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New Approach to Acquired Resistance Enhancement Against *Plasmopara viticola* Using Different Biotic Inducers

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ABSTRACT

Downy Mildew (*Plasmopara viticola*) is a well characterized plant pathogen which is known to infect a large number of grape cultivars (*Vitis* spp.). Downy mildew affects all parts of the plant, including leaves, shoots, buds, stems and fruit, leading to significant decreases in crop quality and on occasions complete loss of yield. This study investigates the ability of three bio-elicitors namely; *Trichoderma harzianum*, *Streptomyces plicatus* and *Pseudomonas fluorescens* to induce elevated levels of resistance in cv. Tifey cultivars in the face of *P. viticola* infection. In addition the study will explore some of the specific biochemical changes occurring within the plant in response to treatment with particular bio-elicitors with particular focus on changes in protein expression and levels of photosynthetic pigments. Enzyme activities such as peroxidase, polyphenoloxidase, chitinase and β -1, 3 glucanase were determined using spectroscopic methods, photosynthetic pigment level were determined in a similar manner. Protein expression patterns were analysed by polyacrylamide gel electrophoresis (SDS PAGE). The greatest reduction for disease severity was observed in response to treatment with *T. harzianum* at 1×10^{-6} spore mL^{-1} and followed by *S. plicatus* at 1×10^{-8} CFU mL^{-1} . Both chlorophyll and carotene levels were increased in response to treatment with *T. harzianum* followed by *P. fluorescens* when compared to an infected untreated control. Significant diversity was observed in terms of enzyme activity depending on the type of elicitor employed. SDS PAGE analysis for grape seedlings showed considerable diversity in protein expression levels between biotic treatments. Pearson cluster analysis showed that protein expression patterns for treatments with *S. plicatus* and *P. fluorescens* treatment were highly similar (95.76%), *T. harzianum* (94.25%) clustered separately to *S. plicatus* and *P. fluorescens* indicating a possible differential response to fungal and bacterial elicitors. This study suggests that the use of bio-elicitors may provide an effective alternative to fungicides in the quest to control downy mildew disease in grapes.

Key words: *Plasmopara viticola*, β -1,3 glucanases, polyphenoloxidase, peroxidase, SDS PAGE

INTRODUCTION

Vitis (grapevines) are a genus of about 60 species of vining plants in the flowering plant family Vitaceae. They are economically important as a source of grapes, both for direct consumption of the fruit and for fermentation to produce wine (Aigrain, 1999; Ali *et al.*, 2010).

Downy mildew of grapevines caused by *Plasmopara viticola*, is a highly destructive disease and is favoured by warm, wet growing seasons and easily causes 50-75% crop losses in one season (Nithyameenakshi *et al.*, 2006; Gokbayrak *et al.*, 2010). The direct loss may be the result of grape clusters rotting and general fruit breakdown, while indirect loss relates to the injury of shoots and premature defoliation. This disease attacks most commercial varieties and wild species of grape (Buonaurio *et al.*, 2009; Schroder *et al.*, 2010). The harmful effect of infection is related to the reduction in both quantity and quality of grapes produced (Agrios, 2005; Atak *et al.*, 2011).

The production of grapevine metabolites is strongly influenced by many factors like environment or pathogen attack (Ali *et al.*, 2010). Synthetic and natural resistance inducing substances may be used to decrease the susceptibility of plants to various pathogens thus reducing the application of pesticides in agriculture. The resistance was determined by the increase of Pathogenesis-related (PR) proteins including peroxidase (PO), polyphenoloxidase (PPO), β -1,3-glucanase, phenylalanine ammonia lyase and stilbene synthase (Chen *et al.*, 2000; Ferreira *et al.*, 2004; Aziz *et al.*, 2007; Magnin-Robert *et al.*, 2007; Yildirim, 2007; Harm *et al.*, 2011), enhanced phytoalexin production (Van Peer *et al.*, 1991; Alkahtani *et al.*, 2011) and induced expression of stress-related genes (Verhagen *et al.*, 2010).

