Impact of Some *Bacillus* spp.,Inducer Resistant Chemicals and Cow´s skim milk on Management of Pepper Powdery Mildew Disease In Saudi Arabia

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ABSTRACT: The aim of this study was detection of the effect of *Bacillus* spp., *B. chitinosporus, B. pumilus, B. subtilis* and *B. thuringiensis*, the inducer resistant chemicals;bion, chitosan, humic acid, oxalic acid, salicylic acid and cow´s skim milk on pepper plants infected with *Leveillula taurica* (the causal of powdery mildew) under laboratory and greenhouse conditions. Results revealed that all treatments resulted in significant reduction to conidial germination of *L. taurica* compared with check treatment. This reduction was gradually increased by increasing the tested concentration. In addition, spraying pepper plants with the bioagent *B. thuringiensis*, the IRC chitosan and cow´s skim milk, each alone or in different combinations, resulted in significant reduction in disease severity with significant increase to the produced pod fruit yield. Furthermore, spraying any of these compounds alone was of less effect in this regard compared with spraying their combinations. However, the fungicide Topas® 200 EW was the superior in this regard; being 3.5 % disease severity and pod fruit yield 244.5 g/plant followed by the mixture of the three treatments, being 4.4% disease severity and fruit yield 223.5 g/plant.

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1.INTRODUCTION

Pepper (*Capsicum annum* L.) is one of the most famous Solanecious crops either for local consumption or exportation. It is liable to infection by many fungal bacterial and viral diseases (Black *et al.,* 1991; Cerkauskas *et al.,* 2011). However, powdery mildew caused by *Leveillula taurica* (Lev.) Arn. (Imperfect stage = *Oidiopsis taurica*) is one of the most damaging diseases that affect greenhouse peppers all over the world (Damicone and Sutherland, 1999; Elad *et al.,* 2007; Sudha and Lakshamanan, 2009; Karkanis *et al.,* 2012). Researches showed a direct correlation between the percentage of powdery mildew infection of the leaves and yield loss, where one percent mildew infection on the leaves would result in a one percent yield loss or more. An early, heavy infection with mildew had about 30% loss of production compared to a later, lighter infection (Cerkauskas and Brown, 2003; Karkanis *et al.,* 2012). Greenhouse pepper growers need to follow an intensive disease prevention plan because it is very important that powdery mildew never gets out of hand (Kiss, 2003; Bettiol et al., 2008). Once pepper leaves are infected with powdery mildew it is difficult to control; if left unchecked the crop can be entirely destroyed (Abdel-Kader *et al.,* 2012). Disease monitoring, early detection and prevention of pepper powdery mildew is critical. By the time pepper powdery mildew is detected in a greenhouse many more leaves are already infected but does not show any disease symptoms. In addition, pepper plants can become defoliated and do not recover as quickly as other greenhouse crops when infected with powdery mildew (Kumar *et al.,* 2006). The disease is most severe on older leaves just prior to fruit set, but it can occur at anytime throughout the season if environmental conditions are favorable, severe infections early in the season can result in heavy yield losses (Peter, 2001).

Chemical control is highly recommended because powdery mildew is an aggressive and destructive disease and satisfactory control without the use of fungicides is unlikely. The role of fungicides in reducing the disease is well known (Mc Grath, 2001;2004). But due to the great hazards on the human health due to the residue of agrochemicals in the consume food,fungicides become unlikely to use. Therefore, great efforts by agro-scientists are carried out to search about alternative safely methods to management plant pests.

This work aims to (i) Evaluate under laboratory conditions the effect of four *Bacillus* spp,*i.e. B. chitinosporus*, *B. pumilus, B. subtilis* and *B. thuringiensis*, cow´s skim milk, inducer resistant chemicals (IRCs) bion, chitosan, humic acid, oxalic acid and salicylic acid against the causal of pepper powdery mildew disease through three laboratory experiments. (ii) Management powdery mildew on pepper plants under greenhous conditions using foliar spray with different concentrations of *B. thuringiensis*, chitosan and cow´s skim milk, either alone or in different combinations compared with the fungicide Topas®200 Ew.

2.MATERIALS AND METHODS

2.1.Materials:

2.1.1.Sample:-

a- Pepper leaves naturally infected by the conidial of *L. taurica* which were collected from a greenhouse located at Abo Arish country, Jazan governorate.

b-Powdery mildew spores from the a baxial side of pepper leaves were also collected.

