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Identification of *Puccinia pimpinellae* on Anise Plant in Egypt and Its Control

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Abstract: An emerging problem for the wider adoption of anise plantation in Egypt is the damage caused by the rust fungus. The detailed description and taxonomic studies (using light and scanning electron microscopy) show that such an obligate parasite fungus (*Puccinia pimpinellae*) is autoecious microcyclic (uredinial-telial stage only). Among tested Apiaceae plants, the host range test proved the specificity of the rust fungus to anise. To the researcher's knowledge, this is the first investigated record of a rust fungus on *Pimpinella anisum* plants in Egypt. The effectiveness of some plant resistance elicitors and two active chitinase producers; *Bacillus subtilis* Bio4 and isolated *Trichoderma harizianum* CH_4 (both of them recorded the highest clear zone/colony size ratio on chitin agar plates) in controlling anise rust disease and on growth and yield of anise were evaluated in two successive growing seasons. Spraying chitosan at 1000 ppm was the most potent in reducing Disease Severity (DS) and Incidence (DI) as well as improving plant height, chlorophyll content, inflorescence No. plant^{-1} (74.2 and 76), 1000-fruit weight (2.94 and 2.83 g) and anise yield (646.8 and 670.0 kg fed^{-1}), during both seasons. *B. subtilis* Bio4 and *T. harizianum* CH_4 showed moderate effect on the tested parameters.

Key words: *Pimpinella anisum*, rust, *Puccinia pimpinellae*, biological control, elicitors, chitinase

INTRODUCTION

Anise (*Pimpinella anisum* L.) is an annual plant belongs to family Apiaceae. The plant is fragrant and widely used in medicine and as food flavoring (Chevallier, 1996). In Egypt, its cultivation has become more widespread in order to cover the increasing of medicinal industries and exportation needs. The most prevalent and destructive disease for anise is the rust which infects not less than 26% of seed lots. This rust was previously suggested to be *Puccinia pimpinellae* Mart. (Ghoneem, 2003).

Plants can be induced to develop enhanced resistance to wide range of microbial pathogen infections by treatment with a variety of biotic and abiotic inducers. Biotic inducers include infection by necrotizing pathogens and plant-growth promoting rhizobacteria and treatment with non pathogens or cell wall fragments. Abiotic inducers include safe chemicals which act at various points in the signaling pathways involved in disease resistance, as well as water stress, heat shock and pH stress. Resistance induced by these agents (resistance elicitors) is broad spectrum and long lasting (Hamiduzzaman *et al*., 2005; Walters *et al*., 2005).

Plant responds to pathogen attack or elicitor treatments by activating a wide variety of protective mechanisms designed to prevent pathogen replication and spreading (Malolepsza and Rózalska, 2005). The defense mechanisms including the fast production and stimulation the accumulation of signal molecule as jasmonic acid, salicylic acid, hydrogen peroxide, reactive oxygen species and protein kinesis, all of which play crucial roles in intracellular signaling pathways (De Gara *et al*., 2003) alterations in the cell wall constitution and accumulation of antimicrobial secondary metabolites known as phytoalexins (Heath, 2000; Agrios, 2005) plus the activation and/or synthesis of defense peptides and proteins (Castro and Fonts, 2005). Another role of elicitors is the induction of local and/or Systemic Acquired Resistance (SAR). This was reported in treated plants protecting against invasions of pathogen due to chitosan (a polymer of β-1,3 linked glucosamine) application (Sharathchandra *et al*., 2004).

Although few microbial species have been tested on *Puccini* species, the deleterious effect of fungicides on the environment has made biological control agents a suitable alternative to control fungal pathogens. Several strains of *B. subtilis* produce a variety of antibiotics by

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which plant pathogens are inhibited (Utkhede, 1984; Rytter *et al*., 1989). The mechanisms for the suppression of pathogens by *Trichoderma* include mycoparasitism, competition for space and resources and antibiosis. The extracellular cell wall degrading enzymes produced by many strains of *Trichoderma* are traditionally included in the concept of mycoparasitism (Abdullah *et al*., 2008). Furthermore, chitinases are well known for their ability to degrade fungal cell walls (Sridevi and Mallaiah, 2008).

To date there is no full description or certified taxonomic studies of the obligate anise rust fungus in Egypt. In this study, detection, description and full identification, as well as host range and improving growth and yield through biotic and abiotic resistance elicitors, were carried as a first full recordation of *Puccinia pimpinellae* on anise plants in Egypt.

MATERIALS AND METHODS

Source of anise seeds, chemical elicitors and microorganisms: Anise seeds were obtained from El-Mers Company, Egypt. The chemical elicitors; Kaolin (KA), Chitosan (CHI), Hydroquinone (HQ), Benzoic acid (BA), Tri-Sodium Orthophosphate (TSOP) and Potassium Sodium (+)-tartrate (PST) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

Strains of *Bacillus subtilis* (Bio1-Bio5) were kindly obtained from Biological Control Department, Pant Pathology Research Institute Agric. Res. Center, Egypt.

Seven *Trichoderma* species (Ch1-Ch7) were isolated (Ellis, 1971) from healthy anise phylloplane.