Acquired resistance is based on stimulating plant defenses against injury causing pathogens by using biotic and/or abiotic elicitors. *Pseudomonas* spp. bacteria have been shown to trigger Induced systemic Resistance (ISR) in different plant species. This ISR is based on multiple mechanisms, including the enhancement of the capacity of plants to mobilize cellular defence responses before or upon pathogen challenge (Farrag *et al.*, 2007; Walters and Fountaine, 2009; Verhagen *et al.*, 2010). There is evidence that some bacterial genotypes may secrete enzymes associated directly with the metabolic processes of plants, and thus play a vital role in the induction of plant resistance against pathogens (Eckardt, 2004). Induction may be due to high concentrations of toxic metabolites triggering increased levels of enzymes which contribute to induced resistance. Also, the acquired resistance may be systemic or localized (Tian *et al.*, 2006; Imran *et al.*, 2007; Barilli *et al.*, 2010).

This study aimed to monitor some of the metabolic changes that may occur inside the plant when treated with bio elicitors and the impact of these biochemical changes on the degree of resistance against downy mildew disease. In addition this study will explore the possibility of using these techniques practically as safe alternative to fungicides.

MATERIALS AND METHODS

All cultivation, inducer treatments and inoculation trials were conducted in a protected green house in Riyadh greenhouse farms, Saudi Arabia in January 2010. Chemical analyses were carried out in same year in plant pathology institute and Faculty of Science, Cairo University, Egypt.

Pathogen inoculum and induction of systemic resistance: Three days after induction, the plants were infected with spore suspension (3×10^5 -conidia mL⁻¹ water) of *Plasmopara viticola*. Three seedlings of highly susceptible grape varieties (Taify) were used in these experiments; three replicates were used for each treatment. Seedling was sprayed at 4th true leaflet growth stage. Inducer treatments in control seedlings were replaced by water only for all replicates.

Disease severity: Three biotic inducers were tested for inducing resistance in grape cv. Taify at three concentrations, including *Streptomyces plicatus* at concentrations 1×10^5 , 1×10^6 and

1×10^7 CFU mL⁻¹, *Pseudomonas fluorescens* at concentration 1×10^6 , 1×10^8 and 1×10^9 CFU mL⁻¹ and *Trichoderma harzianum* at concentration 1×10^6 , 1×10^7 and 1×10^8 spore mL⁻¹. The treatment with tested bio-elicitors was conducted before artificial inoculation with *Plasmopara viticola* about 3 days while, the infected and healthy control were free from elicitors. Disease severity in each treatment was estimated two and three weeks post infection by assessing the scale of leaf break down, brown lesion and disease progress.

Chlorophyll and carotene contents: Photosynthetic pigments were extracted from treated and untreated fresh leaves two and three weeks post inoculation. Ten disks (1 cm⁻²) were taken from each treated leaf and pigments were extracted for 48 h in the dark in a tube containing 10 mL of 85% acetone according to the methods described by Proctor (1981). The total photosynthetic pigment content of the samples was determined from Optical Density (O.D) measurements at 663, 452 and 645 nm, these data were subsequently transformed using formula recorded by Arnon (1949). The treatment concentration of *Streptomyces plicatus* was 1×10^6 , *Pseudomonas fluorescens* 1×10^8 and *Trichoderma harzianum* 1×10^6 .

Peroxidase and polyphenoloxidase assay: Peroxidase and polyphenoloxidase enzymes were extracted according to methods laid out by Maxwell and Bateman (1967) while the peroxidase and polyphenoloxidase activity was determined according to the method described by Allam and Hollis (1972). The treatment concentration of *Streptomyces plicatus* was 1×10^6 , *Pseudomonas fluorescens* 1×10^8 and *Trichoderma harzianum* 1×10^6 .

Chitinase and β -1, 3 glucanase enzyme assay: One gram of plant tissue was homogenized with 0.2 M tris-HCl buffer (pH 7.8) containing 14 mM B-mercaptoethanol at a ratio of 1/3 w/v. The homogenate was centrifuged at 3000 rpm for 15 min under cooling. The supernatant was used to determine enzyme activities (Tuzun *et al.*, 1989). Chitinase activity determination was carried out according to the methods of Monreal and Reese (1969). β -1, 3 glucanase activity was determined according to the method of Jung *et al.* (1995). The treatment concentration of *Streptomyces plicatus* was 1×10^6 , *Pseudomonas fluorescens* 1×10^8 and *Trichoderma harzianum* 1×10^6 .

