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Evaluation of biological control agents for managing squash powdery mildew under greenhouse conditions

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Abstract

Squash (*Cucurbita pepo* L.), is one of the most important vegetable crops for human nutriment in Egypt and the world. One of the most serious diseases that infect squash and cause yield losses was powdery mildew, caused by *Podosphaera xanthisii*. The aim of this study was to investigate the role of *Bacillus subtilis*, *Paenibacillus polymyxa* (10^9 cell ml/1), *Trichoderma harzianum*, *T. album*, *T. viride* and *T. hamatum* (10^7 spore ml/l) for controlling disease under greenhouse conditions. Results indicated that all treatments significantly inhibited the conidial germination of *P. xanthii* than control in vitro and decreased the incidence and disease severity after spraying with the bio-agents on squash plants under greenhouse conditions. The fungicide, Topas-100 (10.0% penconazole "w/v" [(R,S-1-(2-(2,4-dichlorophenyl)-Q pentyl)-1H-1,2,4-triazole]), followed by *B. subtilis* was highly significant for decreasing disease incidence (2.8 and 5.3%, respectively) and disease severity percentage (3.5 and 4.8%, respectively) than the control. The activities of biochemical changes, i.e., peroxidase, polyphenol oxidase, and total phenols, were significantly upregulated as results of most treatments. Also, bio-agent treatments caused significant increase in yield characteristics of squash plants such as fruit number/plant and fruit weight/plant than control. *B. subtilis* recorded the highest increase (110.9% and 98.7%) in fruit number and fruit weight/plant than control.

Keywords: *Bacillus* sp., *Trichoderma* sp., Powdery mildew, Squash, Antioxidant enzyme

Background

Squash (*Cucurbita pepo* L.), is one of the most important vegetable crops for human nutrition. It is a popular vegetable in Egypt and can be produced almost all year-round. Squash plants are infected by many diseases including anthracnose, *Cercospora* leaf spot, mosaic, downy mildew, *Phytophthora* crown rot, *Septoria* leaf spot, squash mosaic, *Verticillium* wilt, and powdery mildew which severely reduce their productivity (Gordon 2018). Powdery mildew (*Podosphaera xanthii*) is considered the most serious disease causing yield losses, as it affects the leaves, stems and fruits of squash grown under different conditions (Hafez et al. 2018). The fruit losses of squash estimated 30–50% by attacking by *Podosphaera xanthii* El-Naggar et al. (2012).

Chemical control alternative for powdery mildews disease by biological ways is environmentally friendly. In recent years, the development and resistance of pathogen cause of less effective of fungicides beside pollution and potentially undesirable effects of the chemical fungicides on human and environment (Manandhar et al. 1988). Successful biological control under greenhouse conditions on foliar diseases has been achieved by a number of researchers using fungal or bacterial bio-against (Hussein et al. 2007).

Gilardi et al. (2008) evaluated the activity of 2 bio-agents mainly *B. subtilis* and *A. quisqualis* alone and in combination with the two fungicides penconazole and azoxystrobin against *P. xanthii* on zucchini (*Cucurbita pepo* L.) under controlled conditions. Abdel-Kader et al. (2012) reported that *Trichoderma viride*, *T. harzianum*, *P. fluorescens*, and *B. subtilis* reduced disease incidence of powdery and downy mildews of cucumber than the control. Use of bio-agents is safely and easily applied and cost-effective control treatment against plant foliar

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diseases. *Trichoderma* isolates have strong antagonistic and mycoparasitic effects against phytopathogens. Therefore, they are able to reduce disease severity in plants (Viterbo and Horwitz 2010; Elsharkawy et al. 2013).

Hafez et al. (2018) found that the bio-agents (*Bacillus* spp. and *Trichoderma* spp.) reduce significant disease severity of squash powdery mildew (*Podosphaera xanthii*). However, the bio-agent role could be attributed to up-regulation of defense-related enzymes catalase, peroxidase, and polyphenol oxidase, which stimulate growth and yield characteristics to control. The fungicide Topas-100 one of systemic fungicides is more efficient in management of cucumber powdery and downy mildews (Abada et al. 2009). Disease severity of squash powdery mildew was reduced with an average of 10.34% when used the fungicide Topas-100 at recommended dose (El-Ghanam et al. 2018).

Here, we investigate the effect of six antifungal and bacterial bio-agents to reduce powdery mildew disease of squash that caused by *P. xanthii* under greenhouse conditions and their effects on the activity of defense-related enzymes as well as the growth and yield of squash plants.

