (Table 1) showed highly significant differences among the isolates.

Table 1. Analysis of variance for growth inhibition zone diameter of 31 rhizobacterial isolates and control (untreated)

Source of variation	df	S.S	M.S	F-value	Prob.
Between treatments	30	8.983	0.299	5.082	0.00
Within treatments	62	3.653	0.059		
Total	92	12.636			
CV%	12.73%				

The inhibitory zones diameters were in the range of 13 - 25mm (Table 2 and Plate 1). The widest inhibition zone diameter (25mm) was recorded for isolate SA40, followed by isolates SA57, SA63, SA67 and SA43 for which 24.7, 24, 23.7 and 23.3 mm inhibition zone diameters were recorded, respectively. The lowest

inhibition zones (13.0 and 13.7 mm) were recorded for isolates SA48 and SA28, respectively (Table 2). Plant Growth Promoting Rhizobacteria (PGPR) is considered as an alternative to chemical pesticides for the management of soil-borne pathogens. Kloepper [18] reported that Pseudomonas and Bacillus strains have great potential in the control of Fusarium wilt disease of chickpea. In addition, Bacterial biocontrol agents belonging to the genera Agrobacterium, Bacillus, Pseudomonas and Streptomyces, have been tested invitro and found to be effective against FOC in many studies [10,11,12].

3.2. In Planta Screening of Antagonistic Bacteria

The effect of the 10 most active rhizobacterial isolates on chickpea Fusarium wilt disease incidence was assessed on cultivars Shendi-1 (highly susceptible) and Burgaig (resistant). Analysis of variance (Table 3) showed significant differences ($P \leq 0.05$), throughout the experiment, among chickpea cultivars only. The overall progress of disease incidence for each cultivar is presented in Figure 1 and Figure 2. Generally, the application of rhizobacterial isolates as biological control agent reduced the disease incidence compared with the control in both cultivars. In cultivar Shendi-1, when the seeds were treated with isolate SA40 the disease symptoms started to appear at the second week after inoculation. Seven of the ten bacterial isolates, compared with the control, had a positive effect on disease incidence throughout the experiment; exceptions were isolates SA67, SA58 and SA9. Similarly, when cultivar Burgaig was treated with SA64 and (SA63; SA32), the incidence started to appear after two and three weeks prior to inoculation, respectively. All bacterial isolates, except SA67 and SA58, had a positive effect on disease incidence throughout the experiment compared with the control.Throughout the experimental period, the highest disease incidence was recorded for cultivar Shendi-1. At the second week after inoculation, incidence of 7.22 and 26.94% were recorded for Burgaig and Shendi-1 cultivars, respectively. However, at the eighth week the disease incidence increased to 57.3% and 78.91% for Burgaig and Shendi-1 cultivars, respectively (Figure 3).

Table 2. Growth inhibition zone diameter (mm) induced by Rhizobacteria isolates on Fusarium oxysporum f.sp ciceris:

Rhizobacteria Isolates	Zone diameter (mm)	% inhibition	Rhizobacteria isolates	Zone diameter (mm)	% inhibition
SA1 (SA40)	25.0 a	55.56	SA17	18.3 efghij	40.67
SA2 (SA57)	24.7 ab	54.89	SA18	18.3 efghij	40.67
SA3 (SA63)	24.0 ab	53.33	SA19	18.0 efghij	40.00
SA4 (SA67)	23.7 abc	42.22	SA20	17.0 fghijk	37.78
SA5 (SA43)	23.3 fghijk	51.78	SA21	17.0 abcd	37.78
SA6 (SA64)	22.3 abcde	49.56	SA22	16.7 ghijk	37.11
SA7 (SA58)	21.7 abcdef	48.22	SA23	16.7 ghijk	37.11
SA8 (SA9)	21.3 abcdefg	47.33	SA24	16.7 ghijk	37.78
SA9 (SA32)	21.0 abcdefgh	46.67	SA25	16.7 ghijk	37.11
SA10 (SA61)	20.7 abcdefghi	46.00	SA26	16.3 hijk	30.22
SA11	20.7 abcdefghi	46.00	SA27	16.0 ijk	35.56
SA12	20.3 bcdefghi	45.11	SA28	16.0 ijk	35.56
SA13	19.3 cdefghi	30.89	SA29	16.0 ijk	35.56
SA14	19.3 cdefghi	42.89	SA30 (SA48)	13.7 jk	30.44
SA15	19.0 defghi	42.22	SA31 (SA28)	13.0 k	28.89