2.1.2.Treatments:-

a-The tested *Bacillus* spp., *B. chitinosporus*, *B. pumilus, B. subtilis* and *B. thuringiensis* (kindly obtained from Agriculture Mirobiolial Deprtment, Faculty of Agriculture Cairo Univirsity) were grown on nutrient broth (NB) medium at 28±1 °C for 48 h. The bacterial suspension was adjusted to contain $1X10^2$, $1X10^3$, $1X10^4$, $1X10^5$, $1X10^6$, $1X10^7$ and $1X10^8$ cfu/ml (El-Gremi, *et al.*, 2011).

b-The inducer resistance chemicals (IRCs) bion, chitosan (β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D- glucosamine (acetylated unit)), humic acid ($C_9H_9NO_6$), oxalic acid anhydrous ($H_2C_2O_4$) and salicylic acid (monohydroxybenzoic acid) were prepared at 5, 10, 25, 50, 75, 100 and 125 mM depending on their molecular weight**.**

c-Cow´s skim milk (not whole milk) was diluted by the water to be 20, 40, 60, 80 and 100 %**.**

2.2. Methods:

2.2.1.Effect of the tested *Bacillus* spp., IRCs and Cow´s skim milk on conidial germination of *L. taurica* under laboratory conditions:

Three laboratory experiments were carried out to study the effect of tested *Bacillus* spp., IRCs and Cow´s skim milk on conidial germination of *L. taurica*. One ml of freshly collected conidia by sterilized brush from the infected leaves were put in 1*st* experiment in seven bacterial suspension ($1X10^2$, $1X10^3$, $1X10^4$, $1X10^5$, $1X10^6$, $1X10^7$ and $1X10^8$ cfu/ml), in 2*nd* experiment in eight concentrations of the tested IRCs and in 3*rd* experiment in five concentrations of cow´s skim milk. One m1 of each conidial suspension was placed on two sterilized slides, hold on two glass rods in a sterilized Petri-dish containing a piece of wet cotton by sterilized distilled water to provide high relative humidity. One m1 of conidial suspension was placed in sterilized distilled water only as check treatment. Preparations were incubated in an incubator at 28 ± 1°C for 48 hrs. One drop from lacto-phenol cotton blue stain was added at the time of slide examination to fix and kill the germinated conidia. Percentage of conidial germination was counted in a total of 100 conidia. The germinated conidia were counted and percentages mean of germination was calculated and recorded for each treatment.

2.2.2.Effect of the *B. thuringiensis*, chitosan and cow´s skim milk on disease inhibition and the produced fruit yield under greenhouse conditions.

Two greenhouse experiments were carried out during 2011 (first trial) and 2012 (second trial). Plastic pots, 30 cm in diameter contained disinfested sandy clay soil (1:1, v:v) by 5% formalin were transplanted with 35 days old pepper seedlings (cv. California wander) at mid of January of 2011 and 2012. Two seedlings were transplanted in each pot. Five replicates were used for each treatment. The plants received all organic agricultural practices as recommended by Ministry of Agriculture. The grown plant was artificially inoculated (forty days after transplanting) by shaking the infected pepper plants. The inoculated plants were kept under humid conditions for two days to encourage the infection by the disease.

The prepared culture of *B. thuringiensis* at $1x10^7$/ml water, chitosan at 100 mM and cow´s skim milk at 60% amended with 30 ml bio-film 1265/l water were sprayed each alone or in different combination onto the upper and the lower leaf surfaces of the plants until run off two days after inoculation by the conidial spore (before appearing the visual symptoms of the disease) as protective treatment and just after appearance of the visual disease symptoms (about three weeks after inoculation) as curative treatment. Plants sprayed with tap water only amended with bio-film 1265 served as check treatment. Plants sprayed with the fungicide Topas®200 EW (Tubaconazole) at the concentration of 20 ml/l were used for comparison. Spraying was repeated every 10 days until the end of the experiments.

The produced fruit yield was harvested periodically, weighed and the final averages weights were recorded.

2.2.3.Disease assessment:-

Plants were examined periodically and disease measures were determined using the devised scale

(0-5) adopted by Horsfall and Barret (1945), *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 grown plants were periodically examined for disease symptoms to estimate the severity of the

disease and the final averages were recorded using the following formula:

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

Where:

n = number of infected leaves in each category.

v = numerical values of each category.

N = total number of the infected leaves.

2.2.4.Statistical analysis*:*

Data were statistically analyzed using the standard procedures for split and split split designs as mentioned by Snedecor and Cochran (1967). The averages were compared at 5% level using least significant differences (LSD) according to Fisher (1948).