Identification of anise rust causative pathogen

Determination of pustule size: Samples of rust infected leaves were taken 8 and 14 weeks after the first appearance of the rust symptoms and examined under a stereoscopic microscope (6-50x magnification) to detect the forming uredinia and telia pustules and study their morphological characteristics. The sampled leaves were boiled in lactophenol:ethanol (1:2 v/v) solution for 3 min for fixation (Shipton and Brown, 1962). The length and width of 60 random pustules were measured using light microscopy; the pustules were measured for at least three leaves. The pustule size was calculated according to Lee and Shaner (1985) formula's:

$$\text{Pustule size} = \text{length (mm)} \times \text{width (mm)} \times B/4$$

Light microscopy observations: To examine morphology and structure of uredinia and telia, freshly infected materials specimens were sectioned freehand under a stereoscopic binocular microscope. Urediniospores and teliospores were scraped from the specimens and mounted in a drop of lacotphenol solution on a microscopic slide. For each specimen, 50 spores were randomly chosen and observed under an Olympus BH 100 microscope for the selected morphological features listed. Measurements were made with a Leica Q-Win Image Analyzer. To observe germ pores in urediniospores, the spores were placed in a drop of lactic acid on a microscopic slide, heated to boil for a few seconds and mounted with an additional drop of lacotphenol solution with aniline blue. The spores on the slide were smashed by applying gentle pressure over a cover slip on the preparation.

Scanning Electron Microscopy (SEM): Rust infected leaves from fresh specimens were marked, cut into ca. 3×3 mm pieces containing a few sori and preserved in glutaraldehyde solution (8% conc., Merk). Sample preparation was performed using the tissue processor model Lynexel, Leica. Where the leaves segments were fixed with osmium oxide and then dehydrated using a serial dilution of ethyl alcohol and finally by acetone. The processed samples were then dried using a critical point drier (EMS 850), coated with gold using a sputter coater (EMS 550). The samples were then examined at The Scanning Electron Microscope Unit, Zagazig Univ., Egypt, using JEOL T100 JSM, SEM.

Pathogenicity and host range tests: To test across infectivity, excised foliage bearing uredinia from anise plants collected were placed in a flask, flooded with distilled water, shaken vigorously for a few minutes and the suspension strained through four layers of cheesecloth. Urediniospores suspended in the filtrate were concentrated to 2.6×10^6 spores mL^{-1}, using the sedimentation technique. The suspension was misted onto 8 week old foliage of anise as well as various Apiaceae plants: (Dill; *Anethum graveolens* L., Celery; *Apium graveolens* L., Khella; *Ammi visnaga* L., Parsley; *Petroselinum crispum* (Mill.) Nym., Carrot; *Daucus carota* L., Coriander; *Coriandrum sativum* L., Cumin; *Cuminum cyminum* L., Caraway; *Carum carvi* L. and Fennel; *Foeniculum vulgare* Mill., which were expected to be hosts of *P. pimpinellae*) grown in 18 cm plastic pots containing clay loam soil until the foliage was completely wet. The plants were then each covered with a clear plastic bag and maintained in a glasshouse to avoid possible natural rust interactions. After 2 days, the bags were removed and the water-filled containers were placed around plants to maintain high ambient humidity. During the remaining 4 week experimental period, the temperature

and relative humidity ranged from 23 to 30°C and 60 to 80%, respectively. The plants were monitored daily for development of symptoms characteristics of rust symptoms.

Screening of microorganism for chitinase activity: The medium used for bacterial screening for chitinase activity had the following composition (g L^{-1}); chitin, 5; yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.3 and KH_2PO_4, 1.36. The pH of the medium was adjusted to 8.0 and sterilized at 121°C for 15 min (Monreal and Reese, 1969). The medium used for screening of fungi for chitinase activity had the following composition (g L^{-1}); chitin, 5; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.3; $(NH_4)_2SO_4$, 1.4; $CaCl_2.2H_2O$, 0.5; peptone, 0.5; urea, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4.7H_2O$, 0.0016; $ZnSO_4.7H_2O$, 0.0014 and $CoCl_2.2H_2O$, 0.002. The pH of the medium was adjusted to 6.0 and sterilized at 121°C for 15 min (Ulhoa and Peberdy, 1991). Colloidal chitin was prepared by the method of Hsu and Lockwood (1975). Each bacterium and fungus was individually inoculated at the centre of the chitin agar plate of the previous media and examined for a Clear Zone (CZ) around the colony after incubation at 30±2°C up to 3 days for bacteria and 5 days for fungi. The diameter of the CZ and Colony Size (CS) was measured. The strain showing the highest chitinase activity was selected based on the CZ/CS ratio (Cody, 1989).

Preparation of inocula: A water suspension of *B. subtilis* Bio4 was made from a 48 h culture maintained on nutrient agar slants. Bacterial density was standardized by adjusting of approximately 6×10^7 cfu mL^{-1}. Seven day old conidia of *T. harzianum* CH_4 grown on PDA plates were suspended in water to obtain 5×10^6 conidia mL^{-1}. These inocula were sprayed on anise in the field trials.