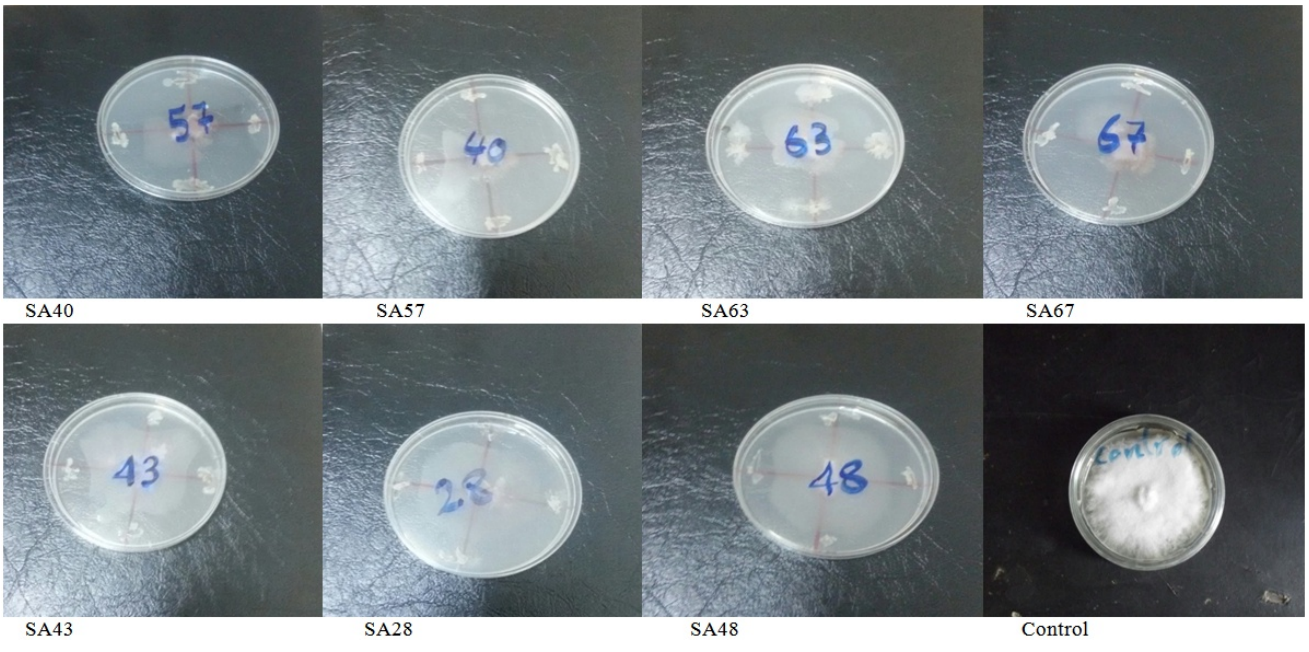

Plate 1. Growth inhibition zone diameters induced by Rhizobacteriaisolates on *F. oxysporium* f.sp.*ciceris*

Table 3. Mean square for disease incidence in Chickpea cultivars, rhizobacterial isolates and rhizobacterial isolates x chickpea cultivar throughout the experiment

Source of variation	df	Mean squares						
		Week2	Week3	Week4	Week5	Week6	Week7	Week8
Rhizobacterial isolates	10	702.35 ns	620.77 ns	802.57 ns	1058.16 ns	782.03 ns	914.70 ns	825.66 ns
Chickpea cultivar	1	3319.04**	4006.26**	4279.02*	8027.35**	5474.40*	5792.49*	4959.60*
Rhizobacterial isolates x Chickpea cultivar	10	737.11 ns	603.67 ns	609.06 ns	100.11 ns	167.00 ns	189.99 ns	226.40 ns
Error	66	421.08	534.50	886.52	909.64	926.58	917.05	941.77
CV%		133.58	120.72	107.43	53.65	52.59	51.45	51.60

df=degree of freedom,*=significant difference, **=highly significant difference and ns= non-significant difference.