3.RESULTS

3.1.Effect of the tested *Bacillus* spp. on conidial germination of *L.taurica* under Laboratory conditions

Data presented in Table 1 indicate that all tested *Bacillus* spp. resulted in significant reduction in germination of *L.taurica* conidia. Reduction % was gradually increased by increasing the tested concentration. Treatments with high concentrations ($1X10^7$&$1X10^8$ cfu/ml) of all *Bacillus* spp. showed the highest inhibition (88.4-100%) in conidial germination. Treatments with two concentrations ($1X10^5$&$1X10^6$ cfu/ml) of all *Bacillus* spp. resulted in (32.0-44.4%) reduction, followed by treatments with low concentrations ($1X10^2$, $1X10^3$ and $1X10^4$ cfu/ml) which showed (5.8-19.0%) reduction all *Bacillus* spp. compared to check treatment (Table, 1).

Table 1. Effect of the tested *Bacillus* spp. on conidial germination of *L. taurica*, 48 h after incubation at 28 ± 1 °C

Treatments	Bacterial suspensions (cfu/ml)													
	$1X10^2$		$1X10^3$		$1X10^4$		$1X10^5$		$1X10^6$		$1X10^7$		$1X10^8$	
	% [x]	% [y]	% [x]	% [y]	% [x]	% [y]	% [x]	% [y]	% [x]	% [y]	% [x]	% [y]	% [x]	% [y]
B. chitinosporus	93.4	*6.6*	90.6	*9.4*	81.4	*18.6*	67.8	*32.2*	56.4	*43.6*	9.4	*90.6*	0.0	*100.0*
B. pumilus	94.2	*5.8*	91.8	*8.2*	82.0	*18.0*	68.0	*32.0*	57.8	*42.2*	11.6	*88.4*	0.0	*100.0*
B. subtilis	94.0	*6.0*	89.6	*10.4*	81.8	*18.2*	67.4	*32.6*	56.4	*43.6*	8.8	*91.2*	0.0	*100.0*
B. thuringiensis	92.8	*7.2*	88.6	*11.4*	81.0	*19.0*	66.4	*33.6*	55.6	*44.4*	0.0	*100.0*	0.0	*100.0*
Check treatment*	95.2	*4.8*	95.2	*4.8*	95.2	*4.8*	95.2	*4.8*	95.2	*4.8*	95.2	*4.8*	95.2	*4.8*

*=Initial germination percentage was 1.8 %. [x] = Average % of conidial germination. [y] = Inhibition of germination%. L.S.D. at 5 % for, c. *Bacillus* spp. = 2.7, Concentrations (C)= 2.5 and B x C = 3.2. L.S.D. at 5 % for: *Bacillus* spp. = 2.1, Concentration (C)=2.7 and I x C = 3.5.

3.2.Effect of IRCs on conidial germination of *L.taurica* under Laboratory conditions

Data presented in Table 2 revealed that all the tested IRCs showed significant inhibition in *L. taurica* conidial germination compared with the check treatment. This reduction was gradually increased by increasing the tested concentration. Treatments with 100&125 mM of all IRCs resulted in the highest inhibition (88.0-100.0%) in conidial germination, followed by treatments with two concentrations 50&75 mM which showed 42.8-67.8% inhibition. Meanwhile, treatments with low concentrations 5, 10 and 25 mM of all IRCs resulted in (11.6-38.0%) iniion in conodial germination in comparison with check treatment (Table 2).

Table 2. Effect of some inducer resistance chemicals (IRCs) on conidial germination of *L. taurica* 48 h after incubation at 28 ± 1 °C

Treatments	IRCs concentrations (mM)													
	5		10		25		50		75		100		125	
	% [x]	% [y]	% [x]	% [y]	% [x]	% [y]	% [x]	% [y]	% [x]	% [y]	% [x]	%[y]	% [x]	% [y]
Bion	86.0	*14.0*	73.6	*26.4*	62.0	*38.0*	50.2	*49.8*	32.6	*67.4*	9.2	*90.8*	0.0	*100.0*
Chitosan	88.4	*11.6*	75.2	*24.8*	64.4	*35.6*	52.0	*48.0*	33.2	*66.8*	11.6	*88.4*	0.0	*100.0*
Humic acid	87.6	*12.4*	77.0	*23.0*	65.2	*34.8*	57.2	*42.8*	34.6	*65.4*	10.6	*89.4*	0.0	*100.0*
Oxalic acid	86.2	*13.8*	77.8	*22.2*	65.8	*34.2*	56.4	*43.6*	35.4	*64.6*	10.0	*90.0*	0.0	*100.0*
Salicylic acid	86.6	*13.4*	73.4	*26.6*	63.2	*36.8*	51.2	*48.8*	32.2	*67.8*	12.0	*88.0*	0.0	*100.0*
Check treatment*	93.4	*6.6*	93.4	*6.6*	93.4	*6.6*	93.4	*6.6*	93.4	*6.6*	93.4	*6.6*	93.4	*6.6*

*=Initial germination percentage was 1.8 %. [x] = Average % of conidial germination. [y] = Inhibition of germination%. L.S.D. at 5 % for: Inducer resistance chemicals (I) = 2.7, Concentrations (C)= 2.5 and I x C = 3.2.

