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Biological control of onion white rot disease using potential *Bacillus subtilis* isolates

M. M. Amin[1] and M. F. A. Ahmed[2*]

Abstract

Background Onion (*Allium cepa* L.) is a very important vegetable crop all over the world, particularly in Egypt for local consumption and exportation. White rot disease is the most serious disease of *Allium* spp. caused by the soil-borne fungus *Sclerotium cepivorum* Berk.

Results In this investigation, five tested isolates of *Bacillus subtilis* showed an antagonistic effect and significantly reduced the linear growth of the pathogen in vitro. *B. subtilis* isolates no. 2 and 4 caused the highest reduction of *S. cepivorum* growth, 75.78 and 74.33%, respectively, while isolate no. 3 was the least effective one, causing 66.67% growth reduction. Under field conditions at two successive seasons (2019/20 and 2020/21), all tested bioagent treatments reduced the percentage of infection than Folicure as an officially recommended fungicide. *B. subtilis* isolate no. 2 showed the highest efficacy (78.57 and 77.78%) followed by isolates no. 4 (74.29 and 72.22%), while *B. subtilis* isolate no. 3 showed the lowest efficacy (42.86 and 50.00%), respectively, for both successive seasons. All treatments increased dry onion bulb yield and improved its quality than the untreated control plants. The chemical changes of total carbohydrates, total nitrogen contents, total soluble solids (TSS %) and enzymes activity related to defense mechanisms in treated plants by biological treatments were considered.

Conclusion This study aimed to find effective biological agents of *B. subtilis* isolates against the white rot of onion and evaluate their effect on yield parameters, components and quality.

Keywords Onion, *Bacillus subtilis*, Biological control, White rot, *Sclerotium cepivorum*

Background

Onion (*Allium cepa* L.) is the most widely cultivated *Allium* spp. It is a very important crop in Egypt for local consumption and exportation. It has been reported that it be rich in phytochemicals especially medicinal flavonoids (Javadzadeh et al. 2009). In the last years, onion production was reduced significantly due to the white rot disease caused by *Sclerotium cepivorum* Berk., which is considered the most aggressive pathogen of *Allium* spp. The pathogen has become widely distributed in Egypt, causing great damage and sometimes total loss of onion crop reached to 100% (Ahmed and Ahmed 2015) and is considered the limiting factor for onion cultivation especially in Upper Egypt (Mohamed 2012).

Recently, humans realized that using many fungicides might harm the environment and human health due to their high toxicity, which may also lead to great disturbance in the biological balance (Ahmed et al. 2017).

The other suggested control methods included soil solarization (Melero-Vara et al. 2000), fumigation (Entwistle 1990) and application of biological control (Shalaby et al. 2013).

Several fungal and bacterial antagonists have proved to control different plant pathogenic fungi (Blaszczyk et al. 2014). The best results to control the white rot of onion are by using *Trichoderma harzianum, T. koningii, T.*

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asperellum, Talaromyces flavus and *Bacillus subtilis* (Mahdizadehnaraghi et al. 2015), increased yield and enhanced biochemical components of onion. Using microbial antagonists as bioagent formulations is considered a suitable ecological method to substitute chemical fungicides.

This study aimed to find effective biological agents of *Bacillus subtilis* isolates against the white rot of onion and to evaluate their effects on yield quantity and quality.

Methods

Sclerotium cepivorum

A preserved pathogenic *S. cepivorum* isolate causing white rot of onion previously isolated from diseased onion plants was tested for its pathogenicity and was used as a source of an aggressive pathogenic isolate in this study (Amin et al. 2016). Cultures of the 7-day-old pathogenic isolate used to inoculate barley seeds medium were incubated at 20 ± 1 °C for 3 weeks to be used as inoculum for further studies (Van der Meer et al. 1983).