Protein profile analysis: The protein extractions were made from 1.5 g (wet mass?) of ten-day old seedlings that were treated with the biotic inducers (The treatment concentration of *Streptomyces plicatus* was 1×10^6 , *Pseudomonas fluorescens* 1×10^8 and *Trichoderma Harzianum* 1×10^6) harvested 1-10 days later, and stored at -80°C. The extraction method used is the one described by Jung *et al.* (1995). SDS-PAGE was performed on the protein extracts according to the method described by Laemmli (1970). The gels were then stained with silver nitrate in order to visualize all proteins.

Data statistical analysis: The data obtained were statistically analyzed using one way Analysis of Variance (ANOVA) with the MSTAT-C statistical package. The Least Significant Difference procedure (LSD) was used at 0.05 level of probability.

RESULTS

Data obtained in Table 1 revealed that treatment of grape seedlings cv. Tifey with different concentration of biotic inducers resulted in a reduction of disease severity comparing with control. The lowest disease severity was recorded by *T. harzianum*, (10.44%) at concentration 1×10^6

Table 1: Effect of different concentrations of biotic inducers (foliar treatment) on disease severity of *Plasmopara viticola* under greenhouse conditions

Treatment	Concentration	Disease severity (%) single spray		Mean	Efficiency (%)
		2 week	3 week		
<i>S. plicatus</i>	1×10 ⁶ CFU	23.80	28.28	26.04	54.58
	1×10 ⁸ CFU	10.10	15.61	12.86	77.58
	1×10 ⁹ CFU	12.61	17.50	15.06	73.74
<i>P. fluorescence</i>	1×10 ⁶ CFU	23.44	26.70	25.07	56.26
	1×10 ⁸ CFU	14.00	17.01	15.51	72.95
	1×10 ⁹ CFU	17.24	22.50	19.87	65.34
<i>T. harzianum</i>	1×10 ⁶ spore mL ⁻¹	8.83	12.04	10.44	81.80
	1×10 ⁷ spore mL ⁻¹	13.47	19.02	16.24	71.67
	1×10 ⁸ spore mL ⁻¹	19.50	22.70	21.10	63.19
Control	-	50.67	64.00	57.33	

LSD at 5% : 3.94

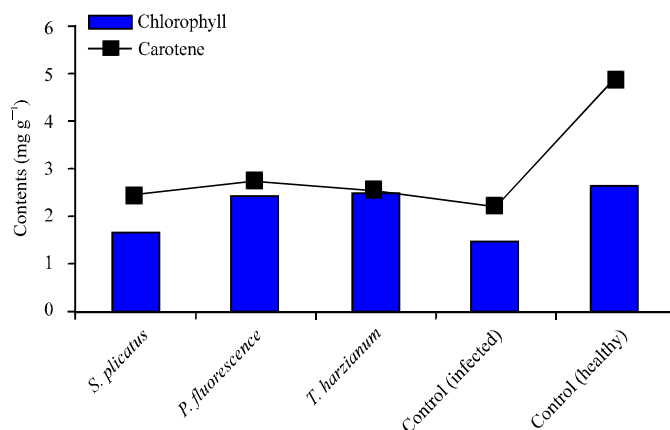


Fig. 1: Total chlorophyll content and carotene content (mg g⁻¹ fresh weigh) in grape seedlings cv. Taify treated with different biotic inducers after inoculation with *Plasmopara viticola* under greenhouse conditions

spore mL⁻¹ followed by *S. plicatus* (12.87%) and *P. fluorescence* (15.51%) at concentration 1×10⁸ CFU for both.

Figure 1 showed an increase in both chlorophyll and carotene content in grape seedlings treated by the tested elicitors compared with infected control. The highest content of chlorophyll was observed with *T. harzianum* and then *P. fluorescence*. The largest value of carotene was recorded by *P. fluorescence* and followed by *T. harzianum*.

Treatment of plants with biotic inducers recorded an increase in polyphenoloxidase activity during the examined periods (0, 1, 2, 4 and 6 days after inoculation with *Plasmopara viticola*) compared with the control (Table 2). The mean of the examined periods revealed the highest increase in enzyme activity (2.89) was the plant treated with *S. plicatus* while, *P. fluorescence* treatment recorded the lowest increase (2.54).