3.3.Effect of cow´s skim milk on conidial germination of *L.taurica* under Laboratory conditions

Data presented in Table 3 revealed that significant inhibition was occurred in conidial germination due to using different cow´s skim milk concentrations. Three concentrations 60, 80 and 100% resulted in the highest inhibition (93.4-100%) in conidial germination, followed by two concentrations 20 and 40% which showed 23.6-68.0% inhibition compared with check treatment (Table 3).

Table(3): **Effect of of different concentrations of cow´s skim milk on conidial germination of *L. taurica* 48 h after incubation at 28±1 ºC**

Treatment	Germination* %	Inhibition %
Cow´s Skim Milk Conc.(%)		
20	76.4	*23.6*
40	32.0	*68.0*
60	6.6	*93.4*
80	2.0	*98.0*
100	0.0	*100*
Check treatment	94.8	*5.2*
LSD at 5 %	3.5	

* = Initial germination percentage was 0.8 %.

3.4.Effect of spraying pepper plants with the combination among *B. thuringiensis*, chitosan and cow´s skim milk on disease severity of powdery mildew under green house conditions

Data presented in Table 4 revealed that spraying pepper plants with *B. thuringiensis*, chitosan and cow´s skim milk, each alone or in different combinations resulted in significant reduced disease severity in both curative and protictive treatments compared with check treatment. However, spraying by any of them alone was of low efficiency in this respect than spraying their different combinations. Combination treatment between *B. thuringiensis*, chitosan and cow´s skim milk resulted in the highest reduction (4.0-5.0%) in disease severity in both curative and protictive treatments nearby the effect of the systemic fungicide Topas® 200 EW (3.1-3.4%). Also, the same combination treatment between *B. thuringiensis*, chitosan and cow´s skim milk resulted in the highest increase (221.2-225.6 g/plant) in pod fruit yield. The highest production in pod fruit yield (242.3-245.8 g/plant) was obtained by spraying the plants by the systemic fungicide Topas® 200 EW (Table 4).

Table 4. Effect of spraying pepper plants with the combination among *B. thuringiensis*, chitosan and cow´s skim milk on the disease severity of powdery mildew and pod fruit yield production under greenhouse conditions

Treatment	Disease severity %				Average pod fruit yield (g)/plant			
	1st trial		2nd trial		1st trial		2nd trial	
	Pro.*	Cur.**	Pro.*	Cur.**	Pro.*	Cur.**	Pro.*	Cur.**
***B. thuringiensis* (BT)**	10.3	12.2	10.7	12.9	175.6	171.0	173.5	172.6
Chitosan (C)	10.0	13.5	10.6	13.5	179.7	176.3	178.6	174.7
Cow´s skim milk (M)	10.5	12.8	10.0	13.2	182.5	179.1	180.5	177.4
BT+ C	8.5	9.2	8.8	10.1	185.5	182.0	185.0	182.7
BT+ M	7.6	9.0	7.9	10.1	192.6	189.0	191.2	188.1
C+ M	7.6	9.0	6.8	9.2	195.4	191.5	194.1	190.2
BT+C+ M	4.0	4.2	4.1	5.0	225.6	222.8	224.3	221.2
Topas® 200 EW	3.1	3.4	3.4	3.2	245.8	242.8	244.3	242.3
Check treatment*	82.1	82.1	84.0	84.0	104.3	103.0	102.4	102.4

* Sprayed with water only. x = Protictive treatment. y = Curiative treatment. **Disease severity experiment** (LSD at 5 % for: Treatments(T)= 3.3, Trial (TR)= n.s., Kind of spray(K) =n.s TxTR=3.2, TxK= 2.7 , TRxK= 1.8 and TxTRxK= 4.1). **Pod fruit yield** (LSD at 5 % for: Treatments(T)= 5.6, Trial (TR)= n.s., Kind of spray (K) =2.4, TxTR= 3.3, TxK= 3.1, TRxK=2.1 and TxTRxK= 4.6).