Bacillus subtilis

Five isolates of *B. subtilis* were obtained from Biological Control Preparation Unit, Central Lab. of Organic Agriculture (CLOA), Agricultural Research Center (ARC), Giza, Egypt, to study their effect in controlling the white rot of onion. Isolates grown in 250-ml conical flasks; each contained 125 ml of Nutrient Glucose broth. The flasks were incubated on a rotary shaker (120 rpm) under complete darkness conditions at 25 ± 2 °C for 3 days. All cultures were individually blended in an electrical blender for 2 min and then used as suspension at concentration of 30×10^6 spores/ml with dilution 1:100 (Ahmed 2013).

Folicure fungicide

Folicure 25% EC (Tebuconazole 25%), a triazole fungicide, was used in the investigation as officially recommended fungicide in Egypt (Amin and Fawaz 2015) as a check control by dipping transplants for 5 min. in 25 ml fungicide/l water just before transplanting and also spraying grown plants at 6 and 12 weeks after transplantation by 187.5 ml/100 L water.

Antagonistic effect in vitro

Sterilized Petri dishes, 9 cm containing 10 ml of nutrient glucose agar as medium, were inoculated 1 cm apart of plate edge with 0.6 cm disk of 5-day-old *S. cepivorum* culture incubated at 22 °C. On apart of 1 cm from the other side of the plate edge, a streak of each bacterial isolate was done. Five Petri dishes were used for each treatment and control (only fungal disk). Plates were incubated at 25 ± 2 °C. Inhibition zone was measured after the full growth of control plates (Ahmed et al. 2017).

Greenhouse experiment

Pot experiments carried out under greenhouse conditions during 2019/20 and 2020/21 seasons. Plastic pots (30 cm diam.) were filled with sterilized sand–clay soil (1:1 v/v) and infected 7 days before transplanting by 2% w/w *S. cepivorum* inoculum grown on barley seeds medium. Four pots were used at each treatment and control. Onion transplants dipped for 5 min in modified bacterial suspension with 1% Arabic gum, Folicure emulsion or water as a control treatment and left to air dry for one hour before transplantation (Ahmed et al. 2017). Five seedlings of cv. Giza 6, 50 days old, were transplanted in each pot on 15 November and irrigated when needed. The percentage of disease infection was determined at the end of each season as follows:-

$$\%\text{of disease infection} = \frac{\text{Number of infected plants}}{\text{Number of cultivated plants in pot}} \times 100$$

Field experiment

Field experiments were carried out during 2019/20 and 2020/21 growing seasons in naturally infested soil having history of high infestation with *S. cepivorum* at Mallawi Agriculture Research Station, Menia Governorate, Egypt. Complete randomized blocks were used. Three plots were used as replicates for each biological treatment, Folicure and control. The area of each plot was 10.5 m^2 (3.0 × 3.5 m).

Onion transplants cv. Giza 6 of 50 days old were transplanted on 20 November after dipping in such antagonistic bacteria or Folicure treatments. All treatments received regular agricultural practices including irrigation and fertilization until harvest in April. Percentage of disease infection was estimated by the end of each growing season as follows:-

$$\%\text{of disease infection} = \frac{\text{Number of infected plants}}{\text{Number of cultivated plants in plot}} \times 100$$

$$\%\text{Efficacy of each treatment} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$$

Biochemical analysis: total carbohydrates, total nitrogen contents and TSS in produced dry onion bulbs

At the end of each season, total carbohydrates and total nitrogen were determined in the aqueous extract of onion bulb according to Dubois et al. (1956), while total soluble solids (TSS%) were measured using a Carl Zeiss hand refractometer.

Enzymatic activity in response to white rot disease development and plant resistance

After 30 days from transplanting, as an indicator of biochemical activities response to white rot infection development, the peroxidase and polyphenol oxidase were determined using the method of Maxwell and Bateman (1967) and Thimmaiah (1999), respectively.

Statistical analysis

Data were statistically analyzed, and the significance of such treatments was assessed by the least significant difference (LSD) at 5% probability using the SAS ANOVA program V.9 (Anonymous 2014).