Table 4. Mean square for disease severity in Chickpea cultivars, rhizobacterial isolates and rhizobacterial isolates x chickpea cultivar throughout the experiment

Source	df	Mean squares						
		Week2	Week3	Week4	Week5	Week6	Week7	Week8
Rhizobacterial isolates	10	0.13	0.28515	0.51852	1.27429	2.07155	2.42	2.70
Chickpea cultivar	1	0.61**	1.20*	1.29	4.09	9.52**	15.02**	19.10***
Rhizobacterial isolates x Chickpea cultivar	10	0.09	0.17	0.46	0.23	0.32	0.44	0.54
Error	66	0.08	0.26	0.52	1.08	1.34	1.49	1.58
CV %		154.28	149.37	138.74	72.29	59.39	55.90	53.04

df = degree of freedom; *and ** denote significant at 5% and 1%.

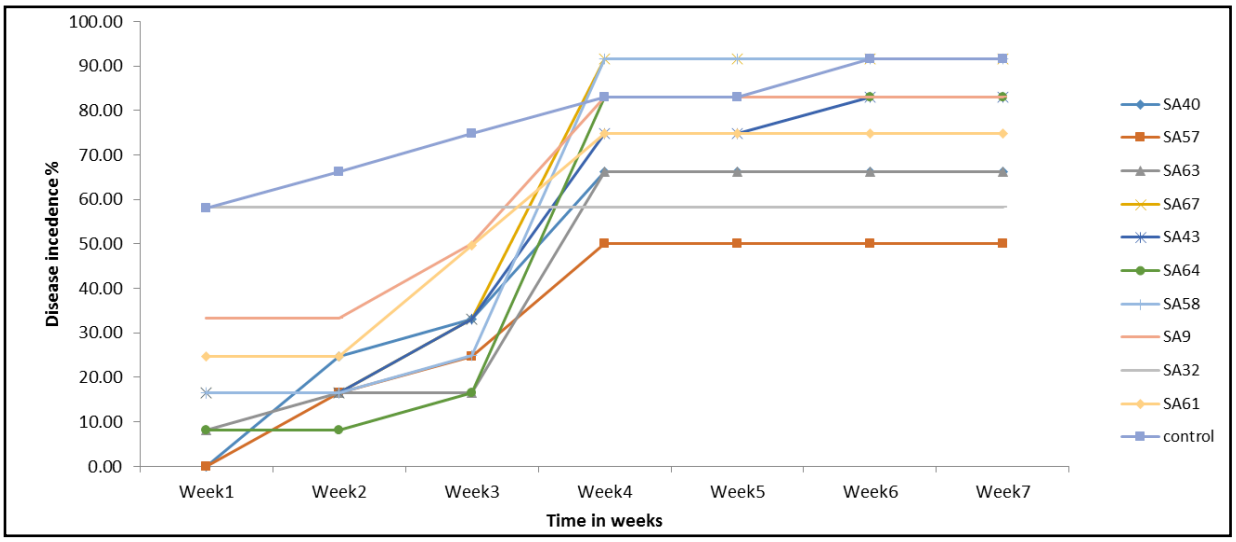

Figure 1. Disease incidence progress during 7 weeks in cultivar Shendi-1 when treated with different rhizobacterial isolates and the control