Field evaluation of the elicitors and bioagents against *P. pimpinellae*

Field trials: Under naturally infected plants at the experimental farm of Tag El-Ezz, Agric. Res. Station, Dakhlia, Egypt, field experiment was carried out during two successive growing seasons. Anise seeds were sown on 1st November in 2006/2007 and 2007/2008 seasons. Each plot was 3.5×1.5 m with four ridges per plot; each ridge has ten hill containing three plants per each. Developed plants were sprayed till dripping with individual chemical elicitor (CHI at 500 and 1000 ppm; KA at 5 and 15 g L^{-1}, BA at 5 and 10 mM and HQ, PST and TSOP at 10 and 15 mM) or the biocontrol microorganisms (*B. subtilis* Bio 4 or *T. harzianum* CH_4) as well as fungicide (Sumi-eight 5% EC), two times with 3 weeks intervals beginning from 6 weeks after sowing. Plants sprayed with tap water only served as check. All other agricultural practices were carried out according to the recommendation of Ministry of Agriculture, Egypt.

Rust disease assessment: Rust disease severity was recorded after complete appearance of rust symptoms by natural infection. The plants were rated for Disease Incidence (DI) as the presence or absence of *P. pimpinellae* infection (percentage of infected leaves on the plant) and Disease Severity (DS) as the severity percentage of disease damage. Five categories were suggested to estimated disease severity on rusted leaves using a scale in which 0, 1, 2, 3, 4 and 5 signified that 0, 1-10, 11-25, 26-50, 51-75 and 76-100% of the leaf surface was covered with pustules, respectively (Fig. 1). Disease severity was calculated as disease index percentage according to the formula adopted by James (1971) as follows:

Fig. 1: Disease severity index of anise leaves infected with *Puccinia pimpinellae*. 0 = Health, 1 = 1-10% infection, 2 = 11-25% infection, 3 = 26-50% infection, 4 = 51-75% infection and 5 = 76-100% infection (100% infection = complete kill)

$$\text{Disease index (\%)} = \frac{\text{Sum of } (n \times v)}{5 \times N} \times 100$$

Where:
n = No. of leaves in each category
v = Numerical value of each category
N = Total No. of leaves in samples

Determination of anise growth, its photosynthetic pigments and yield: After 14 week of sowing, anise plants were randomly selected from the middle part of each plot, leaving two rows from each side to avoid border effects, for the determination of plant height (cm), number of leaves and shoot dry weight plant^{-1} (g). At the same plant age, photosynthetic pigments i.e., chlorophyll (Chl) and carotenoids, were extracted from the 3rd upper leaf (Robinson and Britz, 2000) and measured spectrophotometrically at 452, 650 and 665 μm. The amount of Chl a, Chl b, total Chl and carotenoids were estimated by the equations of MacKinney (1941). After 16 weeks from sowing, the number of inflorescence plant^{-1} was determined. At the end of anise life cycle, 1000-fruit weight (g) and seeds yield (ton fed^{-1}) were recorded. The data was statistically analyzed as completely randomized plot designs with the statistical analysis software CoStat 6.4.

RESULTS AND DISCUSSION

Description of anise rust symptoms: Under the natural infection, rust symptoms initially appeared as small as tiny cream colored flecks on the lower surfaces of anise leaves. These flecks enlarged and formed light-brown or rust-colored sori surrounded by a yellow halo, which originated subepidermally but ruptured the host epidermis as sporulation proceeds. The infection extended to stem, flowering buds, inflorescence and fruit seeds (Fig. 2A-E). The severity of the symptoms increased at the beginning

Fig. 2: Anise rust (*Puccinia pimpinellae*); general view of (A) infected plant symptom, (B) close-up of infected inflorescence, (C) lower surface of leaf shows light-brown pustules with erupted spore masses surround by yellow halos, (D) infected stem and (E) magnified at 100X