Results

Antagonistic effect of Bacillus subtilis isolates in vitro:

Data in Table 1 indicated that all *B. subtilis* isolates as antagonistic bacteria caused a significant reductions in the linear growth of *S. cepivorum*. The reduction ranged between 75.78% with isolate no. 2 and 66.67% with isolate 3. *B. subtilis* isolate no. 2 caused the highest reduction (75.78%), followed by *B. subtilis* isolate no. *4* (74.33%). On the other hand, *B. subtilis* isolate no. 3 showed the lowest effective one, being 66.67% in the reduction of fungal growth.

Table 1 Effect of *Bacillus subtilis* isolates on linear growth of *Sclerotium cepivorum* grown on nutrient glucose agar medium at 25 ± 2 °C

Bacillus subtilis	*Sclerotium cepivorum*	
	Linear growth (mm)	Reduction (%)
Isolate no. 1	26.0	71.11
Isolate no. 2	21.8	75.78
Isolate no. 3	30.0	66.67
Isolate no. 4	23.1	74.33
Isolate no. 5	28.1	68.78
Control	90.0	00.00
L.S.D. at 0.01%	–	0.62

Greenhouse experiments

Efficacy of *B. subtilis* five isolates was evaluated to control onion white rot in comparison with Folicure fungicide under greenhouse conditions. According to the present data in Table 2, all isolates significantly decreased the percentage of the white rot infection than the control in the two successive growing seasons 2019/20 and 2020/21, respectively. *B. subtilis* isolate no. 2 showed the highest efficacy (78.57 and 77.78%), followed by isolate no. 4 (74.29 and 72.22%) in decreasing *S. cepivorum* in both seasons, respectively. On the other hand, *B. subtilis* isolate no. 3 showed the lowest efficacy (42.86 and 50.00%) in controlling onion white rot, than the other treatments and control treatment.

Field experiments

Data in Table 3 demonstrated that all isolates of *B. subtilis* and Folicure treatments significantly reduced disease incidence than the control treatment during the two growing seasons 2019/20 and 2020/21 under field conditions. *B. subtilis* isolate no. 2 showed the highest efficacy, 81.35 and 79.65% followed by isolate no. 4, 72.17 and 75.43% in controlling onion white rot during both successive seasons, respectively. On the other hand, *B. subtilis* isolate no. 3 showed the lowest efficacy in controlling disease incidence at 58.97 and 61.10% in comparison with other treatments and control treatment.

Dry onion bulb yield

Data in Table 4 showed that applying tested *B. subtilis* antagonistic isolates and Folicure, as a recommended fungicide treatment significantly increased dry onion bulb yield than the yield of non-treated onion plants

Table 2 Effect of *Bacillus subtilis* isolates and Folicure fungicide treatments on onion white rot disease incidence under greenhouse conditions during 2019/20 and 2020/21 growing seasons

Treatments	2019/20		2020/21	
	Infection (%)	Efficacy (%)	Infection (%)	Efficacy (%)
Bacillus subtilis Isolate no. 1	30.0	57.14	35.0	61.11
Bacillus subtilis Isolate no. 2	15.0	78.57	20.0	77.78
Bacillus subtilis Isolate no. 3	40.0	42.86	45.0	50.00
Bacillus subtilis Isolate no. 4	18.0	74.29	25.0	72.22
Bacillus subtilis Isolate no. 5	35.0	50.00	40.0	55.56
Folicure 25% EC	25.0	64.29	30.0	66.67
Control (Untreated)	70.0	00.00	90.0	00.00
L.S.D. at 0.05%	2.17	–	2.72	–

Table 3 Effect of *Bacillus subtilis* and Folicure treatments on onion white rot incidence under field conditions at Mallawi, Menia governorate, during 2019/20 and 2020/21 growing seasons

Treatments	2019/20		2020/21	
	Disease incidence (%)	Efficacy (%)	Disease incidence (%)	Efficacy (%)
Bacillus subtilis Isolate no. 1	20.66	63.30	22.67	65.82
Bacillus subtilis Isolate no. 2	10.50	81.35	13.50	79.65
Bacillus subtilis Isolate no. 3	23.10	58.97	25.80	61.10
Bacillus subtilis Isolate no. 4	15.67	72.17	16.30	75.43
Bacillus subtilis Isolate no. 5	22.50	60.04	24.83	62.57
Folicure 25% EC	18.68	66.82	21.80	67.13
Control (Untreated)	56.30	00.00	66.33	00.00
L.S.D. at 0.05%	2.68	1.68	2.85	–

during the two growing seasons 2019/20 and 2020/21. *B. subtilis* isolate no. 2 caused the highest significant increase in dry onion bulb yield followed by isolate no. 4. In addition, the two isolates were superior on Folicure treatment.

