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Effect of Bioagents, Resistance Inducers and Cow Milk on Controlling Cucumber Powdery Mildew

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Four bioagents, *i.e. Bacillus megaterium, B. pumilus, B. subtilis* and *B. thuringiensis*, and four resistance inducers (RI), *i.e.* chitosan, humic acid, potassium monophosphate and salicylic acid as well as cow full cream, skim and milk whey caused significant reduction in the germinated conidia of *Golovinomyces cichoracearum* var. *cichoracearum* (D.C.) Heluta (syn. *Erysiphe cichoracearum* D.C.), the causal of cucumber powdery mildew compared to the check treatment. This reduction was increased with increasing the tested concentration. In greenhouse experiment, spraying the tested bioagents and RI as well as cow full cream, skim and whey milk resulted in different decrements in the disease severity and increments in plant length as well as foliage fresh and dry weight. Field experiments during 2013 and 2014 growing seasons revealed that spraying of *B. subtilis*, potassium monophosphate and cow whey milk, individually or in combinations, caused significant reduction in the disease severity with significant increase in the fruit yield. Spraying these compounds individually was less efficient in this regard compared with spraying them in combinations. Combination of *B. subtilis* + potassium monophosphate + cow whey milk recorded more disease reduction and yield increase. Therefore, the reference fungicide was mostly efficient as the combined treatment.

Keywords: *Bacillus* spp., bioagents, cow milk, cucumber, inducer resistance and powdery mildew.

Cucumber (*Cucumis sativus* L.) is one of the important economic vegetable crops all over the world. Several fungal diseases attack cucumber plants at all growing stages. Powdery mildew, caused by *Golovinomyces cichoracearum* var. *cichoracearum* (D.C.) Heluta (syn. *Erysiphe cichoracearum* D.C.) is the most devastating in production of cucumber and other cucurbits in greenhouses and fields cultivation (Vakalounakis and Klironomou, 2001; Almqvist, 2012 and Cosme *et al.*, 2012). Powdery mildews are commonly serious in cool or warm, humid areas (Agrios, 2005). Synthetic fungicides have been commonly practiced for a long time to check such disease but recently alternative management are taking place to assure human health and safeguard the environment.

The primary initial inoculum is believed to be airborne conidia dispersed over long distances from southern Governorates, where cucurbit crops are grown earlier in the year. Conidia remain viable for 6-8 days according to results from laboratory studies. Possible local sources of initial inoculum include conidia from greenhouse-grown cucurbits and alternative hosts. Cleistothecia have not been reported in Egypt yet, that may be due to the absence of the mating types required for sexual reproduction. Although *G. c.* var. *cichoracearum* is described as having broad host ranges, strains of this fungus has been shown to be host-specific, where the role of non-cucurbit hosts as sources of inoculum has not been investigated (McGrath, 2011).

Cucumber growers need to follow an intensive disease prevention plan because it is very important that powdery mildew never gets out of hand. Once cucumber leaves are become infected, it would be difficult to check; otherwise the crop can be destroyed. There are no powdery mildew resistant cucumber cultivars currently available (Lebeda, 1984). Cucumber powdery mildew is easily seen on the top side of the leaves. Disease monitoring for early detection and sufficient prevention would be critical. Under favourable conditions, the fungus reproduces rapidly and spores can germinate and infect the plant in less than 48 hours. Wind-disseminated spores cause secondary infections and ending with disease outbreak and heavy yield losses (Zitter *et al.,* 1996).

The aim of the current research is to evaluate the efficiency of four bioagents and four RI as well as three cow milk formations against the disease severity and yield production.

Materials and Methods

The causal fungus:

The causal fungus of cucumber powdery mildew was kindly identified in the Mycol. and Plant Dis. Survey Dept., Plant Pathol. Res. Inst., ARC, as *Golovinomyces cichoracearum* var. *cichoracearum* (D.C.) Heluta (syn. *Erysiphe cichoracearum* D.C.).