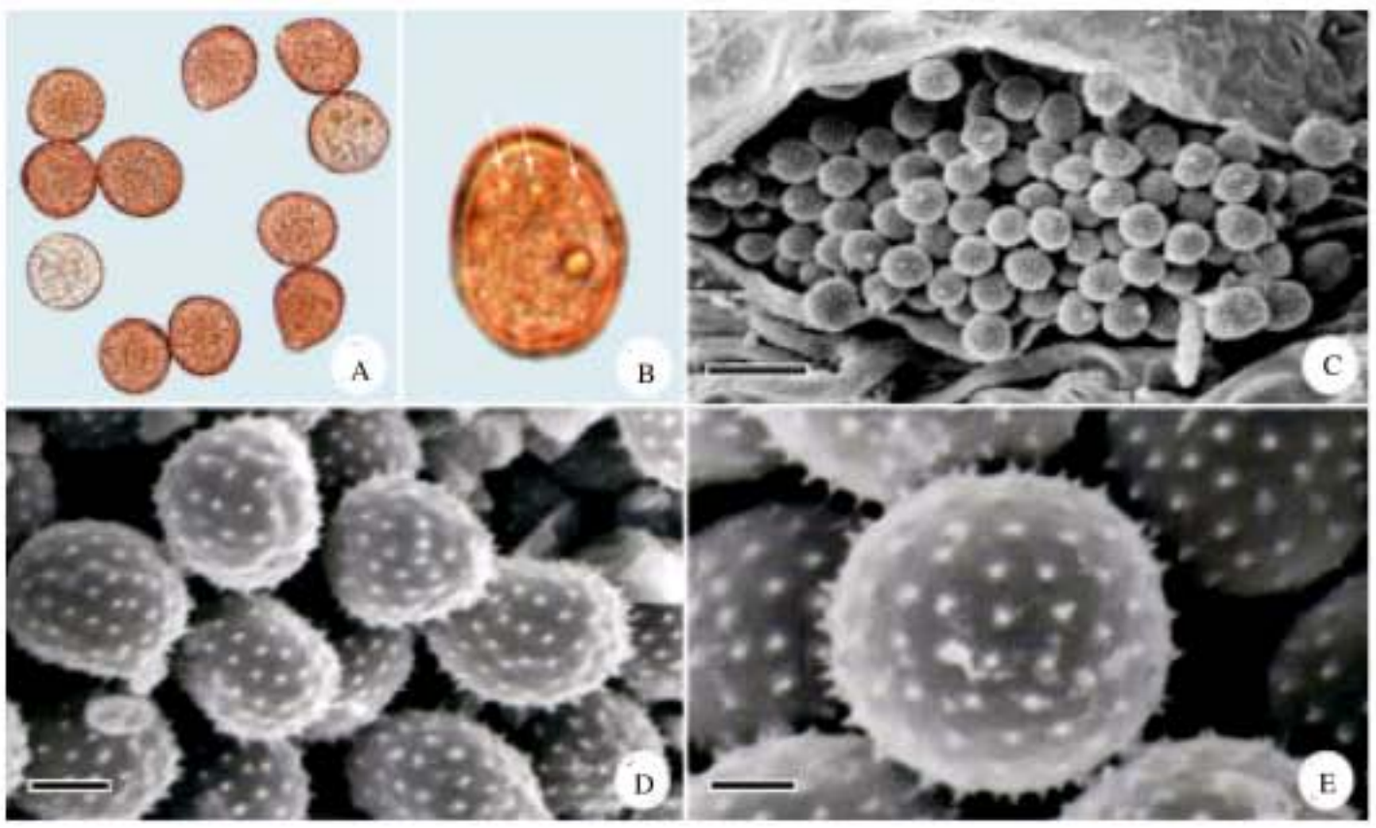

Fig. 3: Anise rust *Puccinia pimpinellae*: (A) urediospores mounted in water (x400), (B) urediniospores with three equatorial germ pores (arrows, x600), (C) SEM of pustules (uredina) showed urediniospores break through the epidermis and (D and E) SEM surface view of an uridinospores, echinulae are over the wall. Scales: C = 20 µm; D = 10 µm; E = 5 µm

of flowering stage which was parallel to the increase in temperature. The greatest number of pustules occurred on the underside of the leaf, although they may also occur on the upper leaf surface. Individual uredinial pustules are minute and brownish colored, but they often occur in groups or clusters which are more conspicuous than individual uredinia would be. Spores are readily released from the pustule and give a rusty appearance to anything they come in contact with.

Severe infection may cause leaves to curl upwards, dry up, turn brown and drop prematurely. Vegetative buds, stems and branches also may become infected and develop typical rust pustules. A severely damaged anise field often looks like it has been scorched. Flowering set, fruit fill and fruit size can be reduced if early infection is severe. Near the end of the season, pustules undergo a subtle change and form brownish-black winter spores (teliospores) that signify the end of the current infection cycles.

Light microscopy and scanning electron microscopy investigations: This is an autoecious and microcyclic rust species (uredial-telial in the life cycle). Spermogonia and aecia unknown. The microscopic investigation reveals that uredinia are 0.057-0.091 mm^2 (0.074 mm^2) in size, mostly hypophyllous, scattered, punctiform, minute, at first covered by the epidermis, later erumpent, pulverulent, cinnamon-brown; urediospores globose or subglobose-oblong and 23-31×22-27 µm in size. The walls were cinnamon-brown, uniformly echinulate and 2-3.5 µm thick at sides and up to 6 µm at the apex, with three equatorial germ pores (Fig. 3A-E).

Telia are 0.047-0.083 mm^2 (0.065 mm^2) in size, mostly hypophyllous or on stems, scattered, naked, surrounded by the torn epidermis, rounded on the leaves, elongated on the stems, sometimes aggregated and confluent in long patches up to 1 cm or longer, later naked, pulverulent, blackish-brown. The telia are also subepidermal in origin and become erumpent as teliospores are formed. Teliospores are formed within the uredinia or exclusively in the telia. The teliospores are two-celled mostly broadly ellipsoid, obovoid-ellipsoid or oblong-ellipsoid, rounded at both ends but less prominently round at the pore, slightly constricted at the septum and 30-43×19-27 µm in size. The walls are chestnut-brown, smooth and 2-3.5 µm thick at sides and up to 4 µm thick at the apex. One germ pore was located in each cell: upper pore apical, lower variable often near the pedicel. The pedicel was 6-16 µm (11 µm) long and basal, fragile, hyaline and persistent (Fig. 4A-D). Comparison of the observed characteristics of the fungus under discussion with the description and morphological characteristics of rust fungi lead to conclusion that this fungus taxonomically identical to *Puccinia pimpinellae*.

