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The Potential of Biodegradable Formulation from Petroleum Waste to Control the Powdery Mildew of Cucumber Singly or in Combination with (Difenoconazole + Azoxystrobin)

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ABSTRACT

A biodegradable formulation from petroleum waste in comparison with the systemic fungicides difenoconazole+ azoxystrobin combination at concentrations of 250 and 325 (µg/ml), respectively was applied as a foliar spray for the control of powdery mildew of cucumber caused by *Sphaerotheca fuliginea.* Plants were grown under the greenhouse conditions and two successive sprayins were carried out. The frist one was applied on 39-day-old plants and the second on the plants of 46days old. The results illustrated that the biodegreable waste has a potential to control the disease and was more effective in controlling the disease than the selected systemic fungicids. The results highlight the potential of this biodegradable formulation as a promising and economic method for controlling powdery mildew on cucumber. Also, it is less toxic on the plants than the used fungicides, while the plant constituents of phenols and chlorophyll were increased which are a clue for increasing the immune system of the treated plants. This biodegradable formulation from petroleum waste may be useful for controlling other diseases of cucumber or other crops.

Keywords: Difenoconazol, azoxystrobin, powdery mildew, cucumber, control, *Sphaerotheca fuliginea*, biodegradable petroleum waste, *Pseudomanas fluorescens, Phanerochaete chrysosporium,* hydrocarbon oxidizers, phloroglucinol, phenazine, benzoquinoline.

INTRODUCTION

Romero *et al*. (2004) reported that the characteristic visual symptoms of the powdery mildew on cucumber leaves is the development of a whitish, talcum-like powdery growth on both surfaces, petioles and stems. Infected leaves usually die prematurely (Zitter *et al*., 1996). The reduction in fruit quality is obvious while the crop yield is significantly reduced.

Stereoscopic microscope identification showed that the isolated fungi are, *Golovinomyces cichoracearum* or *Podosphaera fusca* (syn. *Sphaerotheca fusca*), (Braun *et al*., 2002).

Dai 1979, Tang *et al*., 2003 and Huang, 1999) reported that the fungul spores of these fungi are seed-borne and the relative humidity take part in spreading the spores . They also aded that the incubation period for the spores to germinate and infect leaves is nearly 24h. Seven days later the disease clearly develop and causes a reduction in plant vigor started on seedling while the mature plants produce low quality fruits and decreased yield.

Biodegradation is a natural processes by microbes, which break down petroleum hydrocarbons and change them to other substances (Bragg *et al*., 1994). *Pseudomonas fluorescens is one of the* bacteria capable to degrade the petroleum waste in a process called Hydrocarbon oxidizers while the bacteria is known as Hydrocarbon oxidizers (Atlas, 1981).

Phanerochaete chrysosporium has also the ability to break down this wastes and remove 75-80% of all the total petroleum hydrocarbon in the polluted soil (Yateem *et al.,* 1998).

MATERIALS AND METHODS

The present investigations was conducted during the year 2016. The treatments were designed and seeds were grown at the greenhouses of Plant Pathology Department, and Seed & Tissue Pathology Lab (SEPA), Central Laboratory, Faculty of Agriculture, Mansoura University, Egypt, located on campus.

Source of the pathogen

Sphaerotheca fuliginea fungus was isolated from the infected plants grown in the privet fields of cucumber at Dakahlia governerate. Samples of infected leaves were collected and deliverd to the fungal identification lab of the Institute of Plant Pathology, the Agriculture Research center, Giza, Egypt.

Multiplication and maintenance of cucurbit powdery mildew isolates

Subcultures of powdery mildew were incubated for 7 days in enclosed containers at 22 ± 3°C under ambient lighting, before being transferred to fresh and healthy tissues or used in the bioassays. When sporulation was abundant, conidia were transferred to a new leaves to obtain ample quantity of inoculum to be used in the bioassays of the componants.

Pathogenicity test

The powdery mildew fungus (*Sphaerotheca fuliginea*)was maintained on the top leaves of cucumber plants followed by several successive transfer on new growing plants. Conidia were gently brushed and collected in a small quantity in distilled water containing two drops of Tween 20 and counted with the aid of a hemocytometer to give a suspension of $3x10^4$ conidia ml^{-1}. For inoculation, the upper surface of the leaves of the plants under investigation was uniformly sprayed with a conidial suspension delivered by a hand sprayer then covered with polyethylene bags and kept at 20°C for 20-24 h in dark. Five replicates were used in this treatment. Plants were then transferred back to the greenhouse benchs while the temperature was fluctuated between 20-32°C with days length of nearly 14h. Plants watered when necessary. The number of powdery mildew colonies produced on each leaf was recorded on the plants 10 days after the inoculation.