Table 3 revealed an increase in peroxidase activity in treated plants compared with the control during the examined periods. The most effective biotic inducer, *T. harzianum* treatment

Table 2: Polyphenoloxidase activity 1-6 days after artificial inoculation with downy mildew and inducer treatments on grape seedlings cv. Taify under greenhouse conditions

Treatments	Concentration	Polyphenoloxidase 1-6 days after artificial inoculation and inducer treatments under greenhouse conditions					Mean
		0	1	2	4	6	
<i>S. plicatus</i>	1×10 ⁶ CFU	2.48	2.69	3.21	2.95	3.15	2.89
<i>P. fluorescence</i>	1×10 ⁸ CFU	2.19	2.66	2.86	2.40	2.60	2.54
<i>T. harzianum</i>	1×10 ⁶ spore mL ⁻¹	2.37	2.63	3.07	2.43	2.66	2.63
Control (infected)	-	1.50	1.56	2.04	2.27	2.42	1.96
Control (healthy)	-	1.50	1.35	1.47	1.77	3.34	1.89

**Polyphenol oxidase activity expressed as the change in absorbance at 495 nm/3 g fresh weight min⁻¹. LSD at 5%: 0.918

Table 3: Peroxidase activity 1-6 days after artificial inoculation with downy mildew and inducer treatments on grape seedlings cv. Taify under greenhouse conditions

Treatments	Concentration	Peroxidase activity 1-6 days after artificial inoculation and inducer treatments under greenhouse conditions					Mean
		0	1	2	4	6	
<i>S. plicatus</i>	1×10 ⁸ CFU	17.06	18.73	22.24	24.55	26.20	21.75
<i>P. fluorescence</i>	1×10 ⁸ CFU	16.24	17.49	20.33	22.40	25.71	20.43
<i>T. harzianum</i>	1×10 ⁶ spore mL ⁻¹	22.44	24.50	24.85	28.09	16.57	23.29
Control (infected)	-	13.92	15.54	16.04	19.20	10.43	15.02
Control (healthy)	-	13.92	13.80	15.00	16.96	15.10	14.96

LSD at 5%: 2.043

recorded the highest enzyme activity (23.29) on the other hand the lowest increase in enzyme activity (20.43) was recorded as the plant treated with *P. fluorescence*.

An increase in chitinase enzyme activity was observed in the plants treated with biotic inducers comparing with control as mention in Table 4. The highest mean chitinase enzyme activity was (7.82) from grape seedlings treated with *T. Harzianum*, while *P. fluorescence* treatment recorded the lowest increase (5.50).

Data in Table 5 recorded an increase in β -1, 3 glucanase activity in the plants treated with different biotic inducers when compared with the control. The mean of the examined periods revealed the highest increase in enzyme activity (7.07) as the seedlings treated with *T. harzianum* on the other hand, *P. fluorescence* treatment recorded the lowest increase (5.10).

Cluster analysis (Fig. 2, 3) showed that, there were different numbers of protein fragments per lane, in addition the intensity of comparable bands was observed to vary. The first lane acts as a molecular weight protein marker (M), while the second lane acts as grape seedlings cv. Taify inoculated with *Plasmopara viticola* and treated with *S. plicatus* (K1), third lane treated with *P. fluorescence* (K2), *T. harzianum* (K3) and infected control without any treatment (K4). A high degree of similarity (95.76%) was observed between *S. plicatus* and *P. fluorescens* treatments, treatment with *T. harzianum* yielded a pattern which was similar but distinct from the other two treatments at 94.25% similarity. The untreated infected control lane was observed to cluster entirely separately from the treated samples with a similarity of 92.89%.