Host range of *P. pimpinellae*: After 6 days of artificial infection of Apiaceae family plants, typical rust symptoms were appeared as minute cream flecks appeared on only anise foliage while no symptoms were developed on the other tested plants of Apiaceae family. Within three to five days, these flecked areas expanded, erupted and

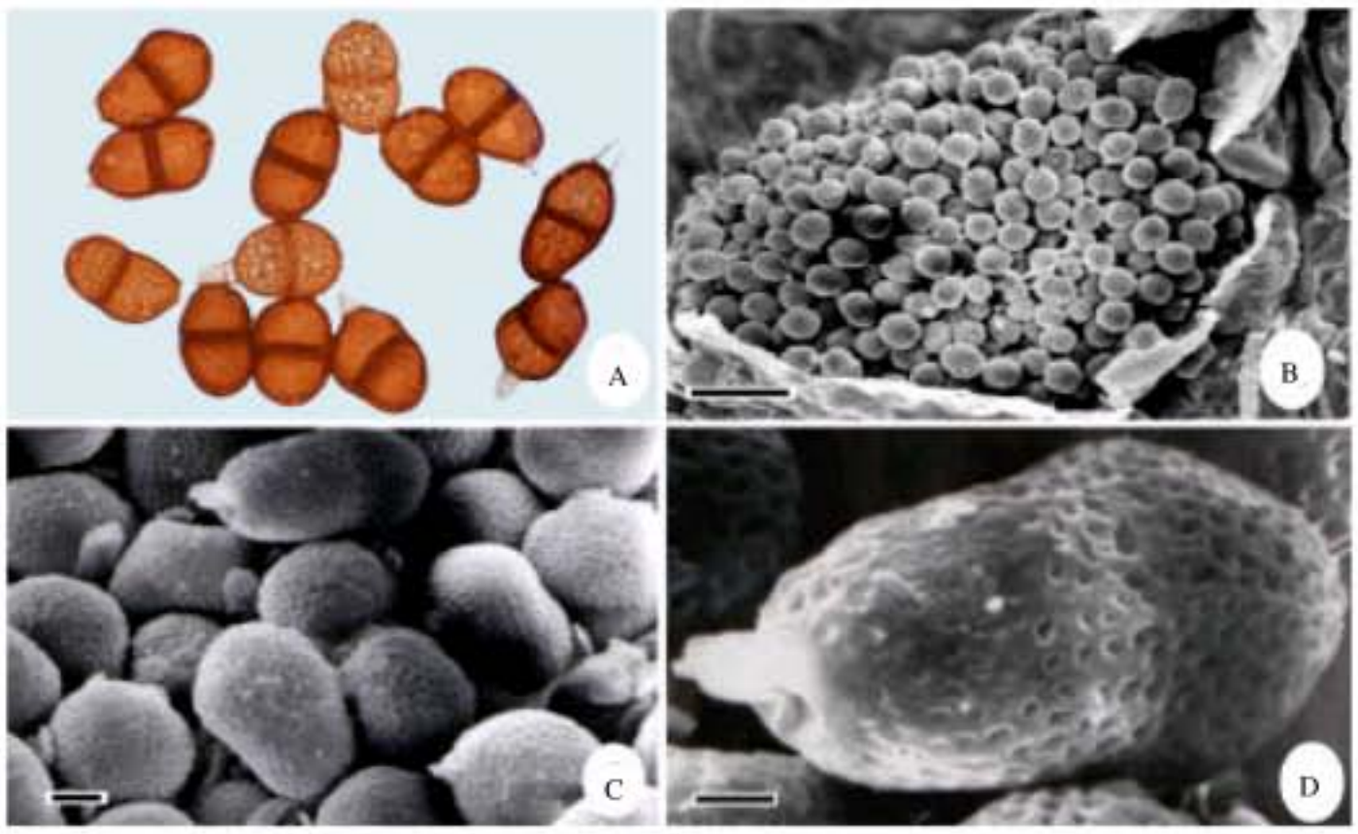

Fig. 4: Anise rust *Puccinia pimpinellae*: (A) teliospores mounted in water (x400), (B) SEM of pustules (telia) showing teliospores the break through the epidermis and (C and D) SEM surface view of an teliospores. Two cells are contained within smooth surface-spore. Scales: B = 8 µm; C = 10 µm; D = 5 µm