Total carbohydrates, total nitrogen contents and TSS in produced dry onion bulbs

Data of biochemical analysis including total carbohydrates, total nitrogen and total soluble solids presented in Table 5 showed a great increase in onion bulb contents from the total carbohydrates and total nitrogen, while some enhancement of TSS contents in treated onion plants with such tested Bacillus isolates and the fungicide measure during both seasons. Total carbohydrates and nitrogen contents in onion dry bulbs achieved by treating onion plants with *B. subtilis* isolate no. 2 followed by isolate no. 4. Folicure and *B. subtilis* isolate no 1 resulted in high contents also followed *B. subtilis* isolate no.3. These results indicated that all treatment kept onion plants healthy and supported its optimal growth, which is emphasized by too low chemical contents in the control treatment. The white rot disease percentages obtained previously for such treatment and the control could clarify these findings. The TSS percentages were also better in dry onion bulbs of plants biologically treated with Bacillus isolates or Folicure than that produced in the control plants.

Enzymatic activities in response to white rot disease development and onion plant resistance

Presented data in Table 6 showed that applying all tested *B. subtilis* antagonistic isolates and Folicure significantly increased the peroxidase and polyphenol oxidase activity of onion plants compared to untreated plants during the two growing seasons 2019/20 and 2020/21 under field conditions. In the two successive seasons compared to control treatment, *B. subtilis* isolate 2 was the highest treatment in increasing the activity of PO more than other treatments including Folicure, followed by isolate 4., while isolate 3 was the lowest one. Concerning the activity of PPO, isolates 1, 2 and 4, respectively, gave the highest activity more than other treatments including Folicure. Activities of

Table 4 Effect of *Bacillus subtilis* and Folicure treatments on onion yield under field conditions at Mallawi, Menia governorate, during 2019/20 and 2020/21 growing seasons

Treatments	2019/20		2020/21	
	Yield (kg/plot)	Efficacy (%)	Yield (kg/plot)	Efficacy (%)
Bacillus subtilis Isolate no. 1	34.80	287.60	33.70	283.19
Bacillus subtilis Isolate no. 2	41.50	342.98	41.30	347.06
Bacillus subtilis Isolate no. 3	32.70	270.25	32.50	273.11
Bacillus subtilis Isolate no. 4	40.90	338.02	40.60	341.18
Bacillus subtilis Isolate no. 5	33.60	277.69	32.90	276.47
Folicure 25% EC	37.70	311.57	36.80	309.24
Control (Untreated)	12.10	00.00	11.90	00.00
L.S.D. at 0.05%	2.41	1.68	2.42	1.93

Table 5 Effect of *Bacillus subtilis* and Folicure treatments on chemical components of yielded dry onion bulbs at the end of the growing season produced under field conditions at Mallawi, Menia governorate, during 2019/20 and 2020/21 growing seasons

Treatments	2019/20			2020/21		
	Total carbohydrates (mg/g dry Weight)	Total nitrogen (%)	TSS (%)	Total carbohydrates (mg/g dry weight)	Total nitrogen (%)	TSS (%)
Bacillus subtilis Isolate no. 1	1.53	13.21	12.85	1.53	13.15	12.83
Bacillus subtilis Isolate no. 2	1.87	15.99	14.55	1.86	15.89	14.46
Bacillus subtilis Isolate no. 3	1.38	12.06	11.93	1.37	11.90	11.90
Bacillus subtilis Isolate no. 4	1.82	14.56	13.90	1.77	14.23	13.85
Bacillus subtilis Isolate no. 5	1.44	12.99	12.65	1.41	12.75	12.53
Folicure 25% EC	1.58	13.38	12.95	1.55	13.36	12.90
Control (Untreated)	0.16	8.88	10.83	0.16	7.95	9.98
L.S.D. at 0.05%	0.12	0.58	0.55	0.10	0.60	0.66

peroxidase and polyphenol oxidase had a remarkable increase in treated plants with bacterial bioagents than the untreated plants.

