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Effect of certain plant extracts and fungicides against powdery mildew disease of Grapevines in Upper Egypt

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ABSTRACT

The efficiency of azadirachtin, jojoba oil, Reynoutria sachalinensis, azoxystrobin + difenoconazole, kresoxim methyl, propiconazole and sulphur in controlling powdery mildew disease of Grapevines caused by Uncinula necator (Schlecht.) was evaluated during 2015 and 2016 seasons. Laboratory study showed that all the tested plant extracts and fungicides significantly ($p < 0.05$) reduced germination of conidia of the causal pathogen. Spraying of the extracts on vine trees significantly ($p < 0.05$) decreased the disease severity (D.S. %) compared with infected control. The tested plant extracts as well as fungicide have high effect in disease reduction; no significant differences ($p < 0.05$) in the disease reduction were found in the effect of the extracts between tested compounds in both seasons. Moreover, treatment of trees with Reynoutria extract exhibited the highest increment in peroxidase activity (PO), polyphenol-oxidase (PPO) activity and phenol content compared to other treatments after 9 days from spraying.

Introduction

Grapevine, Vitis vinifera L., is usually cultivated as fruit crop worldwide. Vineyards covers an about 10 million hectares (Pearson and Geheen 1996). In Egypt, the cultivated area accounted about 72,190 hectares (FAO 2014). Grapevine is an economically important crop for both Egyptian local consumption and exportation. Several arthropod pests and diseases attack grapevine in many regions e.g. black vine thrips, grape phylloxera, grape root worm, grape berry moth, powdery mildew, downy mildew and grey mould (Reineke and Thiéry 2016). Powdery mildew is one of the most important fungal grape diseases worldwide that is caused by Uncinula necator (Schlecht.). It affects leaves, stems and fruits of grapevine grown in the field (Wilcox 2003). Control of U. necator in grapevine is mainly achieved by fungicidal treatment; (Waard et al. 1993; Nasir et al. 2014). Although
Chemical control of pests is very effective, it is the most costly input in production and that developed countries have already encountered very serious undesired side effects from the use of pesticides, such as emergence of secondary pests and environmental health hazards (Cisneros 1984; Ribes et al. 2017). Therefore, using plant extract as alternative candidate to synthetic fungicides would be useful in reducing the undesirable effects of fungicides and it could encourage integrated pest management (IPM) programmes. Plant extracts have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory and field trials and they are considered as the main source of economically bioactive pesticide compounds (Abdu-Allah 2012; Saleem et al. 2012; Varo et al. 2017). Several plant extracts and plant oils have been found effective in controlling obligate diseases, including powdery mildew worldwide (Ashfaq et al. 2016; Rur et al. 2017). Reynoutria sachalinensis extract significantly reduced the incidence of powdery mildew, U. necator on the grape (Avdiushko et al. 1993). R. sachalinensis extract treatment changed plant metabolism in grape infected with powdery mildew, Sphaerotheca fuliginea (Abd-El-Megid et al. 2001; Schmitt 2002). Enzymes activity plays an important role in plant disease resistance through increasing plant defence mechanisms (Takuo et al. 1993). Oxidative enzymes such as peroxidase and polyphenoloxidase catalase the formation of other oxidative phenols as lignin, contributing the defence barriers for reinforcing the cell structure (Avdiushko et al. 1993). Polyphenoloxidase is an important enzyme in the defence mechanism against fungal and bacterial pathogens, through their role in the oxidation of phenolic compounds to quinines (Hassan and Abo-Elyour 2013). In plants, PPO has been associated as well with lignifications of cell walls, thus playing a protective role in injured plants against pathogens, due to reactive quinones produced from phenolic compound catalysis (Morishita et al. 2003). Peroxidase (PO) has been implicated in the hypersensitive response, the formation of papillae and the polymerisation of lignin from monomeric lignols (Sorokan and Maksimov 2013). Therefore, this study was planned to evaluate the potential effects of certain plant extracts and chemical fungicides against grapevine powdery mildew under natural infection of King Roby variety under Assiut climate. The study mainly is focused on the effects of certain plant extracts and fungicides on fungus spore germination and evaluation of the changes in the enzymes activities of the treated vine trees.