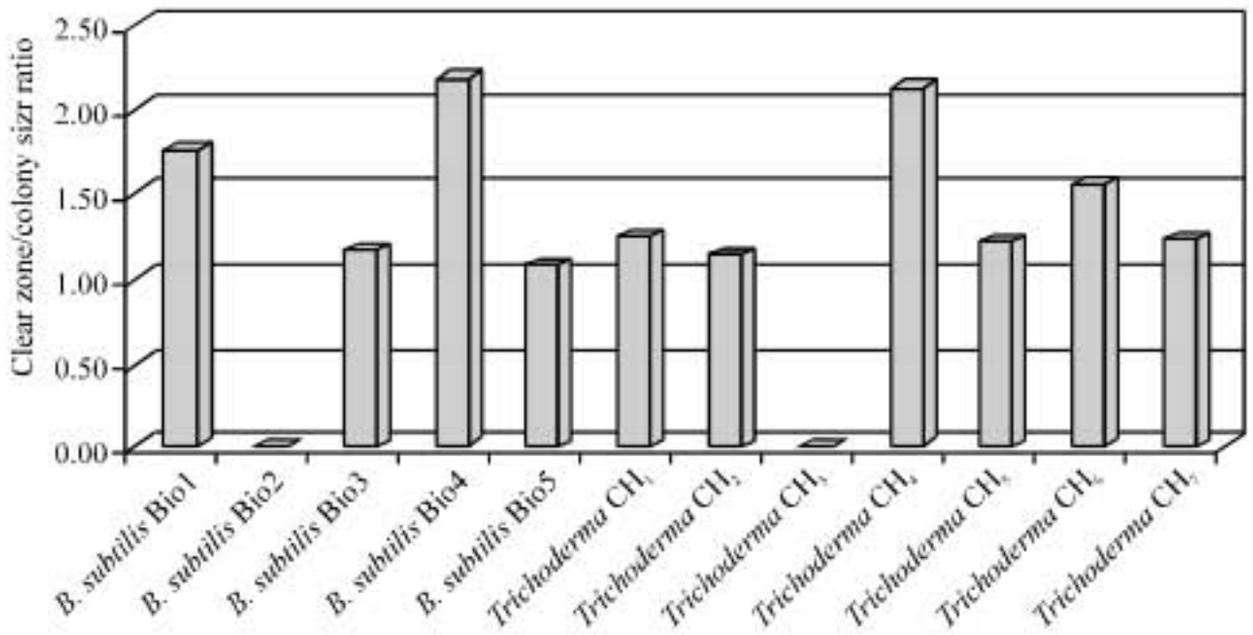

Fig. 5: Chitinase activity as a ratio of clear zone to colony size after 3 days for bacteria and 5 days for fungi

formed uredinia on the lower surface of the symptomatic foliage. The morphology and size range of the uredinia and urediniospores were the same as those of the *P. pimpinellae* applied in this test. This fungus was assumed to be specific on anise, given the negative results upon inoculation of the tested plants of Apiaceae family. The first record of *P. pimpinellae* on anise came from USA in 1960 (United States Department of Agriculture, 1960). Recently, Reichling and Bomme (2004), in UK, reported *P. pimpinellae* as rust causative pathogen of anise.

Chitinolytic activity of the bioagents: Chitin agar plates were used for screening chitinolytic activity of *B. subtilis* strains *Trichoderma* isolates. Each plate was observed for a chitinase activity as a clearing zone surrounding the colony of microorganism. Colonies of both bacteria and fungi showing zones of clearance on chitin agar plates were regarded as chitinase-producing. *Trichoderma* CH_4 and *B. subtilis* Bio4 exhibited chitinase activity on chitin agar plates (Fig. 5), CZ/CS ratio recorded 2.20 and 2.14, respectively. According to Ellis (1971), the fungal isolate was identified as *T. harzianum* CH_4. Both *B. subtilis* Bio4 and *T. harzianum* CH_4 were selected for the biological control of the obligate parasite rust; *P. pimpinellae* under field conditions.

Effect of biotic and abiotic elicitors on disease, growth and yield of anise in field: Since, the obligate parasite *P. pimpinellae* is newly detected in Egypt as rust

Table 1: Efficacy of elicitors and biological treatment applied to anise leaves on rust disease development under natural infection

		1st season				2nd season			
		8 week		14 week		8 week		14 week	
Treatments	Conc.	DS	DI	DS	DI	DS	DI	DS	DI
Check		23.26	48.86	61.44	55.31c	20.47	41.80	50.40	52.49
Sumi-eight 5% EC	0.175 ml L^{-1}	2.62	6.50	8.12	24.76	2.60	6.00	5.68	27.18
	0.35 mL 10 L^{-1}	1.60	2.51	5.44	20.32	1.76	2.34	4.68	26.52
Kaolin	5 g L^{-1}	19.72	30.05	31.87	34.27	12.79	35.45	27.00	31.42
	15 g L^{-1}	10.29	22.19	22.42	29.63	8.45	22.48	16.00	33.73
Chitosan	500 ppm	13.64	27.90	30.42	45.43	10.71	26.89	25.74	52.25
	1000 ppm	7.34	15.50	11.58	26.82	5.87	13.90	8.72	25.25
Hydroquinone	10 mM	10.88	19.26	28.58	57.17	9.40	18.11	23.96	39.18
	15 mM	9.36	16.22	19.62	42.50	8.78	15.73	19.47	36.80
Benzoic acid	5 mM	10.47	19.33	26.58	37.04	12.43	18.99	24.79	38.44
	10 mM	9.00	27.08	16.70	35.01	8.30	25.44	12.30	37.86
Tri-Sod. orthrophosphate	10 mM	16.31	31.12	39.33	47.41	14.69	29.43	29.70	46.76
	15 mM	12.60	28.72	30.48	33.08	11.26	27.77	21.66	32.30
Pot. Sod. (+)-tartrate	10 mM	9.10	12.47	20.61	30.80	7.51	11.93	16.30	29.56
	15 mM	15.63	21.10	31.55	52.92	12.90	18.23	30.10	51.31
B. subtilis	6×10^7 cfu mL^{-1}	17.55	23.66	29.64	49.40	14.74	26.84	25.42	30.69
T. harizianum	5×10^6 spore mL^{-1}	19.93	35.06	46.00	59.16	17.24	35.74	35.34	58.07
LSD at $p\leq0.05$		5.35	13.00	8.31	18.31	3.83	12.71	8.56	17.41

Mean is the average of five replicates

causative pathogen on anise plants and because of the ordinary fungicides it is not recommended for the medicinal plants, such as anise. The following investigation is a trial for replacing biotic (*B. subtilis* Bio4 and *T. harizianum* CH_4) and abiotic elicitors with the ordinary fungicide; Sumi-eight 5% EC, which is already applied in controlling the majority of rust diseases in Egypt.