Discussion

The present work designed to reduce using toxic chemicals in agriculture processes to produce food of high quality in sufficient quantity, enhance biodiversity system, maintain and increase the long-term fertility of soils. In addition, we find out the most suitable non-chemical methods to protect onion plants by using potential *Bacillus subtilis* isolates against white rot disease.

Table 6 Effect of *Bacillus subtilis* isolates and Folicure treatments on peroxidase and polyphenol oxidase activity of onion plants after 30 days from transplanting under field conditions at Mallawi, Menia governorate, during 2019/20 and 2020/21 growing seasons

Treatments	2019/2020		2020/2021	
	Peroxidase mg/ml	PPO (unit/mg protein)	Peroxidase mg/ml	PPO (unit/mg protein)
Bacillus subtilis Isolate no. 1	0.187	0.170	0.189	0.190
Bacillus subtilis Isolate no. 2	0.225	0.035	0.233	0.036
Bacillus subtilis Isolate no. 3	0.146	0.012	0.151	0.013
Bacillus subtilis Isolate no. 4	0.201	0.030	0.226	0.032
Bacillus subtilis Isolate no. 5	0.158	0.015	0.160	0.017
Folicure 25% EC	0.195	0.022	0.198	0.024
Control (Untreated)	0.065	0.009	0.067	0.010
L.S.D. at 0.05%	0.044	0.005	0.033	0.006

Data obtained indicated that all *B. subtilis* isolates caused a significant reduction in the linear growth of *S. cepivorum.* The present results proved that all tested antagonists indirectly inhibited the in vitro growth of *S. cepivorum* indirectly through the production of toxic metabolites. These results are in harmony with those obtained by Ouf et al. (2008) who found a commercial product of *B. subtilis* reduced the growth rate of *S. cepivorum* in vitro, where this reduction increased with increasing biocide concentration up to 2% of a commercial product of growth medium. However, the effectiveness of *B. subtilis* was less than that obtained by *Trichoderma viride*.

Obtained differences among *B. subtilis* in this investigation were also found by Dinu et al. (2012) who tested certain bioagents and found that two *B. subtilis* isolates and one isolate of *Pseudomonas chlororaphis* were superior to *B. pumilus* and *B. amyloliquefaciens* to suppress *S. cepivorum* growth in vitro, and all tested isolates produced two bioproducts suppressed differently fungus mycelial growth. In vitro inhibition of *S. cepivorum* growth obtained by *Bacillus* spp. attributed to the production of a large number of peptides antibiotics and antifungal secondary metabolites representing at least 25 different chemical structures that inhibit mycelial growth of various pathogens by diffusion in culture medium (Al-Ajlani and Hasnain 2006). In addition, (Ahmed 2013) reported different antibiotics such as bacteriocin and subtilisin and volatile compounds.

Five isolates of *B. subtilis* were used to evaluate their efficacy to control onion white rot in comparison with Folicure fungicide under greenhouse conditions. According to the present data, all isolates significantly decreased the percentage of white rot infection than the control in the two successive seasons 2019/20 and 2020/21, respectively. *B. subtilis* isolate no. 2 showed the highest efficacy (78.57 and 77.78%).