Isolation and identification of tested Bacillus spp. as bioagents:

Bacteria naturally occurring on cucumber leaf surface were isolated from the phylloplane of healthy leaves, collected from Embaba, Giza Governorate. Serial dilution plate technique was used to isolate native bacteria on nutrient agar medium (Oedjijono and Dragar, 1993). Isolated *Bacillus* spp. were purified and identified according to the description of Parry *et al.* (1983) and Holt *et al.* (1994).

Four *Bacillus* species, *i.e. Bacillus megaterium, B. pumilus, B. subtilis* and *B. thuringiensis,* were grown on nutrient broth (NB) medium at 28±1°C for 48 h. The bacterial suspension was adjusted to five concentrations, *i.e.* 1x10; $1x10^2$; $1x10^3$; $1x10^4$ and $1x10^5$ cfu/ml water. Distilled sterile water was used as check treatment.

Tested resistance inducers (RI):

Chitosan, humic acid, potassium monophosphate and salicylic acid were prepared at six concentrations, *i.e.* 0.0, 20, 40, 60, 80 and 100 mM, on base of their molecular weight.

Tested cow full cream, skim and whey milk:

Cow full cream, skim and whey milk were diluted by distilled sterile water (v:v) to get six concentrations, *i.e*. 0.0, 20, 40, 60, 80 and 100%.

Effect of the bioagents, RI and cow milk on the fungal spore germination:

Using sterilized brush, freshly conidia were collected from the infected cucumber leaves and put in each concentration of the tested bioagents, IRCs and cows full cream as well as skim and whey milk. One m1of conidial suspension was placed on two sterilized slides, born on two glass rods inside a sterilized Petri-dish containing a piece of wetted cotton by sterilized distilled water to provide high relative humidity. Spore suspension put in distilled sterilized water only was used as check treatment. Preparations were incubated at 28±1°C for 24 h. One drop from lacto-phenol cotton blue stain was added at the time of slide examination to fix and killing the germinated conidia. Conidial germination (%) was counted in a total of 100 conidia.

Effect of the bioagents, RI and cow milk on the disease severity and some plant growth parameters under greenhouse conditions:

Plastic pots (30-cm-diam.) contained disinfested Nile silt soil (by 5% formalin) were sown with cucumber seeds (cv. Amira) at mid of August 2013. Four seeds were sown in each pot and thinned into two plants in each pot, 15 days after sowing. Tested bioagents ($1x10^6$cfu/ml water), RI (100 mM) and cow (80%) full cream, skim and whey milk (amended with calculated aliquots of an adhesive surfactant Seda film as recommended; 30 ml/100 l water) were sprayed on the tested plants three days before inoculation with the causal fungus. The grown plants were inoculated by shaking cucumber leaves severely infected by powdery mildew. Plants sprayed with tap water amended with the adhesive surfactant (Seda film) and inoculated with the causal fungus were kept as check treatment. The inoculated plants were kept under humid conditions (by spraying the floor of the greenhouse by water) for two days to encourage the infection by the disease, then examined periodically after spraying the treatments to assess the severity of the disease (30-day after inoculation) using the devised scale (0-5) adopted by Jasinski *et al.* (2010). Also, plant length (cm) as well as foliage fresh and dry weight were assessed and recorded after assessing the disease severity.

Effect of the tested bioagents, RI and cow whey milk on the disease severity and the produced fruit yield under field conditions:

Two field experiments were carried out during 2013 and 2014 growing seasons at Embaba, Giza Governorate. The first one was sown at June, 8 and the second at June, 12. The land was prepared for sowing cucumber as usual. The land was divided into plots (42 m^2) with ridges of 7 m. long and 120 cm. width. Four seeds of cv. Amira were sown in each hill with 40 cm. distance. Two weeks after sowing the emerged seedlings were thinned into two seedlings in each hill. The grown plants were left to the natural infection by cucumber powdery mildew.