Disease development of rust: The follow up of DS and DI during the two growing seasons (Table 1) show that foliar application of CHI at 1000 ppm is the most effective among all tested biotic and abiotic elicitors in reducing DS and DI. The reduction of DS, in comparison to check treatment, reached 68.4, 81.2, 71.3 and 82.7% after 8 and 14 weeks of anise sowing during the 1st and 2nd seasons, respectively. Benzoic acid at 10 mM came next in this respect.

CHI was reported to inhibit germination and growth of several fungi. CHI reduces the germination of uredospores of *P. aruchidis* by complete inhibition of all the RNA synthesis (Hadwiger *et al*., 1986) by sensitizing the plant to respond more rapidly to a pathogen attack through a combination of isoforms of chitinases and glucanases that may affect the growth of *P. uruchidis* in the intercellular space (Sathiyabama and Balasubramanian, 1998) and CHI may be referred as hydrophobic materials, thus creating a low water potential on infected leaves which prevent spore germination, infection and growth of the pathogens (Hsieh and Huang, 1999). The present inhibitory action of CHI on anise rust causative *P. pimpinellae* is another example.

Significant reductions in DS were recorded by *B. subtilis* Bio4 and *T. harizianum* CH_4, especially on the long term (14 week) of both seasons, compared with the check treatment. *B. subtilis* Bio4 was found to be more effective than *T. harizianum* CH_4. *B. subtilis* was reported to inhibit spore germination and reduce the incidence of rust pustules on inoculated geranium leaves in the greenhouse. The inhibitory agent was present in its culture filtrate (Rytter *et al*., 1989). On the other hand, Govindasamy and Balasubramanian (1989) reported the ability of *T. harzianum* conidial suspensions to inhibit the germination and germ tube growth of urediospore suspensions of *P. arachidis* of groundnut, a phenol-like antifungal compound inhibitory to *P. arachidis* which was isolated from the germination fluid of *T. harzianum*. Also, chitinase produced by *T. harzianum* showed antifungal activity against a wide range of fungal species (Nampoothiri *et al*., 2004).

Growth attributes of anise: After 14 weeks from sowing, the response of anise growth to the foliar application of elicitors and biocontrol agents was determined by means of measuring height, number of leaves and shoot dry weight of anise plants. Data of both seasons (Table 2) revealed that the majority of elicitors significantly increased growth parameters to different extents. In this respect CHI at 1000 ppm came at the top of other elicitors in increasing plant height. The number of leaves per plant was maximized by the application of 15 mM TSOP in the 1st season and 1000 ppm CHI in the 2nd season. Finally, PST recorded the highest significant increment of shoot dry weight (12.39 and 12.86 g) in both seasons.

Table 2: Anise growth as affected by elicitors and biological treatments under natural infection by *P. pimpinellae* after 14 week from sowing

		Plant height (cm)		Leaves No. plant^{-1}		Shoot dry weight (g plant^{-1})	
Treatments	Conc.	1st season	2nd season	1st season	2nd season	1st season	2nd season
Check		52.20	55.9	39.2	35.2	8.89	9.27
Sumi-eight 5% EC	0.175 ml L^{-1}	61.20	64.5	51.4	56.0	11.19	11.73
	0.35 ml L^{-1}	60.00	62.9	49.8	54.2	11.23	11.46
Kaolin	5 g L^{-1}	62.20	60.7	49.6	53.0	10.75	10.84
	15 g L^{-1}	61.60	65.9	53.4	59.4	11.65	12.01
Chitosan	500 ppm	60.20	60.0	46.2	50.0	11.25	11.56
	1000 ppm	65.80	70.0	59.4	64.0	12.12	12.67
Hydroquinone	10 mM	60.00	66.3	56.8	63.0	11.60	12.04
	15 mM	61.20	65.4	58.8	63.2	11.89	12.12
Benzoic acid	5 mM	59.00	62.2	53.2	57.8	11.32	11.80
	10 mM	62.00	66.0	55.0	59.4	12.03	12.36
Tri-Sod. orthrophosphate	10 mM	58.00	59.2	49.4	49.4	11.50	10.82
	15 mM	62.00	59.8	62.2	60.2	11.80	11.43
Pot. Sod. (+)-tartrate	10 mM	64.80	66.7	56.4	61.8	12.39	12.86
	15 mM	58.60	60.2	51.0	54.0	11.67	11.95
B. subtilis	6×10^{7} cfu mL^{-1}	57.60	59.1	52.2	55.0	10.24	11.32
T. harizianum	5×10^{6} spore mL^{-1}	55.90	56.8	45.4	47.2	9.19	9.85
LSD at p≤0.05		5.85	6.5	7.6	6.7	0.73	0.62

Mean is the average of five replicates

Table 3: Variation in photosynthetic pigments (mg g^{-1} fresh weight) of treated anise under natural infection by *P. pimpinellae* after 14 week from sowing