4.DISCUSSION

Production of healthy and safe food free from toxic substances is the desire of consumer, especially that consume freshly like pepper. Therefore, to avoid the use of hazard chemicals against diseases, certain protective or curative procedures could be conducted using different non-chemical methods to control such diseases. In this regard, *Bacillus* spp., inducer resistant chemicals (IRCs) and cow´s milk were evaluated for management pepper powdery mildew. However in most cases, using such untraditional management methods did not give adequate results when used alone. In this respect, the use of these methods is preventable to use as a mixture.

The present effort indicated that treatments with *Bacillus* spp., IRCs and cow´s skim milk caused significant reduction to conidial germination of *L. taurica* compared with check treatment. This reduction was gradually increased by increasing the tested concentration. In addition, spraying pepper plants with the bioagent *B. thuringiensis*, the IRC chitosan and cow´s skim milk, each alone or in different combinations, resulted in significant reduction to the severity of the disease with significant increase to the produced fruit yield, more than spraying any of these compounds alone. However, the fungicide Topas® 200 EW was the superior in this regard followed by the mixture of the bioagent *B. thuringiensis*, the IRC chitosan and cow´s skim milk.

Larcke (1981) found that unlike elicitors of phytoalexines accumulations, which are elicited at the site of application may be responsible for localized protection and induces systemic acquired resistance that sensitizes the plant response rapidly after infection. These responses induced phytoalexines accumulation and lignifications and induce enhance activities of chitinase and β-glucanase (Dean and Kuc, 1985; Metranx and Boller, 1986). Doubrava *et al.* (1988) mentioned that induced acquired resistance is persistent and generally is pathogen nonspecific. Furthermore, Kessmann *et al*., (1994) reported that the mechanism of systemic acquired resistance is apparently multifaceted, likely resulting in stable broad spectrum disease control and they could be used preventatively to bolster general plant health, resulting in long lasting protection. Iriti and Faoro (2003) reported that bion was used to induce resistance in bean against rust caused by *Uromyces appendiculatus*. Histochemical and cytochemical investigations showed that BTH causes hydrogen peroxide (H_2O_2) accumulation in the treated tissues. H_2O_2 deposits were localized in situ for the first time in the apoplast of the leaf epidermis. No cell death was detected at BTH concentrations below the phyto-toxicity threshold, suggesting that acquired resistance against bean rust is mainly related to the enhanced activity of anionic peroxidases, promoted by H_2O_2 accumulation, thereby leading to cell wall strengthening. This hypothesis is also supported by the long induction phase required to establish complete resistance.

Chitosan is an anti-transpirant compound that has proved to be effective in many crops (Khan *et al*., 2002; Karimi *et al*., 2012; Abu-Muriefah and Sharifa, 2013) and was used to protect plants against oxidative stress (Guan *et al.,* 2009) and to stimulate plant growth (Farouk *et al.,* 2008; 2011; 2012). Chitosan is a natural, low toxic and inexpensive compound that is bio-degradable and environmentally friendly with various applications in agriculture. It was found that foliar applications with chitosan resulted in higher vegetative growth and improvement in fruit quality of pepper, radish and cucumber (Farouk *et al.,* 2008; Ghoname *et al.,* 2010).Ghoname *et al.* (2010) also observed that foliar application of chitosan on sweet pepper increased significantly the number of fruits per plant and the mean weight of fruit, as well as, quality characteristics of the fruit. The role of chitosan in all eviating the harmful effect of water stress on yield may be due to an increase in stomata conductance,net photosynthetic CO2-fixation activity under water stress (Khan *et al.,* 2002), and to its role in reducing transpiration to save water.

In report given by Trankner (1992) he mentioned that in case of powdery mildew disease, *Bacillus subtilis* grow on the treated surfaces and utilize available nutrient substances and prevent pathogenic spores to establish germinate and invade healthy tissues. *Bacillus* sp. also grows very fast and occupies the court of infection and preventing pathogen spores to reach susceptible tissues in competition for spaces (Wolk and Sorkar, 1994). This might be due to that treatments with bio-preparation induce systemic resistance as the main mechanism of activity on the plant (Ramamoorthy *et al*., 2001, Xing *et al.,*2003; Abdel-Kader *et al*., 2012;).

Bettiol (1999) indicated that numerous small studies from around the world have validated the use of milk sprays on powdery mildew on a wide range of plants. Most recently, a spray made of 40% milk and 60% water was as effective as chemical fungicides in managing powdery mildew of pumpkins and cucumbers grown in mildew areas. Like other fungicides, milk sprays work best when used preventatively, before the disease can gain a foothold. He added that it does not matter if the milk you use is skim or whole because it is the protein rather than the milk fat that is working on your behalf. Also, Pleasant (2012) used milk in managing many powdery mildew diseases on different hosts.

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