In another experiment, the desingend formulation from biodegradable petroleum wast was applied on the fungal inculated leaves 10 days after the inculation. The

effect of the formulated degradable wast were recorded after 60 days as described by (Reuveni *et al*., 1996).

Disease assessment

Disease incidence

Percentage of disease incidence was recorded as recorded by the following equation:

$$\%\ \textbf{Powdery mildew incidence} = \frac{\textbf{No. of powdery mildew symptoms bearing plants}}{\textbf{Total no. of plants}} \textbf{ X 100}$$

Disease severity

Powdery mildew severity was assessed following standard severity scale: 0 (No disease symptom), 1 (1-25% leaf area infected), 2 (26-50% leaf areas infected), 3 (51-75% leaf areas infected), and 4 (76-100% leaf areas infected (Cohen *et al*., (2004). The percentage of disease severity was recorded by the following equation:

$$\%\ \textbf{Powdery mildew severity} = \frac{\textbf{Sum of individual ratings}}{\textbf{Total no. of rating x maximum disease grade}} \textbf{ X 100}$$

Source of fungicides

The chemicals viz., azoxystrobin 20 SC and difenoconazol 12.5 SC were obtained from Syngenta Pvt. Ltd., Egypt.

Biodegradation of petroleum wastes (lubricating oil)

Two biodegradable isolates for petroleum wastes were used in this study. First is white rot fungi *Phanerochaete chrysosporium* and the second is bacteria *Pseudomonas fluorescens* NRRL 340. Both isolates were kindly obtained from Dr. Essam Eldin Sallam Ibrahim Sallam, Soil, Water and Environment Research Institute (SWERI), Agricultural Research Centre (ARC), Egypt.

A 10 Erlenmeyer flasks (500 ml) were prepared with 90 ml lubricant (spent car oil, petroleum waste)pH 6.8 in each one and sterilized at 121^{o}C for 20 min. All flasks were inoculated with 2ml of *Phanerochaete chrysosporium* ($1x10^{5}$ ml^{-1}), 6 ml of peptone 1% as a carbon source. Flasks were incubated at 35±2^{o}C in dark with shaking at 200rpm. After 10 days, two ml of *Pseudomonas fluorescens* NRRL 340 ($1x10^{9}$ ml^{-1})were added, then incubated at 25±2^{o}C in dark with shaking 200 rpm for 3 days. After fermentation, 20ml 0.01M phosphate buffer pH 7.0 were added and flasks were shacked for 20min. The biodegradable lubricant was filtrated through two layers of sterile cheesecloth and Whatman No.1 filter papers. The supernatant was kept under 4^{o}C for further study.

High performance liquid chromatography (HPLC)

An HPLC system (Agilent Technologies, model 1050, Waldbronn, Germany)combined with quaternary pump, auto-sampler, diode array detector (HP–1050), fluorescence detector (HP-1046A), and data analysis software, was used. UV detection (214 nm)plus fluorescence detection λ ex 260 nm)was applied.

Mass spectrometry (MS)

The Electron Impact Mass Spectra (EI-MS) had a delay of 3 min. to avoid the solvent plead and then scanned from m/z 50 to m/z 300. Ionization energy was set at 70 eV. The compounds were identified using Wiley and Nist 5.0 mass spectral database.

Statistical analysis

A one-way analysis of variance was conducted to analyze the data, by completely randomized design (CRD). Data collected from all experiments were statistically analyzed using the Statistical Analysis System package (SAS institute, Cary, NC, USA). Differences between treatments were determined using Fisher's least significant difference (LSD) test by Duncan's multiple range test (Duncun, 1955). All comparisons were performed at $P \leq 0.05$.

RESULTS

Pathogenicity test

Three *Sphaerotheca fuliginea* isolates were tested for their pathogenicity, under greenhouse conditions, using susceptible cucumber plants Super Dalila F1 Hybrid, Data presented in Table (1)show that, the disease incidence caused by gamsa isolate was significantly higher than that of belqusa and badway isolates (100, 76.85 and 62.43%, respectively). No significant difference between both isolates with regard to their disease severity was shown.

Table 1. Pathogenicity test of three *Sphaerotheca fuliginea* isolates (gamsa, belquas & badway)on cucumber plants, 35 days old, under controlled greenhouse conditions.

