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Full Length Research Paper

Identification of newly detected *Puccinia pimpinellae* on anise plant in egypt and its control using biotic and abiotic elicitors in relation to growth and yield

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An emerging problem for the wider adoption of anise plantations 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 authors' knowledge, this is the first investigated record of a rust fungus on *P. anisum* plants in Egypt. The effectiveness of some plant resistance elicitors and two active chitinase producers, i.e. *Bacillus subtilis* Bio4 and *Trichoderma harzianum* Ch4 (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 was 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. harzianum* Ch4 showed a moderate effect on the tested parameters.

Key words: *Pimpinella anisum,* rust, *Puccinia pimpinellae,* biological control, biotic and abiotic elicitors, chitinase.

INTRODUCTION

Anise (*Pimpinella anisum* L.) is an annual plant and belongs to the family *Apiaceae*. The plant is fragrant and widely used in medicine and as food flavorant (Chevallier, 1996). In Egypt, its cultivation has become more widespread in order to cover the increasing 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* (Ghoneem, 2003).

Plants can be induced to develop enhanced resistance to a 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).

Plants respond to pathogen attack or elicitor treatments by activating a wide variety of protective mechanisms designed to prevent pathogen replication and spreading (Malolepsza and Rózalaska, 2005). The defense mechanisms include the fast production and accumulation of signal molecule such 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

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cell wall constitution and accumulation of antimicrobial secondary metabolites known as phytoalexins (Heath, 2000; Agrios, 2005), as well as 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. 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 *Bacillus subtilis* produce a variety of antibiotics by 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 work, 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 record of *P. 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, i.e. 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 *B. subtilis* (Bio1 – Bio5) were kindly obtained from Biological Control Department, Pant Pathology Research Institute Agricultural Research 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 to study their morphological characteristics. The sampled leaves were boiled in a 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 the formula of Lee and Shaner (1985):

Pustule size = length (mm) x width (mm) $\times \pi/4$.

Light microscopy observations

To examine the morphology and structure of uredinia and telia, freshly infected materials specimens were sectioned free-hand 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. 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 x 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. The leave segments were then fixed with osmium oxide and 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) and coated with gold using a sputter coater (EMS 550). The samples were examined at The Scanning Electron Microscope Unit, Zagazig University, Egypt, using a JEOL T100 JSM scanning electron microscope.

Pathogenicity and host range tests

To test for across-infectivity, excised foliage bearing uredinia from anise plants 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 four-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 characteristic of rust symptoms.

Screening of microorganism for chitinase activity

The medium used for screening of bacterial chitinase activity had the following composition (gL^{-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