The prepared microbial culture of *B. subtilis* at $1x10^6$/ml water, the RI potassium monophosphate at 100 mM and cow whey milk at 100 % (amended with calculated aliquots of an adhesive surfactant Seda film as recommended; 30 ml/100 L. water) were sprayed (thirty days after sowing), each alone or in different combinations, onto the grown plants at the first appearing of symptoms. Plants sprayed with tap water only served as check treatment (check). Also, the fungicide Punch (flusilazole) was sprayed at the rate of 6 ml/100 l. water as recommended and efficient one for management the disease. Spray was repeated 4 times every 14 days. The plants received all agricultural practices as recommended by Min. of Agric. and Land Reclamation. The produced fruit yield was harvested periodically and the final average weight was recorded.

Disease assessment:

Plants were examined periodically and disease measures were determined using the devised scale (0-5) adopted by Jasinski *et al.* (2010), whereas 0= No symptoms appear; 1= 0.1 to 0.9% of leaf area covered by the infection; 2= 1.0 to 3.9% of leaf area covered by the infection; 2= 4.0 to 15.9% of leaf area covered by the infection; 4= 16 to 63.9% of leaf area covered by the infection; 5= more than 64% of the plant growth covered by the infection and the plants turned to be burned. The grown plants were periodically examined for disease symptoms to estimate the disease severity according to the following formula:

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

Whereas: n = Number of infected leaves in each category.
v = Numerical values of each category.
N = Total number of the infected leaves.

Statistical analysis:

Data were statistically analyzed using the standard procedures of randomized complete block design. Statistics were performed by MSTATC package. The means were compared at 5% level (Fisher, 1948) using least significant differences (L.S.D).

Results

Effect of Bacillus species in different concentration on the conidia germination of G. c. cichoracearum:

Data presented in Table (1) indicate that all of the bioagent concentrations significantly reduced, with different degrees, the conidial germination of *G. c. cichoracearum* when compared with the check treatment. Inhibition of spore germination was increased with the increase of tested concentration. Generally, *B. subtilis* was the most effective bioagent in this respect, followed by *B. thuringiensis* and *B. megaterium* then *B. pumilus*, when conidial germination reached 53.1, 57.9, 58.7 and 63.7, respectively.

Effect of RI on conidial germination:

Results in Table (2) reveal that all of the tested RI resulted in significant reduction in conidial germination of the causal fungus when compared with the check treatment. Potassium monophosphate seemed to be the most effective in reducing the conidial germination. Moreover, increasing the tested concentration led to decrease in conidial germination. On the general, potassium monophosphate was the most effective RI in this regard, followed by salicylic acid and humic acid then chitosan, when conidial germination reached 51.7, 58.5, 59.4 and 60.5, respectively.

Table 1. Effect of different concentrations of four *Bacillus* spp. on conidial germination of *G. c.* var. *cichoracearum*, 24 h after incubation at 28±1ºC

Tested bioagent	Conidial germination at different concentrations						Mean
	0.0	1x10	$1x10^2$	$1x10^3$	$1x10^4$	$1x10^5$	
B. megaterium	93.8	92.0	88.4	50.0	28.0	0.0	58.7
B. pumilus	93.8	92.8	90.0	59.2	36.0	10.4	63.7
B. subtilis	93.8	86.0	81.0	42.6	15.0	0.0	53.1
B. thuringiensis	93.8	91.0	86.8	49.0	26.8	0.0	57.9
Mean	93.8	88.4	83.2	40.2	21.2	2.1	---
L.S.D.at 5% for: Bioagents (B)= 2.1, Concentrations (C)= 2.7 and B x C= 3.5.							