Materials and methods

Source of bio-agents

The efficacy of 2 bacterial bio-agents and 4 fungal strains comparing with the fungicide Topas-100 (10.0% penconazole) for controlling squash powdery mildew disease under greenhouse conditions was estimated. Squash seeds 'cv. Eskandarani' were grown one seed per 15 cm pot. *Bacillus subtilis* and *Paenibacillus polymyxa* were isolated from the surface of healthy cucumber and squash leaves and identified according to Kamel (2003). The bacterial isolates were grown on nutrient broth (NB) for 48 h while fungal strains (*Trichoderma harzianum*, *T. album*, *T. hamatum*, and *T. viridi*) were obtained kindly from the Plant Pathology Research Institute., Agricultural Research Center (ARC), Giza, Egypt, and cultured on potato dextrose agar (PDA). Fungal strains (*Trichoderma* spp.) were grown for 10 days on PDA medium separately, then prepared spores and mycelial suspensions and adjusted to about 10^7 spore ml/l with sterilized water. while, *B. subtilis* and *P. polymyxa* were separately grown in (NB) medium in 250-ml flasks and kept on shaker for 3 days at 150 rpm. The pellets of used bacterial bio-agent were suspended in tap water and modified the number of cells to 10^9 cell ml/1 by using a hemocytometer slide (Hafez et al. 2018). For assessment with 6 bio-agents, plants were sprayed by distilled water as a negative control and sprayed with Topas-100 at the recommended dose of 0.25 ml/l for comparison.

Disease management

In vitro experiment

The experiments were carried out in the laboratory of the Department of Plant Pathology, Faculty Agriculture, Benha University, Egypt. Conidial spores of *P. xanthii* were obtained from young sporulating lesions, the lesions were softly shaken by glass bar, and new conidia were dropped on glass slides according to Nair et al. (1962): "Slides were previously cleaned by ethyl alcohol and air dried before covering with thin smears of 2% water agar, amended with filter-sterilized culture filtrate of the tested antagonist. Slides were placed on V-shaped glass rods in sterilized Petri-dishes, containing several layers of water-moistened filter papers". Slides with conidia were incubated at 25 °C for 24 h under continuous light (Reifschneider et al. 1985). Conidia were considered to have germinated, if a germ tube, at least as long as the width, was produced (Menzies et al. 1991). Percentages of germination were calculated for 100 conidia on a slide at × 100 magnification. Three slides were examined for each treatment. Agar-free culture filtrate slides were used as a control treatment.

Greenhouse experiments

Squash seeds (*Cucurbita pepo* L., cv. Eskandarani) were sown in a clay soil at the rate of 3 seeds per hill under the experimental greenhouse conditions (28 °C, 85% RH, and 12 h photoperiod). The experiment was conducted in a randomly complete block design, with 3 replicated plots for each treatment. Squash plants in all plots received all the recommended agricultural practices. Natural infection with *P. xanthii* conidia, the causal agent of squash powdery mildew, was conducted under greenhouse conditions. Infected plants used as inoculum source (susceptible host Eskandarani) were uniformly inoculated by freshly collected conidia by placing heavy infected plants of squash which are sensitive to *P. xanthii* inoculation according to the method described by (Hafez et al. 2018). In addition, plants were sprayed with 2 bacterial bioagents, 4 fungal strains and the fungicide Topas-100 at recommended dose as mentioned before. Plants sprayed with sterilized tap water were used as control treatment.

Disease assessment

Squash plants after 45 days were examined periodically and the disease measures were determined using the devised scale (0–5) according to El-Ghanam et al. (2018) where 0 = no symptoms appear, 1 = 0.1 to 3% of leaf area covered by the infection, 2 = more than 3 to 10% of leaf area covered by the infection, 3 = more than 10 to 25% of leaf area covered by the infection, 4 = more than 25 to 50% of leaf area covered by the infection, 5 = more than 75% of the plant growth covered by the infection and the plants turned to be stunted.

The disease incidence and the severity of the disease were recorded using the following formula:

$$\text{Disease severity } (\%) = \Sigma\ (\text{nxv})/5\text{N} \times 100$$

where n = number of infected leaves in each category, v = numerical values of each category, and N = total number of the infected leaves.

Disease incidence (%) = no. of infected plants/total no. of the plants assessed × 100

Biochemical assay

Estimation of total phenolic compounds

One gram of squash leaves sample was extracted by 10 ml of 80% methanol at 70 °C for 15 min. Then phenolic compounds were determined using the colorimetric method of analysis by Folin-Ciocalteu reagent described by Zieslin and Ben-Zaken (1993).

Determination of peroxidase

Peroxidase activity was determined according to the method described by Allam and Hollis (1972). Peroxidase activity was expressed as the increase in absorbance at 430 nm/gram fresh weigh/15 min.