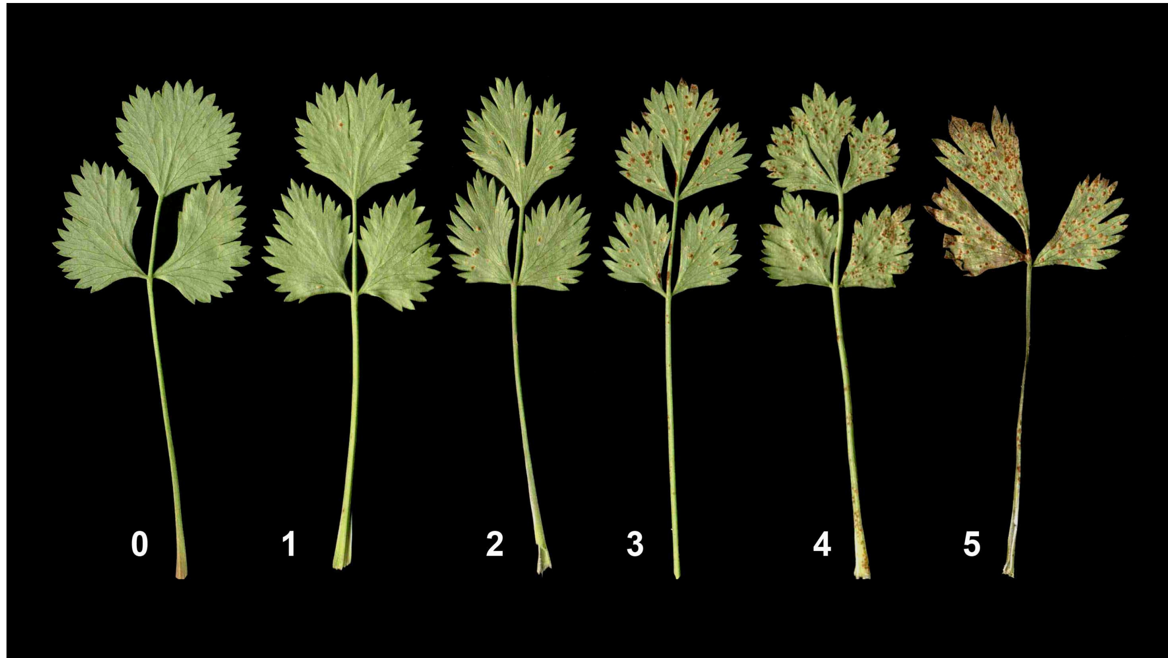

Figure 1. Disease severity index of anise leaves infected with *Puccinia pimpinellae*. 0, Health; 1, 1-10% infection; 2, 11-25% infection; 3, 26-50% infection; 4, 51-75% infection; and 5, 76- 100% infection (100% infection=complete kill).

fungi for chitinase activity had the following composition (gL^{-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 medium 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 to approximately 6×10^7 cfu ml^{-1}. The 7-day old conidia of *Trichoderma harzianum* Ch4 grown on PDA plates were suspended in water to obtain $5x10^6$ 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, Agricultural Research Station, Dakhlia, Egypt, the field experiment was carried out during two successive growing seasons. Anise seeds were sown on first of November in the 2006/2007 and 2007/2008 seasons. Each plot was 3 × 3.5 m with four ridges per plot; each ridge had ten hill containing three plants per each. Developed plants were sprayed until dripping with the individual chemical elicitor (CHI at 500 and 1000 ppm; KA at 5 and 15 gL^{-1}, BA at 5 and 10mM and HQ, PST and TSOP at 10 and 15 mM) or the biocontrol microorganisms (*B. subtilis* Bio 4 or *T. harzianum* Ch4), as well as fungicide (Sumi-eight 5% EC), two times with three-week intervals beginning from sixth week after sowing. Plants sprayed with tap water only served as controls. All other agricultural practices were carried out according to the recommendation of the 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 estimate 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 (Figure 1). Disease severity was calculated as disease index percentage according to the formula adopted by James (1971):

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

Where: n = Number of leaves in each category, v = Numerical value of each category and N = Total number of leaves in samples