Table 2. Effect of some RIs on the fungus conidial germination, 24 hour after incubation at 25±1°C

Tested resistance inducer	Conidial germination (%) at different concentrations (mM)						Mean
	0.0	20	40	60	80	100	
Chitosan	94.4	90.2	78.4	56.0	32.0	12.0	60.5
Humic acid	94.4	89.4	78.0	54.8	30.0	10.0	59.4
Potassium monophosphate	94.4	88.0	72.2	42.0	13.6	0.0	51.7
Salicylic acid	94.4	92.0	80.0	51.8	23.4	9.2	58.5
Mean	94.4	89.9	77.2	51.2	24.8	7.8	-----
L.S.D. at 5%: Inducer resistance chemicals (I)= 2.7, Concentrations (C)= 2.5 and IxC= 3.2							

It is worth to note that there were significant interactions among the bioagents and the concentrations of the resistance inducers. This means that different tested materials would give different effects on the conidial germination.

Effect of cow milk on conidial germination:

Data presented in Table (3) indicate that all the tested formulas of cow's milk caused significant reduction in the conidial germination. Increasing the tested concentration led to decrease in conidial germination. Conidial germination was completely inhibited at concentration of 80 and 100% in case of full cream milk; meanwhile in case of skim milk complete reduction was recorded at concentration of 100%. On the mean, full cream milk was the most effective followed by skim then whey milk (being 46.6, 56.1 and 57.3% of germination, respectively).

Table 3. Effect of cow milk on conidial germination of *G. c.* var. *cichoracearum*, 24 h after incubation at 28±1°C

Tested milk	Conidial germination (%) at different concentrations						Mean
	0.0	20	40	60	80	100	
Full cream	95.0	84.2	68.0	32.4	0.0	0.0	46.6
Skim	95.0	90.4	75.0	51.6	24.8	0.0	56.1
Whey	95.0	91.0	76.2	52.6	2 9.0	12.0	57.3
Mean	95.0	88.2	73.1	45.5	17.6	4.4	--
L.S.D. at 5% for: Kind of milk (K)= 2.7, Concentrations (C)= 1.9 and K x C= 3.1							

Effect of four Bacillus spp., resistance inducer and cow milk formula on the disease severity under greenhouse conditions:

Results in Table (4) indicate that spraying of cucumber plants with either of the tested bioagents, the IR or the three kinds of cow milk resulted in significant reduction in the disease severity compared with the check treatment. The most reduction in the disease severity (13.8%) was recorded with *B. subtilis*, while the check treatment gave 52.1%. Other compounds came thereafter with significant differences among them. Concerning the growth parameters and yield weight, results indicate that all of the applied materials caused significant increases in comparison with the check treatment. Concerning the three kinds of milk, the full cream was the superior in dry weight per plant (181.1g), followed by skim (167.5g) then whey (158.9g). It is worth to note that potassium monophosphate as resistance inducer also gave the highest figure for fresh weight and dry weight (920g and 182.8g), respectively. Although, whey milk was the lowest one of cow milk in reducing the disease severity and increasing plant length as well as foliage fresh and dry weights, but it was used in managing the disease under field conditions due to its low price as it is a secondary product in manufactories of processing cheese.

Table 4. Effect of the four species of Bacillus, resistance inducer (RI) and cow milk formula on the disease severity in greenhouse experiment

Treatment	Disease severity (%)	Plant length (cm)	Fresh weight (g/plant)	Dry weight (g/plant)
B. megaterium	14.6	54.7	896.8	179.3
B. pumilus	17.0	50.0	849.7	171.2
B. subtilis	13.8	56.9	915.5	180.7
B. thuringiensis	16.1	52.6	870.1	168.5
Chitosan	17.9	54.0	900.7	176.8
Humic acid	20.3	50.7	848.5	172.0
Potassium monophosphate	14.2	56.0	920.0	182.8
Salicylic acid	16.0	51.8	863.4	173.1
Full cream milk	15.0	55.0	905.7	181.1
Skim milk	17.4	50.2	844.0	167.5
Whey milk	20.0	46.0	831.0	158.9
Check	52.1	40.0	721.5	117.0
L.S.D. at 5 %	1.8	1.1	4.7	2.3

Effect of combined treatments of B. subtilis, potassium monophosphate and cow whey milk on the disease severity and cucumber fruit yield under field conditions:

Results in Table (5) indicate that the combined treatment gave better results. Disease severity pointed out under field experiment in two successive seasons of 2013 and 2014. In this concern, all the tested compounds reduced disease severity, meanwhile, the tested fungicide (Punch) was the superior and the combination of *B. subtilis* with potassium monophosphate and cow milk whey came on the second rank of efficacy. These superior records reflected on the yield during the two tested seasons.