Determination of polyphenol oxidase

Polyphenoloxidase activity was determined according to a modification of Ishaaya (1971). The phenol oxidase activity was determined as O.D. units $\times 10^3$ at an absorbency of 405 nm.

Effect of use bio-agents on total yield/plant of squash plants

Average numbers and weights of fruits plant were recorded after harvesting fruits at marketable size. The squash fruits from each replicate of each treatment were collected twice a week, after 45 to 90 days from sowing, and the accumulated yield was expressed as the number and weight of fruits per plant.

Statistical analysis

Data were statistically analyzed using the (F) test and the value of LSD (at 5%) according to Gomez and Gomez (1984).

Results and discussion

Efficacy of bio-agents on spore germination of *Podosphaera xanthii*

The efficacy of *Bacillus subtilis*, *Paenibacillus polymyxa*, *Trichoderma album*, T. *harzianum*, *T. viride*, and *T. hamatum* on percentage of *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea*) spores' germination was evaluated under laboratory conditions compared with the fungicide Topas-100.

Data in Table 1 indicate that all tested treatments reduced significantly the percentage of conidial germination than the control. The high reduction was induced by Topas-100, which recorded 98.5%, followed by *P. polymyxa* 88.0% and *B. subtilis* 82.0%, whereas, *T. viride* and *T. hamatum* reduced conidial germination by 79.2% and 78.9%, respectively. On the contrary, *T. album* was the least effective treatments. The production of antimicrobial metabolites was another biocontrol mechanism that can be associated with mycoparasitism. The *Trichoderma* isolates produced metabolites in liquid medium. This extract inhibited pathogen growth on plate in low percentages (Sanchez et al. 2019). Obtained results are in agreement with many researchers who found that the bio-agents studied were effective for controlling powdery mildew disease (Kamel 2003; García-Gutiérrez et al. 2013; Tanaka et al. 2017).

Effect of using bio-agents under greenhouse conditions

Results in Table 2 show that all tested treatments significantly reduced the percentage of the disease incidence and severity than the control. In this respect, treatments with the fungicide Topas-100 followed by *B. subtilis* were the highest significant ones for decreasing disease incidence (2.8 and 5.3%, respectively) and disease severity percentage (3.5 and 4.8%, respectively). Moreover, *T. harzianum* treatments caused significant decrease in

Table 1 Effect of some bio-agent on spores' germination of *Podosphaera xanthii*, in vitro

Treatment	Concentration	Germination (%)	Efficiency (%)
B. subtilis	10^9 cfu	12.0	82.0
Paenibacillus polymyxa	10^9 cfu	8.0	88.0
Trichoderma album	10^7 spores ml^{-1}	20.3	69.6
Trichoderma harzianum	10^7 spores ml^{-1}	19.2	71.3
Trichoderma viride	10^7 spores ml^{-1}	13.8	79.2
Trichoderma hamatum	10^7 spores ml^{-1}	14.1	78.9
Topas-100	0.25 ml l^{-1}	0.9	98.5
Control	-	66.9	0.0
L.S.D. at 5%		14.8	-

Table 2 Effect of spraying bio-agent to control powdery mildew disease on squash plants under greenhouse conditions

Treatment	Disease incidence %	Efficacy %	Disease severity %	Efficacy %
B. subtilis	5.3	93.6	4.8	94.0
Paenibacillus polymyxa	27.7	66.7	14.5	81.9
Trichoderma album	13.9	83.3	10.0	87.5
Trichoderma harzianum	8.3	90.0	6.6	91.8
Trichoderma viride	15.9	80.9	11.6	85.6
Trichoderma hamatum	25.0	69.9	18.3	77.2
Topas-100	2.8	96.6	3.5	95.7
Control	83.3	0.0	80.5	0.0
L.S.D. at 5%s	4.10	-	3.96	-

disease incidence percentage (8.3%) and efficacy was 90.0%. Meanwhile, the bioagent *P. polymyxa* was the lowest efficient ones, with 66.7%. These results agree with the findings of Gilardi et al. (2008) and El-Sharkaway et al. (2014) who used *Bacillus* sp. for controlling cucurbit powdery mildew. Hence, *B. subtilis* played an important role on plant growth-promoting rhizobacteria that is widely applied to management the highest reduction of disease severity of many crop diseases (Liu et al. 2014).

Also, *Trichoderma* spp. produce antifungal compounds which acted synergistically for reducing the disease severity of powdery mildew disease of squash plants (El-Kot and Derbalah 2011). Priming of plants with *Trichoderma* spores or *Trichoderma* secondary metabolites produced rapid and effective defense responses against pathogen attack by acting as immunity stimulants (Contreras-Cornejo et al. 2011; Tucci et al. 2011; Elsharkawy et al. 2012; Yoshioka et al. 2012).