Table 4: Chitinase activity 1-6 days after artificial inoculation with downy mildew and inducer treatments on grape seedlings cv. Taify under greenhouse conditions

Treatments	Concentration	Chitinase activity after days of artificial inoculation and inducer treatments under greenhouse conditions					Mean
		0	1	2	4	6	
<i>S. plicatus</i>	1×10 ⁸ CFU	6.98	7.38	5.59	6.13	5.54	6.32
<i>P. fluorescence</i>	1×10 ⁸ CFU	4.31	4.72	5.18	7.24	6.06	5.50
<i>T. harzianum</i>	1×10 ⁶ spore mL ⁻¹	6.89	7.10	7.75	8.42	8.96	7.82
Control (infected)	-	0.96	1.03	1.16	2.30	4.26	1.94
Control (healthy)	-	0.96	0.99	1.10	1.89	2.40	1.47

LSD at 5%: 1.12

Table 5: β -1, 3 glucanase activity 1 -6 days after artificial inoculation with downy mildew and inducer treatments on grape seedlings cv. Taify under greenhouse conditions

Treatments	Concentration	Chitinase activity after days of artificial inoculation and inducer treatments under greenhouse conditions					Mean
		0	1	2	4	6	
<i>S. plicatus</i>	1×10 ⁸ CFU	5.57	5.70	5.94	4.81	5.64	5.53
<i>P. fluorescence</i>	1×10 ⁸ CFU	4.49	5.41	5.62	3.98	6.01	5.10
<i>T. harzianum</i>	1×10 ⁶ spore mL ⁻¹	6.16	7.14	7.31	6.88	7.88	7.07
Control (infected)	-	1.83	2.03	2.21	2.59	3.23	2.38
Control (healthy)	-	1.83	1.90	2.03	2.56	2.74	2.21

LSD at 5%: 0.987

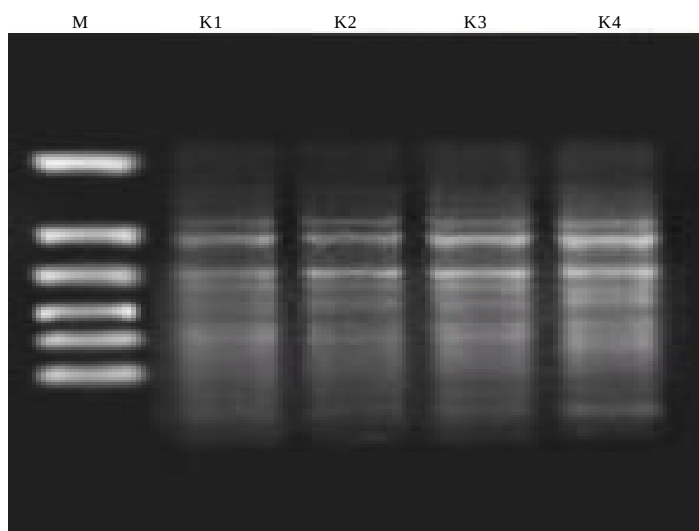


Fig. 2: SDS PAGE gel of grape after treatment with bio-elicitors; *S. plicatus* (K1), *P. fluorescence* (K2), *T. harzianum* (K3) and Control (K4)

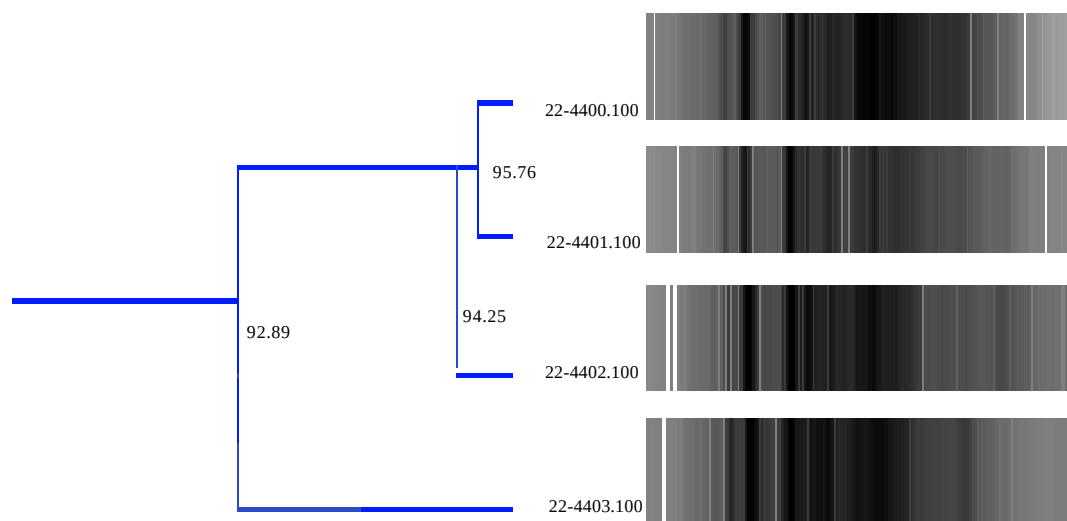


Fig. 3: Cluster analysis using gel documentation for SDS PAGE gel of grape after treated with bio-elicitors. Upper lane (400) *S. plicatus*, (401) *P. fluorescence*, (402) *T. harzianum* and (403) Control