Table 5. Effect of combined treatment on the disease severity and yield production in 2013 and 2014 growing seasons under field conditions at Giza Governorate

Treatment*	Disease severity (%) during		Mean	Average fruit yield/plot (kg/42 m^2) during		Mean
	2013	2014		2013	2014	
BS	11.8	12.0	11.9	68.8	67.0	67.9
PMP	10.6	10.4	10.3	70.0	71.0	70.5
CWM	15.0	14.4	14.7	65.6	66.9	66.3
BS+PMP	9.8	10.0	9.9	72.2	71.0	71.6
BS+CWM	10.6	10.8	10.7	71.7	71.5	71.6
PMW+CMP	9.7	9.0	9.4	73.8	73.8	73.8
BS+PMP+CWM	3.5	3.1	3.3	80.0	81.0	80.5
Punch	2.8	3.0	2.9	82.8	82.0	82.4
Check	54.0	53.1	53.6	35.0	32.7	33.9
Mean	15.9	15.6	---	67.5	67.4	---
L.S.D. at 5% for:						
Treatments (T)=	2.4			3.1		
Season (S)=	n.s			n.s		
T x S=	3.4			4.2		

* BS= *B. subtilis;* PMP= Potassium monophosphate and CWM= Cow whey milk.

Discussion

Powdery mildew is a widespread and seriously diverted to many crops particularly fruit and vegetables. The traditional measurement to put the disease under practical check has been applying specific fungicides. Recently alternative measurements have been come into consideration to avoid the hazardous impact of the fungicide to human health and environment. Four alternatives tool thoroughly investigated and these were biological check (Trankner, 1992; Oedjijono and Dragar, 1993; Romero *et al*., 2004 and Almqvist, 2012), resistance inducers (Metranx and Boller, 1986 and Ramamoorthy *et al*., 2001) and very recently cow milks (Westney, 2002; Crisp *et al*., 2006 and Pleasant *et al*., 2012) and growth (nutrient) promotions in compare with one specified fungicide, *i.e.* Punch (flusilazole), (Larcke, 1981; McGrath, 2001 & 2004 and Jasinski *et al*., 2010).

The main objective of the current research is to compare these alternatives, either individually or in combinations. The study was conducted under *in vitro*, greenhouse and field conditions. Applying *Bacillus subtilis* as individual bioagent proved to be the most effective *in vitro* and greenhouse but not under the field conditions. In this concern, other tested *Bacillus* spp., *i.e. B. megaterium, B. pumilus* and *B. thuringiensis,* gave lesser potentially than *B. subtilis.* Such results had been reported by many investigators on many crops, *i.e.* cucumber, zucchini, pumpkins, pepper and others (Bettiol, 1999; Raaijmakers *et al*., 2002; DeBacco, 2013 and Alharbi and Alawlaqi, 2014).

From the obtained results, it seems that *B. subtilis* as a bioagent came on the top as broad spectrum (Fiddaman and Rossal, 1993 and Xing *et al*., 2003).

Resistance inducers (RI) have been characterized by their ability to stimulate plant target preventive ability of the disease infection afterward when the pathogen starts to invade the host. Hence, they should be applied firstly before any pathogen invasion (Fiddaman and Rossal 1993 and Xing *et al*., 2003). Moreover, RIs are known mostly to have no antifungal toxicity in the laboratory studies.