Treatment	Disease Incidence (%)[a]	Disease Severity (%)[b]
Gamsa isolate	100.00 a [c]	62.31 a
Belquas isolate	76.85 b	46.26 b
Badway isolate	62.43 c	42.83 b
Not inoculated (no fungus)	0.00 c	0.00 d

[a] **% Powdery mildew Incidence = (No. of Powdery Mildew Symptoms bearing plans/Total no. of Plants) x 100**

[b] **% Powdery mildew Severity = [Sum of Individual Ratings/ (Total No. of Rating x Maximum Disease Grade)] x 100.**

[c] **Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (*P*=0.05).**

Two biodegradable isolates for petroleum wastes were used in this study, *Phanerochaete chrysosporium* and *Pseudomonas fluorescens* NRRL 340. After fermentation conditions in 13 days, the active ingredients in final supernatant were identified by the aid of HPLC technique, which showed the presence of three compounds namely: phloroglucinol, phenazine, and benzoquinoline. These compounds were characterized by comparing their mass spectra with those obtained by the NIST and WILEY libraries. The obtained results were tabulated in Table (2).

Table 2. Chemical composition of biodegradable petroleum wastes (lubricating oil).

No.	RT	Compounds	Molecular formula	Molecular weight	Peak area (%)
1	1.325	Phloroglucinol	$C_6H_6O_3$	126.1	8.7955
2	1.383	Phenazine	$C_{12}H_8N_2O$	180.2	11.2279
3	1.464	Benzoquinoline	$C_{13}H_9N$	179.2	9.023

Data presented in Table (3)show that, disease incidence on infected plants by isolate *sphaerotheca fuliginea* in the control treatment increased by 41.75 % on the plants of 60 days old. Also the combination of azoxystrobin and difenoconazol recorded 17.64% reduction in disease incidence on the plants of 60 days, while the disease incidence for biodegradable lubricant at a concentration of 400 µg/ml decreased the disease incidence down to 14.90 %.

Table 3. Effect of biodegradable formulation, and premix of azoxystrobin and difenconazol concentrations as treatments on disease incidence and disease severity of cucumber plants on 60 days old inoculated with *Sphaerotheca fuliginea*.

Treatment	Conc. (µg/ml)	Disease Incidence (%)[a]	Disease Severity (%)[b]
Control (check)	0	41.75a [c]	30.44b
Azoxystrobin + Difenoconazol	325	17.64e	28.67c
Biodegradable lubricant	300	18.28 d	20.04e
	350	17.16f	18.81f
	400	14.90g	11.72g
Not inoculated (no fungus)		38.69b	27.66d

[a]% Powdery mildew Incidence = (No. of Powdery Mildew Symptoms bearing plans/Total no. of Plants) x 100

[b]% Powdery mildew Severity = [Sum of Individual Ratings/ (Total No. of Rating x Maximum Disease Grade)] x 100.

[c] Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (*P*=0.05).

The disease severity on the untreated plants showed 30.44% of on the 60 days old plants, while treatment with biodegradable lubricant at 400 µg/ml reduced disease severity down to 11.72 % on the 60 days old plants. The disease severity of the combination of azoxystrobin + difenoconazol recorded 28.67%.

Data presented in Table (4)recorded the total chlorophyll at a rate of 1.72 mg/g in the treatment in which combination of azoxystrobin 20% + difenoconazol 12.5% was applied on 60 days old plants infested with *S. fuliginea*. The check presented 1.14 only. On the other hand, there were significant differences in total phenol content between the treatment and the control plants which show 36.40 catechol/100 g fresh weight)in the check, while the treatment of azoxystrobin 20% + difenoconazol 12.5% recorded 55.03 (mg catechol/100 g fresh weight), respectively.

The treatment in which the the biodegradable waste was applied showed 15% reduation in the disease incidence. The total chlorophyll recorded 2.18 mg/g compair to 1.1 in the check. In case of the total phenol it recorded 69.70 (mg catechol/100 g fresh weight), while it was 36.4 in the check.

Table 4. Effect of combination of premix of azoxystrobin + difenoconazol as well as the biodegradable formulation on the contents of Chlorophyll A, B (Chl. A, Chl. B), total chlorophyll (T. Chl.), carotenoids (Carot.), and total phenol (T. Phenol) in leaves of 60 days old plants inoculated with *Sphaerotheca fuliginea*.

Treatment	Conc. (µg/ml)	Chl. A (mg/g)	Chl. B (mg/g)	T.Chl. (mg/g)	Carot. (mg/g)	T. Phenol (mg catechol /100 g fresh weight)
Control (check)	0	0.664 f [a]	0.475 d	1.139 e	0.066 d	36.396 d
Azoxystrobin + Difenoconazol	325	1.004 b	0.718 b	1.722 b	0.1 b	55.03 b
Biodegradable lubricant	300	0.825 de	0.589 cd	1.415cd	0.082c	45.21 c
	350	0.875 cd	0.625 bc	1.501 d	0.087 bc	47.95 bc
	400	1.272 a	0.909 a	2.181 a	0.126 a	69.69 a
Not inoculated (no fungus)		0.786 e	0.562 cd	1.348 d	0.078 cd	43.076 cd

[a] Values within a column followed by the same letter (s) are not significantly different according to Duncun's multiple range test (*P*=0.05).