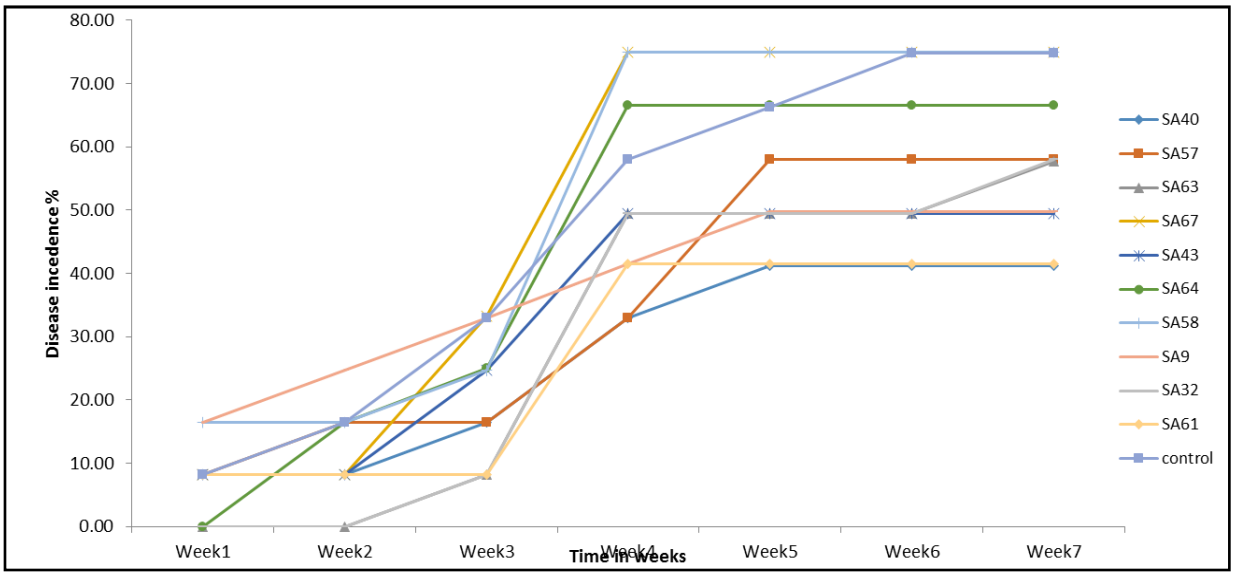

Figure 2. Disease incidence progress during 7 weeks, in cultivar Burgaig when treated with different rhizobacterial isolates

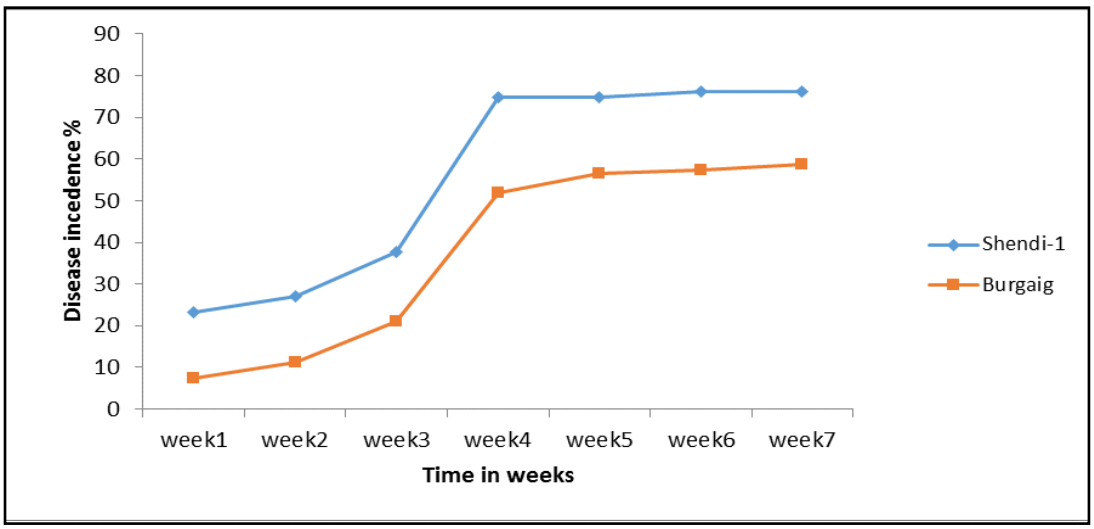

Figure 3. Main effect of cultivars on disease incidence throughout the experiment