Treatments	Conc.	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids
Check		1.26	1.19	2.55	0.116
Sumi-eight 5% EC	0.175 ml L^{-1}	2.14	1.68	3.75	0.172
	0.35 ml L^{-1}	1.60	1.49	3.19	0.129
Kaolin	5 g L^{-1}	1.30	1.09	2.47	0.114
	15 g L^{-1}	1.96	1.82	3.78	0.114
Chitosan	500 ppm	1.90	1.67	3.57	0.189
	1000 ppm	2.63	2.24	4.88	0.192
Hydroquinone	10 mM	1.59	1.41	2.99	0.128
	15 mM	1.71	1.55	3.39	0.447
Benzoic acid	5 mM	1.48	1.35	2.84	0.145
	10 mM	2.11	1.78	3.90	0.178
Tri-Sod. orthrophosphate	10 mM	2.23	0.81	3.04	0.117
	15 mM	2.63	1.50	3.23	0.116
Pot. Sod. (+)-tartrate	10 mM	2.06	1.76	3.83	0.137
	15 mM	1.99	1.65	3.65	0.128
B. subtilis	6×10^{7} cfu mL^{-1}	1.52	1.32	2.81	0.112
T. harizianum	5×10^{6} spore mL^{-1}	1.43	0.78	2.71	0.198
LSD at p≤0.05		0.51	0.40	0.65	NS

NS: Not significant, Mean is the average of three replicates

However, neither *B. subtilis* Bio4 nor *T. harizianum* CH_4 exerted any significant impact on anise growth except increasing the number of leaves per plant during the second season. These increases may be attributed to the act of elicitors which have effects on the physiological processes in plants such as ion uptake, cell elongation, cell division, enzymatic activation and protein synthesis (Shakirova *et al*., 2003; Amin *et al*., 2007).

Photosynthetic pigments of anise: In the second season only, photosynthetic pigments of anise were determined simultaneously with growth attributes after 14 weeks from sowing. Among the tested elicitors (Table 3), spraying CHI at 1000 ppm on anise plants significantly increased leaf content of Chl a (2.63 mg g^{-1}) and Chl b (2.24 mg g^{-1} fresh weight) consequently, total Chl. It is obvious to note that these increases were superior to those treated with the fungicide. The statistical analysis of the data reveals non significant differences among treatments in carotenoids. This increment may be due to stimulating pigment formation and enhancing the efficacy of photosynthetic apparatus with a better potential for resistance and decrease in photophosphorylation rate usually occurring after infection. On the other hand, none of the tested microbes recorded any significant variation in photosynthetic pigments by comparison.

Anise yield: Data of Table 4 show that the tested inducers and bioagents which were applied to control *P. pimpinellae*, have direct effect on anise net yield. Spraying anise plants with 1000 ppm of CHI significantly improved inflorescence No. plant^{-1} (72.4 and 76), 1000-fruit weight (2.94 and 2.83 g) and anise yield (646.8 and 670.0 kg fed^{-1}), in both seasons. Among the bioagents, *B. subtilis* was better than *T. harizianum* in all tested parameters. The role of CHI in increasing anise yield may

Table 4: Inflorescence number, 1000-fruit weight and anise yield as influenced by biotic and abiotic elicitors under natural infection by *P. pimpinellae*

		*Inflorescence No. plant^{-1}		1000-fruit weight (g)		Fruits yield (kg fed^{-1})	
Treatments	Conc.	1st season	2nd season	1st season	2nd season	1st season	2nd season
Check		37.8	40.4	2.00	2.11	265.0	300.0
Sumi-eight 5% EC	0.175 ml L^{-1}	60.0	63.0	2.53	2.40	556.6	572.8
	0.35 ml L^{-1}	58.0	61.2	2.62	2.48	487.4	502.4
Kaolin	5 g L^{-1}	53.4	59.2	2.15	2.39	400.0	380.2
	15 g L^{-1}	61.8	65.8	2.69	2.52	524.6	560.4
Chitosan	500 ppm	50.6	56.8	2.19	2.03	475.2	505.2
	1000 ppm	74.2	76.0	2.94	2.83	646.8	670.0
Hydroquinone	10 mM	59.8	61.4	2.56	2.42	528.8	557.2
	15 mM	56.8	59.2	2.51	2.62	502.4	541.4
Benzoic acid	5 mM	63.6	60.2	2.40	2.22	450.8	497.4
	10 mM	67.6	70.2	2.89	2.77	609.2	641.4
Tri-Sod. orthrophosphate	10 mM	48.6	52.2	2.40	2.15	362.6	411.6
	15 mM	51.8	57.4	2.44	2.21	424.0	455.8
Pot. Sod. (+)-tartrate	10 mM	65.2	68.0	2.86	2.69	589.6	625.0
	15 mM	55.6	53.8	2.41	2.02	432.6	461.2
B. subtilis	6×10^{7} cfu mL^{-1}	51.8	57.0	2.59	2.22	494.4	514.4
T. harizianum	5×10^{6} spore mL^{-1}	44.6	47.8	2.10	2.02	353.2	365.2
LSD at p≤0.05		10.3	9.1	0.33	0.42	78.5	88.0

*Data of inflorescence number were recorded after 16 week from sowing, Mean is the average of five replicates

be due to its interaction with the cellular DNA, leading to stimulation of physiological processes and multiple biochemical reactions in the plant followed by active translocation of the photoassimilate. This in turn leads to increasing photosynthetic pigments and growth parameters and consequently dry matter accumulation (Hadwiger *et al*., 1986; Umar and Bansal, 1995). Moreover, rust infection causes considerable losses in anise yield, so, any treatment that protects anise plants from this infection directly increases its yield.

Since the growth parameters and photosynthetic pigments were well built, that was a parallel with the reduction in DS and DI. The effects of CHI suggest its potential as a protectant against *P. pimpinellae*, which finally serves to increase the net yield of such medicinal plants. Treatment of anise with abiotic and/or biotic inducers may reduce the input of fungicides against *P. pimpinellae*.

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