Materials and methods

Plant extracts and fungicides

Azadirachtin (nimbecidine*, 0.03% EC, T. Stanes and Company LTD, India) was purchased from Stanes and Company LTD, India. Pure jojoba oil was personally supplied by one producer; it was extracted under cold conditions. Giant knotweed, Reynoutria sachalinensis, Milsana* 5%, KHH Biosci, Raleigh, NC was got as a gift
from an Egyptian friend. Azoxystrobin + difenoconazole (Amistar Top®, 32.5% SC, was bought from Shoura Chemicals, Egypt. Kresoxim methyl (Kreso®, 30% SC, Chema Egypt, Anhelo Acting Chemical Limited China), propiconazole (Tilt®, 25% EC, Syngenta in Egypt), sulphur (Kumulus-s®, 80.0% WG, Shoura Chemicals, Egypt) were brought from Egyptian local market.

Conidiospores germination

The fungal inoculum of *U. necator* used here was obtained from King Roby variety naturally infected with powdery mildew at Assiut governorate, Egypt. Conidia from leaves of plants were gently brushed with distilled water (100 ml) containing two drops of Tween-80 and the spore was adjusted to 5 × 10⁵/ml.

Leaf discs (10 cm) of the cv. King Roby were placed in Petri dishes on wet filter papers. Leaf discs were sprayed with 2 ml of a 5x10⁵ conidia/ml suspension and then sprayed with azadirachtin (500 μl/100 ml), jojoba oil (350 μl/100 ml), reynoutria extract (1000 μl/100 ml), azoxystrobin + difenoconazole (75 μl/100 ml), kresoxim methyl (50 mg/100 ml), propiconazole (14 μl/100 ml), sulphur (250 mg/100 ml). Sterile water was used as a control treatment. Ten leaf discs were included in a replicate. The dishes were incubated at 25°C in darkness for 24 h after that the spore germination was determined under a light microscope and the percentage of germination was calculated as follows:

\[
\text{Percentage of germination} = \frac{\text{No. of germinated spores}}{\text{Total number of spores}} \times 100
\]

Field experiment

The field experiments were carried out on a King Roby commercial vineyard variety. This variety has been cultured in clay loam soil since 10 years at Plant Pathology Experimental Farm, Assiut University, Assiut Governorate, Egypt. Vines were planted 1.5 m apart in rows 3 m apart and a double fence was used as the training system leaving about 80-buds per vine, in the first week of January of each experimental season. The vigour of selected vines was almost uniform. The infections of trees with *U. necator* are increased in May and June months of 2015 and 2016 seasons. In this time, the most effective treatment from the laboratory study were tested.

Forty-eight vine trees were used and divided into eight groups in randomised complete block design with three replicates. Two vine trees were used for every replicate. In June 2015 and 2016 seasons, sunny days without wind, plant extracts and fungicides dilutions were applied using a one nozzle knapsack sprayer, one litre of spraying solution was sufficient to good cover of one vine tree. For each treatment, one concentration of azadirachtin (5 ml/litre), jojoba oil (3.5 ml/litre), reynoutria extract (2.5 ml/litre), azoxystrobin + difenoconazole (0.75 ml/litre), kresoxim methyl (0.5 g/litre), propiconazole (0.15 ml/litre), sulphur (2.5 g/litre)
was applied, this concentration have not any phytotoxicity on plants. Tap water and TritonX-100 at 0.05% was used in dilutions and also used as control. Culture practices as irrigation, fertilisation and pest control were applied uniformly across the vineyard. After one week, disease severity, the percentage of leaf area covered with sporulating colonies, was estimated.

**Disease assessment**

Disease severity, the percentage of leaf area covered with sporulating colonies, was estimated according to (El-Morsi et al. 2012; Morishita et al. 2003). The diseases symptoms evaluation was classified into five categories based on the following scale:

0 = no visual infection, 1 = 1–5% infection, 2 = 6–25%, 3 = 26–50%, 4 = more that 50% of leaf area covered by fungal colonies. Final disease assessment was calculated after one week from inoculation using the following formula:

\[
\text{Disease severity (\%) = } \left( \sum (n \times v) / 5N \right) \times 100
\]

Whereas: \(n\) = number of infected leaves in each category, \(v\) = numerical values of each category = total number of the infected leaves.

**Determination of enzymes activity and total phenol in the field treated trees**

The effect of selected plant extracts and fungicides on peroxidase, polyphenol-oxidase and ß-Glucosidase activity was determined and compared with control. The fresh leaves were randomly taken as samples from treatments and control before treatment and nine days after treatment. The leaves were immediately frozen and stored at −80 ºC until extraction of enzymes.

**Leaves extraction**

Fresh frozen leaf samples were ground with sodium phosphate buffer (0.1 M, pH 7.0) using a glass homogeniser at a concentration of 100 mg/1 ml buffer. Homogenates were centrifuged at 10,000 r.p.m. for 30 min at 4 ºC. The supernatant was used to determine enzyme activities (Tuzun et al. 1989).