DISCUSSION

Powdery mildew is one of the most economically important disease attacking cucumber world-wide. The disease is caused by the air-bone fungus *Sphaerotheca fuliginea*. The awareness of this disease was increased as its impact on the yield is drastic and cause higher loss in the fruit yield of cucumber; one of the important vegetable crop and mandatory in our daily dites. Chemical control of disease especially in the vegetables of short life span including cucumber has hazard effect on the human health when accumulated in the edable parts of the crop in return to the improper and carelessness application. Moreover, their direct effect on the labors health during the application process especially in the third world while safty regulations are never followed. Care and exercise for the proper time of appliying the toxic chemicals (fungicids)on the growing plants is also never happened. So far, the present investigation was carried out to evaluate the effect of the biodegradabel petroleum wast as a less toxic products for controlling the powdery mildew of cucumber since the intensive spray of fungicids are commonly used in greenhouses. This study was carried out during two successive growing season (2016)and the research was designed to use the soil-borne bacterium *Pseudomanas fluorescens* to biodegrade the petroleum waste which nearly take 7 days. The research data when statistically analized has proved the efficacy of the degradable waste by *Pseudomanas fluorescens* for controling the disease as it give 61.49% reduction in the disease incidence in compar to 5.8% when the compination of azoxystrobin + difenoconazol was applied while the check plants showed 100% infection. The presented results clearly demonastrate the possible use of the formulation gained from the biodegradation of petroleum waste as an alternative and safe method for controling the powdery mildew occures on cucumber and other edable vegetable crops. While the chemical constituents of chlorophyll and total phenols are taken as indicator and clue for increasing the immune system of the plants and produce healthy edible parts of the crop. Unless the proper timing of the systemic fungicids used for controling the diseases their residues in the plants and fruits is commonly found. From an economic stand point, the biodegrdable formulation produced from petroleum wast has proved to be a promising for controling this disease which has a lower risk on human and their diet and also to the environment as well. In this respect, it is recommended to expand applying this formulation on other vegetables and field crops not

only under greenhouse condition but also on a large scale in the open fields.

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تقيم كفاءة مخلفات البترول المحللة بيولوجيا في مقاومة مرض البياض الدقيقى في الخيار بمفردها أومدعمة بتوليفة من المبيدات الفطرية الجهازية دايفينوكونازول وأزوكسيستروبين

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تناولت هذه الدراسة مقدرة المركبات الناتجة عن تحلل مخلفات البترول بيولوجيا بواسطة البكتيره سيدوموناس فلورسنس والمعزولة من التربة في مقاومة مرض البياض الدقيقى فى الخيار والمتسبب عن الاصابة بالفطر SPHAEROTHECA FULIGINEA وقد تم عمل مقارنة لكفاءه تاثيرها في مقاومة الفطر مقارنة بتاثير المبيدات الفطرية الجهازية دايفينوكونازول وأزوكسيستروبين مجتمعين وذلك في موسم ٢٠١٦ تحت ظروف الزراعة المحمية. وقد اثبتت النتائج بعد تحليلها احصائيا تفوق هذه المخلفات المحللة بيولوجيا في مقاومة المرض بنسبة ٦١.٤٩% مقارنة بـ ٥.٨% عند استخدام دايفينوكونازول وأزوكسيستروبين في ذات الوقت الذى كانت فيه نسبة الإصابة ١٠٠% في نباتات المقارنة والغير معاملة باى من المبيدات إضافة الى أن تلك المواد المحللة بيولوجيا لم تظهر أى تأثير سام على النباتات المنزرعة وقد اكدت التحليلات الكيماوية لمحتوى الأوراق النامية من الكلورفيل والفينولات الكلية زيادتها بدرجة معنوية مما يشير إلى عدم تراكم أى مادة سامه داخل النبات نتيجة المعاملة بهذه المركبات المحللة بيولوجيا. وبذلك فإن هذه النتائج تلقى الضوء على إمكانية استخدام التكسير الحيوي لبعض المخلفات البترولية في مقاومة أمراض البياض الدقيقى كبديل آمن ليس فقط على محصول الخيار بل قد يمتد إلى باقى الخضروات التى تصاب بالبياض الدقيقى.