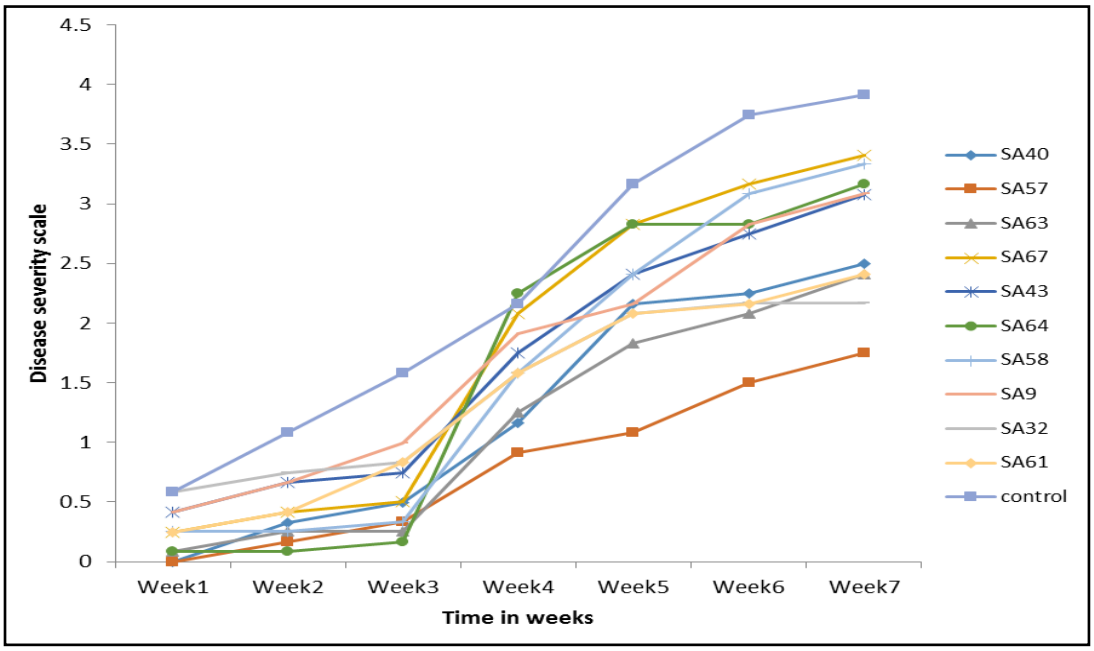

Figure 4. Disease severity progress during 7 weeks, in cultivar Shendi-1 when treated with different rhizobacterial isolates

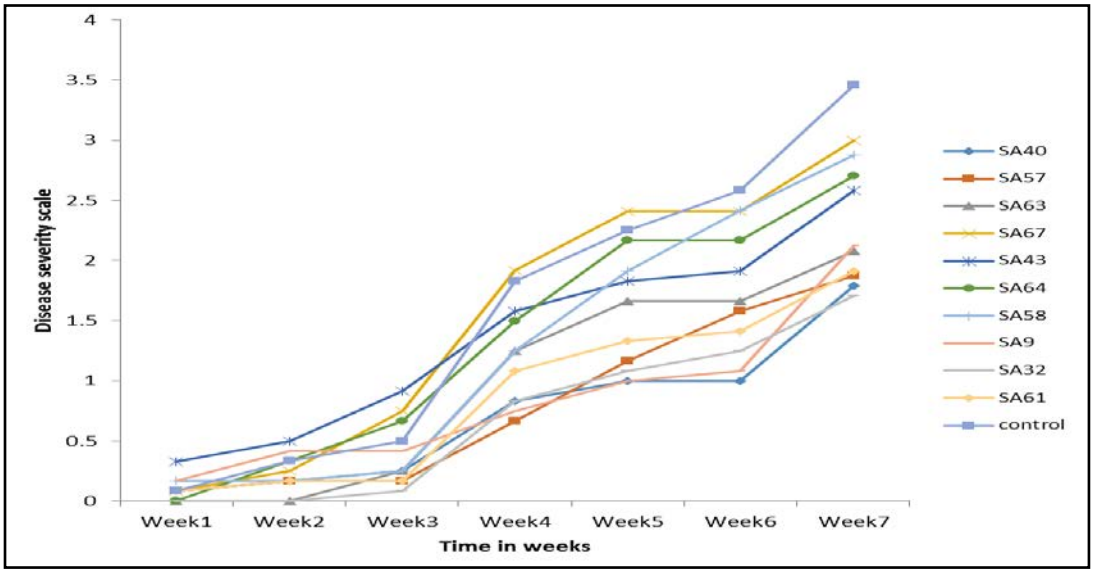

Figure 5. Disease severity progress during 7 weeks, in cultivar Burgaig when treated withdifferent rhizobacterial isolates