**Peroxidase (PO) assay**

Peroxidase activity was assayed according to the method described by (Allam and Hollis 1972). The cuvette contained 0.5 ml of 0.1 M potassium phosphate buffer at pH 7.0, 0.3 ml of enzyme extract, 0.3 ml of 0.05 M pyrogallol, 0.1 ml 1.0% \(H_2O\), and distilled water was added to bring cuvette contents to 3.0 ml. The reaction mixture incubated at 25 ºC for 15 min, then the reaction was inactivated by adding 0.5 ml of 5.0% (v/v) \(H_2SO_4\) (Kar and Mishra 1976). Peroxidase activity
was expressed as the increase in absorbance at 425 nm/gram fresh weigh/15 min. Peroxidase activity = OD 436 nm/mg protein.

**Polyphenol oxidase (PPO) assay**

The polyphenoloxidase activity was assayed according to the method described by (Matta and Dimond 1963). The reaction mixture contained 0.2 ml enzyme extract, 1.0 ml of 0.2 M sodium phosphate buffer at pH 7.0 and 1.0 ml of 10⁻³ M catechol and the volume was completed with distilled water up to 6.0 ml. The reaction mixture was incubated at 30 °C for 30 min. Polyphenoloxidase activity was expressed as the increase in absorbance at 420 nm/g fresh weigh/30 min.

PPO activity = OD 410 nm/mg protein.

**ß-Glucosidase (ß-GL) activity**

ß-glucosidase activity was determined using the method described by (Abo-Elyousr et al. 2012). The reaction mixture was as follows:0.5 ml supernatant, 1.5 ml of Sörensen Phosphate buffer pH 6.5, 6.8 gm KH₂PO₄ and 8.99 gm Na₂HPO₄ x 2H₂O were dissolved in 1000 ml water, after addition of 0.372 g/l ethylene diamine tetra- acetic acid (EDTA) the pH was adjusted to 6.5, and 0.5 ml 5 mM p-nitrophenylglucopyranosid. The mixture was incubated at 30 °C for 5 min and measured at 400 nm. ß-Glucosidase activity was determined according to the following formula and expressed as enzyme unit/mg protein:

ß-Glucosidase activity = mM p-Nitrophenol/mg protein,

The protein levels were determined in the leaves extraction using biuret solution according to the method of (Gornall et al. 1949).

**Determination of total phenol content**

One ml of the supernatant extraction, which was prepared as described before, was added to 5 ml of distilled water and 250 μl of Folin–Ciocalteu reagent (Merck), and the solution then was incubated at room temperature. After 3 min, 1 ml of a saturated solution of Na₂CO₃ (LOBA) and 1 ml of distilled water was added and the reaction mixture was incubated for 1 h. The absorbance of the developed blue colour was measured at 725 nm using a blank, water and reagent only. Caffeic acid (Fluka) was used as reference. The total phenolic compounds of samples were expressed as milligram caffeic acid per gram of fresh leaves (Malick and Singh 1980).

**Statistical analysis**

All experiments were performed in duplicate. Analyses of variance were carried out using the SPSS (SPSS Inc., Chicago, IL) statistical software package). Least
significant difference (LSD) was calculated at $p \leq 0.05$ according to (Gomez and Gomez 1984).

**Results**

**Effect of plant extracts and fungicides on conidiospores germination**

Microscopic observation showed that all treatments inhibited conidial germination (Figure 1). The highest effect was found for the treatment with sulphur and azoxystrobin + difenoconazole fungicides (77.4 and 70.9%) followed by reynoutria extract (64.2%), while azadirachtin and kresoxim methyl extracts gave the lowest reduction of conidial germination (9.7 and 11.5%) compared to other treatments. The treatment with jojoba extract and propiconazole fungicide showed lower effect (52.7 and 55.9%).

**Effect of certain fungicides and plant extracts on powdery mildew disease development under field conditions**

Data presented in Table 1 showed that all treatments caused significant reduction in the severity of grapevine powdery mildew during 2015 and 2016 seasons compared to the control. The highest reduction levels of 95 and 97.6% were found

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**Figure 1.** Percentage of spore germination of *Uncinula necator* (the causal pathogen of grapevine powdery mildew) by four fungicides and three plant extracts. Values within the column that are associated with different letters indicate significant differences ($p \leq 0.05$).
after the treatment with propiconazole during both the two seasons, respectively, followed by sulphur treatment (71.9% and 94.4%); while, azadirachtin showed the lowest reduction level. Based on the reduction percentages, the tested compounds can be asendingly arranged as follows: propiconazole > kresoxim methyl > sulphur > azadirachtin > reynoutria extract > jojoba oil > azoxystrobin + difenoconazole. No phytotoxicity on the vine leaves were recorded at the tested doses of compounds.