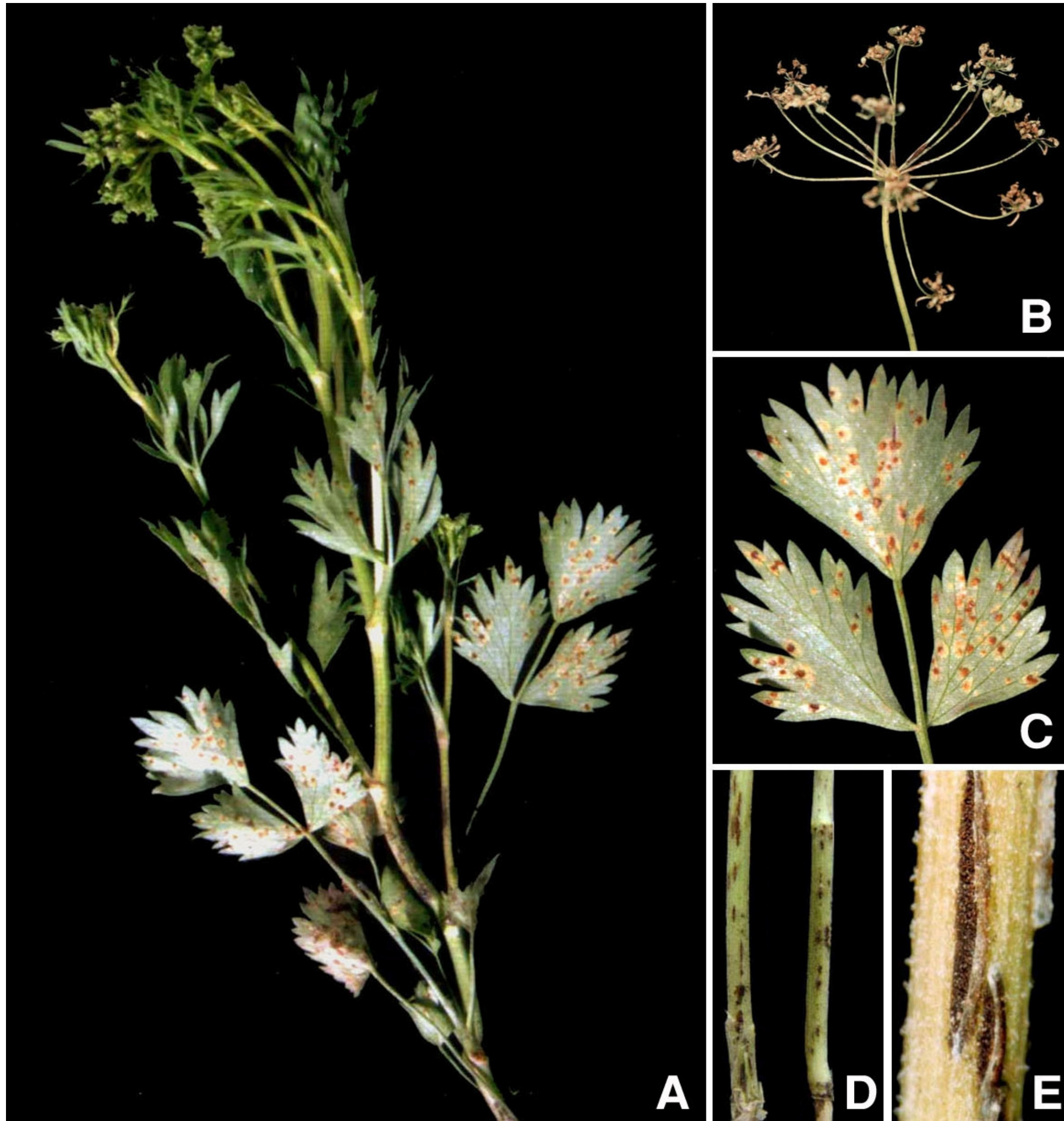

Figure 2. Anise rust (*Puccinia pimpinellae*). General view of infected plant symptoms (A), close-up of infected inflorescence (B), lower surface of leaf showing light- brown pustules with erupted spore masses surrounded by yellow halos (C), infected stem (D) and magnified at 100X (E).

Determination of anise growth, its photosynthetic pigments and yield

Fourteen (14) weeks after 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 third upper leaf (Robinson and Britz, 2000), and measured spectrophotometrically at 452, 650 and 665 nm. The amount of Chl a, Chl b, total Chl and carotenoids were estimated by the equations of Mackinney (1941). At 16 weeks after sowing, the number of inflorescence plant^{-1} was determined. At the end of the anise life cycle, 1000-fruit weight (g) and seeds yield (ton fed^{-1}) were recorded. The data were statistically analyzed as completely randomized plot designs with the statistical analysis software CoStat 6.4.

RESULTS AND DISCUSSION

Description of anise rust symptoms

Under natural infection, rust symptoms initially appeared as small 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 proceeded. The infection extended to the stem, flowering buds, inflorescence and fruit seeds (Figure 2). The severity of the symptoms increased at the beginning of the flowering stage, which was parallel to the increase in temperature. The greatest number of