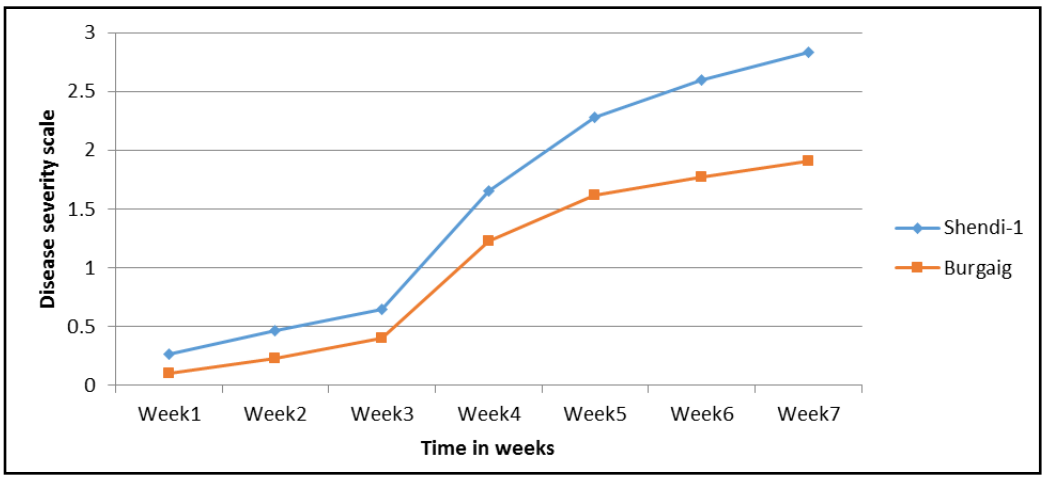

Figure 6. Main effect of cultivars on disease severity throughout the experiment

This result confirms the finding obtained by Ahmed and Adam [21] who reported 75% disease incidence for cultivar Shendi-1 which indicates its high susceptibility to *Fusarium* wilt disease. The lowest disease incidence of less than 10% have been scored for the cultivar Burgaig throughout the experimental period.

The overall development of disease severity in each of the two cultivars is presented in Figure 4 and Figure 5. For cultivar Shendi-1, all bacterial isolates, compared with the control, had significantly decreased disease severity from the 4th week onwards; except for isolates SA67 and SA64. However, for cultivar Burgaig, five isolates (SA40, SA57, SA9, SA32 andSA61) had a positive effect on disease severity, compared with the control.

Regarding the main effect of both cultivars and bacterial isolates on disease severity, significant differences ($p \leq 0.05$) were observed between cultivars during the 2nd, 6th, 7th and 8th weeks. However, no-significant differences were detected among the isolates throughout the experimental period (Table 4). Concerning the main effect of cultivars, the highest disease severity, throughout the experiment, was observed for cultivar Shendi-1. At the beginning of the experiment, the disease severity recorded was 0.26 and 0.1 for cultivar Shendi-1 and Burgaig, respectively. While at the end, the recorded severity was 2.84 for cultivar Shendi-1 and 1.91 for Burgaig (Figure 6). Previous studies have also reported an antagonistic activity of *Pseudomonas* sp. against *Fusarium* sp. [19]. Also, Kumari and Khanna [20], reported that twenty-eight, out of forty, rhizobacterial isolates showed antagonistic activity against *Fusarium oxysporum* f.sp. *ciceris*.

3.3. Effect of Rhizobacteria on Disease Index

The disease index for the two tested cultivars was measured in terms of disease incidence and severity. The first disease's symptoms appeared 12 days after inoculation. As shown in Table 4, isolate SA40 recorded the highest incidence (44.82) and the highest severity reduction of 63.89% with cultivar Burgaig. For cultivar Shendi-1, the highest incidence and severity reduction values were 36.34 and 55.36%, respectively, obtained after treatment with isolate SA61and SA57, respectively. On the other hand, isolate SA67 had the lowest incidence and severity reduction values in both cultivars.

4. Conclusions

Based on the results of this study, the in planta application of rhizobacterial isolates, reduced the disease incidence of the Fusarium Wilt disease of chickpea (*Cicer arietinum* L.). The study concluded that, the rhizobacteria can be used as biological control agents to control the Fusarium Wilt disease of the crop in the field. Further studies could be carried out in order to buttress the result obtained in this experiment

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