### Effect of certain fungicides and plant extracts on enzymes activities

Data in Figures 2–4 indicated that the vine trees treated with reynoutria extract exhibited the highest increment in PPO, PO activities compared to other treatments, while kresoxim methyl gave the highest amount of β-Glucosidase (β-GL) after 9 days from spraying. Increased activity of PPO was also observed after treatment of grapevine plants with propiconazole, kresoxim methyl and azoxystrobin + difenoconazole. The highest increase in PO activity was caused by reynoutria extract, propiconazole and kresoxim methyl. The highest activity of β-Glucosidase (β-GL) was found after treatment with kresoxim methyl; while, the reynoutria extract, azadirachtin and Jojoba oil nearly showed similar effect on enzyme activity. Sulphur did not increase the enzyme activity compared to control plants. Treatment with propiconazole, sulphur, kresoxim methyl, azoxystrobin + difenoconazole and reynoutria significantly increased the phenol in the leaves of grapevine plants after 9 days, while other treatments showed no change in phenol level compared to control plants (Figure 5).

### Table 1. Disease severity (DS%) and reduction percentages of three extracts and four fungicides field treatments against grapevines powdery mildew, Uncinula necator (Schlecht.) in King Ruby variety during 2015 and 2016 seasons.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>2015</th>
<th></th>
<th>2016</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DS% ± SE % Reduction ± SE</td>
<td>DS% ± SE % Reduction ± SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.35 ± 6.41 A –</td>
<td>15.78 ± 5.28 A –</td>
<td></td>
<td></td>
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<tr>
<td>Plant extracts</td>
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<tr>
<td>Azadirachtin</td>
<td>5.1 ± 0.6 AB 66.4 ± 22.18 A</td>
<td>5.4 ± 1.8 B 81.0 ± 9.5 AB</td>
<td></td>
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</tr>
<tr>
<td>Jojoba oil</td>
<td>4.3 ± 1.5 AB 65.0 ± 14.7 A</td>
<td>2.0 ± 0.1 B 83.9 ± 5.4 AB</td>
<td></td>
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<tr>
<td>Reynoutria extract</td>
<td>8.5 ± 4.3 AB 66.2 ± 17.1 A</td>
<td>0.8 ± 0.8 B 94.7 ± 5.3 A</td>
<td></td>
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<tr>
<td>Fungicides</td>
<td></td>
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<tr>
<td>Azoxystrobin + Difenocona-</td>
<td>6.9 ± 3.1 AB 57.4 ± 12.6 A</td>
<td>3.0 ± 1.1 B 60 ± 18.6 B</td>
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<td>zole</td>
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<tr>
<td>Kresoxim methyl</td>
<td>2.7 ± 1.6 B 87.3 ± 6.6 A</td>
<td>1.6 ± 0.8 B 91.4 ± 4.7 A</td>
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<td></td>
</tr>
<tr>
<td>Propiconazole</td>
<td>1.3 ± 0.7 B 95 ± 2.9 A</td>
<td>0.5 ± 0.3 B 97.6 ± 1.6 A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphur</td>
<td>2.5 ± 1.3 B 71.9 ± 16.7 A</td>
<td>0.5 ± 0.5 B 94.4 ± 5.6 A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Means, in the same column, followed by the same letter are not significantly different from each other at 5% probability level, LSD test.
Figure 2. Effect of three plant extracts and four fungicides on the peroxidase (PO) enzyme activity. Values within the column that are associated with different letters indicate significant differences ($p \leq 0.05$).

Figure 3. Effect of three plant extracts and four fungicides on the polyphenol oxidase (PPO) enzyme activity. Values within the column that are associated with different letters indicate significant differences ($p \leq 0.05$).
Figure 4. Effect of three plant extracts and four fungicides on the leaves β-glucosidase (β-GL) enzyme activity. Values within the column that are associated with different letters indicate significant differences ($p \leq 0.05$).