DISCUSSION

Reduction in the severity of downy mildew disease of grapevine plants due to treatment with different biotic inducers is related to stimulation of induced resistance. Plants respond to pathogen attack or elicitor treatments by activating a wide variety of protective mechanisms designed to prevent pathogen invasion, replication and propagation (Malolepsza and Roaska, 2005).

The disease severity was decrease with all biotic elicitors especially *T. Harzianum* followed by *S. plicatus* and finally *P. fluorescence*, across a range of concentrations. The defense mechanisms induced by these elicitors may be the rapid production of antimicrobial secondary metabolites known as phytoalexins (Agrios, 2005; Kumar *et al.*, 2006) and possibly the activation and/or synthesis of defense peptides and proteins (Castro and Fontes, 2005; Lohani *et al.*, 2007). In various plant species, resistance can be induced with elicitors against a wide range of pathogens (Yao and Tian, 2005; Amin *et al.*, 2007). The inducers take the form of increased phenolic content and (PR) proteins such as peroxidase, polyphenoloxidase, chitinase and β -1,3-glucanase, these results are in agreement with those reported in Ferreira *et al.* (2004), Joseph and Jini (2010) and Alkahtani *et al.* (2011).

The application of elicitors increased the total chlorophyll and carotene content of grape seedlings. This increase may be due to stimulation of pigment formation and enhanced efficacy of photosynthetic apparatus thus generating a better potential for resistance and in addition a decrease in photophosphorylation rate usually occurring after infection (Chandra and Bhatt, 1998). Elicitors may increase the number of chloroplast per cell, number of cells per leaf and consequently leaf area (Possingham, 1980; Farouk, 2005; Farouk *et al.*, 2008).

All the changes in protein profile reflect the changes in structural and functional (enzymes) protein according to type of elicitors applied. These observed changes in protein expression may be related to acquired resistance against downy mildew. In this investigation the protein pattern differed according to the type of elicitor, as evidenced by the similarity calculations. In the case of

the grape seedlings treated with *S. plicatus* and *P. fluorescens* the protein profiles were highly similar (95.76%) suggesting a common mode of action. The treatment with *T. harzianum* displayed a lower level of similarity in comparison to both *S. plicatus* and *P. fluorescens* (94.25%), this may be due to their nature and mode of action also. Studies on protein profile have shown that proteins are probably the most important Pathogenesis-related (PR) factor and are likely to play a central role in induced resistance to downy mildew. SDS-PAGE gels stained with silver nitrate did not show any additional protein induction in the treated samples when compared to the control, these results are in agreement with those reported in Ryals *et al.* (1996), BeHcot *et al.* (2000) and Gautam and Stein (2011).

CONCLUSION

Many grape varieties grown throughout the world for the purposes of direct consumption, drying or beverage manufacturing, may be exposed to attack by many diseases; principal among these is the downy mildew pathogen *Plasmopara viticola*. Downy mildew may lead to a reduction in crop quantity and quality. There are several measures and protocols to control this disease which are dependent on the use of fungicides. In an effort to limit the use of fungicides in agriculture inducing elevated levels of natural resistance is seen as an effective alternative. In this investigation treatment with differed bio-elicitors increased the innate resistance of the plant and decrease downy mildew disease severity. In addition a number of biochemical changes were observed specifically elevated levels of oxidative enzymes and photosynthetic pigments. Furthermore significant differences in protein profile and enzyme activities was interpreted as evidence of improved pathogen resistance. Based on the results reported in this study the authors believe there is strong evidence for continued investigation and development of bio-elicitors as an effective tool for the protection of grape and other fruit crops against downy mildew and related pathogens.

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