These results can be explained by the conclusion obtained by Ahmed (2013) who stated that the efficacy of microbial antagonists depends on their capacity to compete with other pathogenic microorganisms by occupying rhizosphere area under different environmental conditions, as well as the production of phytohormones enhancing plant disease resistance. Other study explains the role of some species related to group of Bacillus as well as producers of variable chemical compounds with different structures that have antagonistic effects against many pathogens. Most bioactive, compounds produced by *Bacillus* spp., are bacteriocins, polyketides, lipopeptides and siderophores. Generally, they have a wide range of antagonistic activity against different plant pathogenic fungi, bacteria and viruses (Fira et al. 2018). It is worth mentioning that during this investigation Folicure fungicide treatment gave 64.29 and 66.67% efficacy in reducing onion white rot disease during both investigating seasons. This finding indicates that *B. subtilis* isolates no. 2 showed more suppressive activity of white rot disease than the recommended fungicide, Folicure.

Data of the present study demonstrated that all isolates *B. subtilis* and Folicure treatments significantly reduced disease incidence than the control treatment during the two growing seasons 2019/20 and 2020/21 under field conditions. *B. subtilis* isolate no. 2 showed the highest efficacy. Many studies proved that *B. subtilis* is one of the plant growth-promoting rhizobacteria (PGPR), which suppresses a variety of root and vascular diseases caused by soil-borne pathogens (Mishra et al. 2013). Ahmed (2013) also showed that bioagents with different striations own different defense mechanisms against enzymes which dissolve the cell wall of the pathogen, antibiotics such as bacteriocin and subtilisin, volatile compounds and phytotoxic substances.

Obtained data showed that *B. subtilis* isolate no. 2 caused the highest significant increase in dry onion bulb yield, followed by isolate no. 4. In addition, the two isolates were superior on Folicure treatment, which is attributed to their capability as plant growth promoters as phosphate solubilizes and producers of phytohormone indole 3-acetic acid, which affect plant growth and yield, surplus producing phenolic compounds resulting in higher yield when adopted on different field crops (Khan et al. 2020). On the other hand, *B. subtilis* isolate no. 3 was the least effective one during the two growing seasons.

These results are in agreement with those reported by Fayzalla et al. (2011) who mentioned that the biocontrol agents effectively managed the pathogen and simultaneously increased the growth of plants and its production as yield increase in both onion bulb and seed crop. The increase in yield also may be due to either healthy root system that absorb and supply adequate amount of mineral nutrient or the syntheses of these raw nutrient materials effectively in the presence of high amount of chlorophyll and protein that led to more fruit yield (Mahmoud et al. 2016).

Data of biochemical analysis including total carbohydrates, total nitrogen and total soluble solids showed a great increase in onion bulb contents. These results indicated that all treatment kept onion plants healthy and supported its optimal growth, which is emphasized by too low chemical contents in the control treatment. The white rot disease percentages obtained previously for such treatment and the control could clarify this finding. Therefore, biological isolates and Folicure treatments resulted in increased yield and enhanced quality of dry onion bulbs. These results are in harmony with those obtained by Ahmed et al. (2017) who concluded that treatments affect positively plant protection and disease reduction is accompanied with increase in amount of total carbohydrates, total nitrogen and TSS.

Presented data showed that applying all tested *B. subtilis* antagonistic isolates and Folicure significantly increased peroxidase and polyphenol oxidase activity of onion plants than untreated plants during the two growing seasons 2019/20 and 2020/21. Activities of peroxidase and polyphenol oxidase had a remarkable increase in treated plants with bacterial bioagents. More activity of these enzymes is well associated with the defensive system of host plants (El-Meneisy et al. 2019). It was concluded that the positive control of white mold disease on onion plants was achieved by *B. subtilis* isolates attributed to the direct suppressive effect on fungal pathogen, *S. cepivorum*, and due to increasing plant resistance through activation of defensive enzymes.

Conclusion

Five isolates of *B. subtilis* were tested in this investigation under laboratory, greenhouse and field conditions to evaluate their capability to control white rot disease on onion incited by *S. cepivorum*. All tested isolates reduced linear growth of *S. cepivorum*, highly significantly decreased disease incidence under greenhouse and field conditions. All isolates increased onion yield, total carbohydrate, total nitrogen, TSS and activity of PO and PPO. *B. subtilis* isolates no. 2 and 4 gave best results than the control as well as Folicure fungicide.

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