Figure 5. Effect of three plant extracts and four fungicides on the leaves phenol content. Values within the column that are associated with different letters indicate significant differences ($p \leq 0.05$).
Discussion

Based on Pesticides Egyptian Protocols when the powdery mildew symptoms reached up to 5% naturally infection on trees (the economic infection), it requires curative chemicals therefore, this work was planned to test certain plant extracts and fungicides against powdery mildew of grapevine under field conditions. The tested plant extracts as well as fungicide showed high potential effect on disease reduction. The field reduction results were compatible with the high inhibition in germination spore in *R. sachalinensis* extract as well as azoxystrobin + difenoconazol (systemic fungicide). *R. sachalinensis* extract showed high field efficacy and also it exhibited high spore germination inhibition of *Sphaerotheca fuliginea* (Seddon and Schmitt 1999). Conversely, azadirachtin and jojoba oil extracts showed low inhibition of *U. necator* spore and high field reduction, this could be explained by it may be these extracts need more long time (more 24 h) to penetrate into spore to give the fungicidal effects. In general, the reduction of disease was higher in 2016 than 2015 in all treatments; these can be explained by climate changing in two years, where the temperature, sunny period and water evaporation rate values were higher in June 2016 than the same period in 2015. The recorded mean temperature, sunny period, water evaporation rate were 34 ± 0.61, 29.79 ± 2.26°C; 11.87 ± 0.19, 11.06 ± 1.32 h; and 9.84 ± 0.83, 8.29 ± 1.00 ml in the first 10 days of June 2016 and 2015 (while the compounds sprayed on the field), while in the same period, the relative humidity was higher in this period in 2015 than 2016 years, it was recorded 33.2 ± 4.89, 22.7 ± 6.85 respectively, according to Assiut University Metrology Station. Our data reported herein convinced by other investigators such as (Daayf et al. 1995), who found that *R. sachalinensis* extract significantly reduced the severity of powdery mildew on cucumber compared with control; it was effective as benomyl fungicides. Abd-El-Kareem et al. (2002) reported that aqueous neem kernel extract was as effective as sulphur against powdery mildew, *Sphaerotheca fuliginea* on courgettes. Pasini et al. (1997) verified that organic JMS Stylet oil, canola oil, synertrol and neem extract provided 55% control of powdery mildew on rose plants. Also, Zhang et al. (2016) reported that Regalia extract from giant knotweed (*R. sachalinensis*), SC alone significantly reduced powdery mildew compared to the non-treated control, and was as effective as the chemical standard Procure 480SC (triflumizole)

The high preventive effect (97.74%) recorded by neem oil followed by Jojopa oil (89.82%) and *R. sachalinensis* extract (82.77%) against okra powdery mildew diseases was reported by Moharam and Ali (2012), on the other hand our results disagree with the results reported by Haroun (2002) who found that high reduction in disease incidence treated by garlic extract and clove extract, these reductions were more higher than synthetic Topas-100 fungicide and the control treatments.

The potential of *R. sachalinensis* extract in reduction in the present study could be explained by the higher amount of peroxidase (PO) and polyphenol oxidase
(PPO), the defence oxidative enzymes, by around eight, nine folds in treated leaves compared with control. The mechanisms of PPO to infected plants depend on two ways, first way; by direct action of PPO on the pathogen inhibition by suppressing its life cycle. Secondly; induces mediated phenolic compounds which restrict the pathogen and enhance the biocontrol action (Mayer 2006, Saleem et al. 2012). The present results concerning the increase in peroxidase, polyphenol-oxidase enzymes activity are in agreement with those reported by Abd-El-Kareem et al. (2002), El-Habbak (2003), and Abo-Elyousr et al. (2008).

Increasing the total phenol levels in kresoxim methyl and sulphur-treated grapevine plants by four folds compared with untreated plants gave surely an increase in the capability of plants in resistance of infection process and disease development, The present results concerning the increase in total phenol contents are in agreement with those reported by Ahmed (2004) and Daayf et al. (1997). They reported that R. sachalinensis extract stimulated the production of fungi-toxic phenolic compounds as a result of elicitation with the extract and this being particularly evident when the cucumber plant was stressed by the pathogen of powdery mildew (S. fuliginea). Moreover, the amounts of these compounds in treated plants were nearly five times the level found in the control plants. Since the role of secondary metabolic substances, such as phenolic compounds, on disease resistance mechanisms are well known (Kalaichelvan and Nagarajan 1992).

Although PO, PPO, β-GL activities of sulphur-treated grapevine plants were not high as the other treatments, total phenol levels were increased by four folds as compared to control. These can be explained by inorganic sulphur have multi-discipline activity with no signs of resistance developing.

The present study can be concluded that azadirachtin, jojoba oil, and Reynoutria sachalinensis extracts had high potential effect against the powdery mildew disease of grapevines under Assiut, Egypt field conditions and the effect is comparable with that of the synthetic fungicides. These extracts could be used with fungicides or as alternatives in integrated grapevines powdery mildew management programmes (IPM) with the consideration of climate changes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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