The fungicide (Topas-100) as a systemic fungicide is more efficient in management of many fungal diseases, including powdery and downy mildews in Cucurbits (Abada et al. 2009; Hafez et al. 2018).

Effect of some bio-agents on biochemical changes of squash plants

Results in Table 3 revealed that all treatments increased the activity of peroxidase than in control treatment. The highest activity of peroxidase was induced by *P. polymyxa* (262.3), followed by *T. viride* and *B. subtilis* recording 194.3 and 180.7, respectively. Meanwhile, Topas-100 was the least effective one. On the other side, the highest level of polyphenol oxidase was recorded after treatments by *T. album*, *P. polymyxa*, and *T. viride* recording 131.3, 123.7, and 114.0, respectively. While *T. harzianum* was the least effective one in respect of the effect of different bio-agents on total phenols content, the highest total phenols content was recorded in plants treated with *T. viride* (75.9), followed by Topas-100 (61.6) and *T. harzianm* (54.6), while *T. hamatum* showed the least effect on total phenols content. Sprayed plants recorded best results for most growth characters, peroxidase, polyphenol oxidase, and total phenols enzymes activity compared with untreated one)Hegazi and El-Kot 2010). Many investigators supported this idea since they stated that there are positive relationships between peroxidase enzyme and resistance developed in plants (Nawar and Kuti 2003; Emeran et al. 2006).

Table 3 Effect of some bio-agent on biochemical changes of squash plants

Treatment	Peroxidase (Δ_{405}. D. × 10^3/min/g fresh weight)	Polyphenol oxidase (O.D. units × 10^3/min/g fresh weight)	Total phenols (μg GAE/g fresh weight)
B. subtilis	180.7	84.3	50.8
Paenibacillus polymyxa	262.3	123.7	48.6
Trichoderma album	151.7	131.3	45.7
Trichoderma harzianum	179.0	82.3	54.6
Trichoderma viride	194.3	114.0	75.9
Trichoderma hamatum	159.3	108.3	44.9
Topas-100	147.3	84.3	61.6
Control	56.1	23.4	14.0
L.S.D at 5%	84.9	27.6	11.6

Table 4 Effect of bio-agents on the total yield/plant of squash plants under greenhouse

Treatment	No. fruits plant	Total yield/ plant (kg)	Efficacy %	
			No. fruits plant	Total yield/plant (kg)
B. subtilis	19.8	1.5	110.9	98.7
Paenibacillus polymyxa	16.1	1.2	71.3	61.3
Trichoderma album	13.6	1.0	44.7	36.0
Trichoderma harzianum	18.3	1.4	95.0	81.3
Trichoderma viride	14.5	1.0	54.3	45.3
Trichoderma hamatum	15.4	1.2	63.8	54.7
Topas-100	17.5	1.3	86.2	74.7
Control	9.4	0.8	0.00	0.00
L.S.D at 5%	2.2	0.05	-	-

Effect of bio-agents on the total yield/plant of squash plants under greenhouse

Data in Table 4 indicate that all tested treatments significantly increased the fruit number and fruit weight/ plant. The highest increase was recorded by *B. subtilis* (110.9 and 98.7%), followed by *T. harzianum* (95.0 and 81.3%) and Topas-100 (86.2 and 74.7%), respectively. *T. album* was the least effective treatment in this respect. The biological activity mechanisms of bioagents and chemical fungicide that reduced disease severity, suppressed and improved the total yield of squash (Cardwell et al. 1997).

B. subtilis induces plant resistance to stress and produces several plant hormones that can improve plant growth (Han and Lee 2005). Several researches have showed that plants grown particularly under salt stress conditions and inoculated with *B. subtilis* had a higher plant growth, yield, and nutrient uptake (Bochow et al. 2001; Saleh et al. 2005).

Hafez et al. (2018) reported that bio-agent treatments significantly increased growth and yield attributes of squash plants. Also, they stated usage of *B. subtilis* could suppress squash powdery mildew and increase yield up to 92% and *T. harzianum* increased total yield/plant up to 75%. Meanwhile, the fungicide increased total yield up to 45%. Overall, the results showed an alternative use of fungicides and bio-agents to control squash powdery mildew disease and the increase in total yield per plant.

Conclusions

Based on obtained data it could be concluded that the bio-agent could significantly inhibited the conidial germination of *P. xanthii* than control in vitro and decrease the incidence and severity disease after spraying with the bio-agents on squash plants under greenhouse conditions, with stimulating growth and yield attributes in comparison to control.

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