Salicylic acid (SA) has been known as the superior of all the RIs (Abada and Ahmed, 2014). Other natural RIs have been introduced such as potassium monophosphate BTH (Iriti and Faoro, 2003), chitinase and humic acid (Dean and Kuc, 1985 and Metranx and Boller, 1986). The most effective RI in the current study was potassium monophosphate when compared with the rest of tested RIs.

The cow milk forms tested in this study were full cream, skimmed and whey. All of those forms succeeded to put the disease under sufficient check with the full cream on the top. Such results had been reported by Bettiol (1999) and Westney (2002).

Inclusively, combination trials were took place under field conditions during the two successive seasons of 2013 and 2014. The very successful check occurred in case of applying the combined treatment of *B. subtilis* + potassium monophosphate + whey milk. The obtained potentiality was disease reduction stimulating plant growth and the yield of cucumber. Such results had been reported by many investigators (Vogt and Buchenauer, 1997; Belanger *et al*., 1998 and Romero *et al*., 2004). This combined treatment was almost effective as the tested fungicide (Punch).

It is worth to note that, in spite of the superiority of full cream milk, whey was selected for combined treatment because it is mostly available, economically cheap and free of main nutritional assets.

However, the obtained results indicate to the necessity of animation and improvement in field of alternatives putting in mind the human and animal health and environmental safe, towards the economic aspects, and social impacts for sake of the farmers and the social public community while sustainable agriculture production.

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تأثير الرش بالكائنات المضادة والكيماويات المستحثة للمقاومة واللبن البقري على مكافحة البياض الدقيقى في الخيار

هالة على محمد الدكر* و نهير عبد النظير محمود**
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أدى استخدام البكتريا *Bacillus megaterium, B. pumilus, B. subtilis* and *B. thuringiensis* ككائنات مضادة والكيتوزان وحامض الهيوميك وفوسفات البوتاسيوم الأحادية وحامض السالسيلك ككيماويات مستحثة للمقاومة واللبن البقري كامل ومنزوع الدسم والشرش إلى حدوث انخفاض معنوي للنسبة المئوية للجراثيم الكونيدية النابتة للفطر جلوفينوميسس سكيكوراسيرم نوع سيكوراسيرم المسبب لمرض البياض الدقيقي في الخيار مقارنة بمعاملة المقارنة . هذا الانخفاض كان يزداد مع زيادة تركيز هذه المركبات.

في تجربة الصوبة أدى رش أى من هذه المركبات إلى حدوث درجات متباينة من الانخفاض فى شدة الإصابة بالمرض مع زيادة طول النباتات وكذلك الوزن الطازج والجاف مقارنة بمعاملة المقارنة. بالإضافة لذلك ، أظهرت تجارب الحقل موسمى ٢٠١٣ و ٢٠١٤ بمحافظة الجيزة ، أن الرش بالبكتريا *B. subtilis* وفوسفات البوتاسيوم الأحادية وشرش اللبن البقري ، كل على حدة أو بمخاليط مختلفة ، إلى حدوث انخفاض معنوي في شدة الإصابة بالمرض مع حدوث زيادة معنوية في محصول الثمار الناتج مقارنة بمعاملة المقارنة. علاوة على ذلك ، فقد كان الرش بأى من هذه المركبات بمفردها الأقل فعالية في هذا الصدد مقارنة برش هذه المركبات بمخاليطها المختلفة. وأكثر من ذلك ، فقد كان الرش بكل من البكتريا *B. subtilis* وفوسفات البوتاسيوم الأحادية وشرش اللبن البقري مع بعضهم هو الأكثر فعالية في إحداث انخفاض في شدة الإصابة بالمرض و زيادة محصول الثمار الناتج بقيم قريبة من نتائج رش المبيد الكيماوى (بلانش) ، حيث كانت شدة الإصابة ٣.٣ ، ٢.٩ % فى حين وصل المحصول الى ٨٠.٥ ، ٨٢.٤ كجم / ٤٢ متر مربع ، على الترتيب.