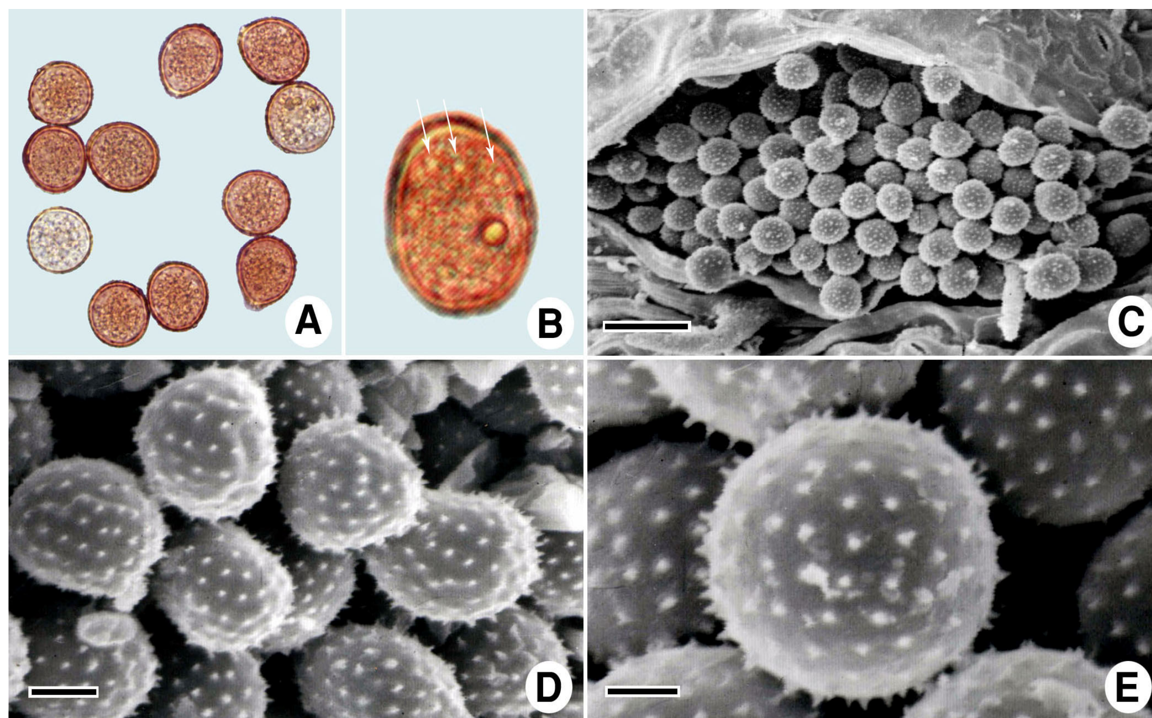

Figure 3. Anise rust *Puccinia pimpinellae*. (A) Urediospores mounted in water (X400), (B) urediniospores with three equatorial germ pores (arrows, X600), (C) SEM of pustules (uredina) showing urediniospores breaking through the epidermis and (D and E) SEM surface view of uridinospores, echinulae are over the wall. Bars: C, 20 µm; D, 10 µm; E, 5 µm.

pustules occurred on the underside of the leaf, although they may also occur on the upper leaf surface. Individual uredinial pustules were minute and brownish-colored, but they often occurred in groups or clusters that were more conspicuous than individual uredinia. Spores were readily released from the pustule and gave a rusty appearance to anything that they came into 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 had 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 cycle.

Light microscopy and scanning electron microscopy investigations

This is an autoecious and microcyclic rust species (uredial-telial in the life cycle). Spermogonia and aecia are 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 were globose or subglobose-oblong and 23-31 X 22-27 µm in size. The walls were cinnamon-brown, uniformly echinulate, and 2-3.5 µm thick at the sides and up to 6 µm at the apex, with three equatorial germ pores (Figure 3).

Telia were 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 were also subepidermal in origin and became erumpent as teliospores were formed. Teliospores were formed within the uredinia or exclusively in the telia. The teliospores were 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 X 19-27µm in size. The walls were chestnut-brown, smooth, and 2-3.5 µm thick at the 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 (Figure 4). Comparison of the observed characteristics of the fungus under discussion with the description and morphological characteristics of rust fungi leads to conclusion that this fungus was taxonomically identical to *P. pimpinellae*.

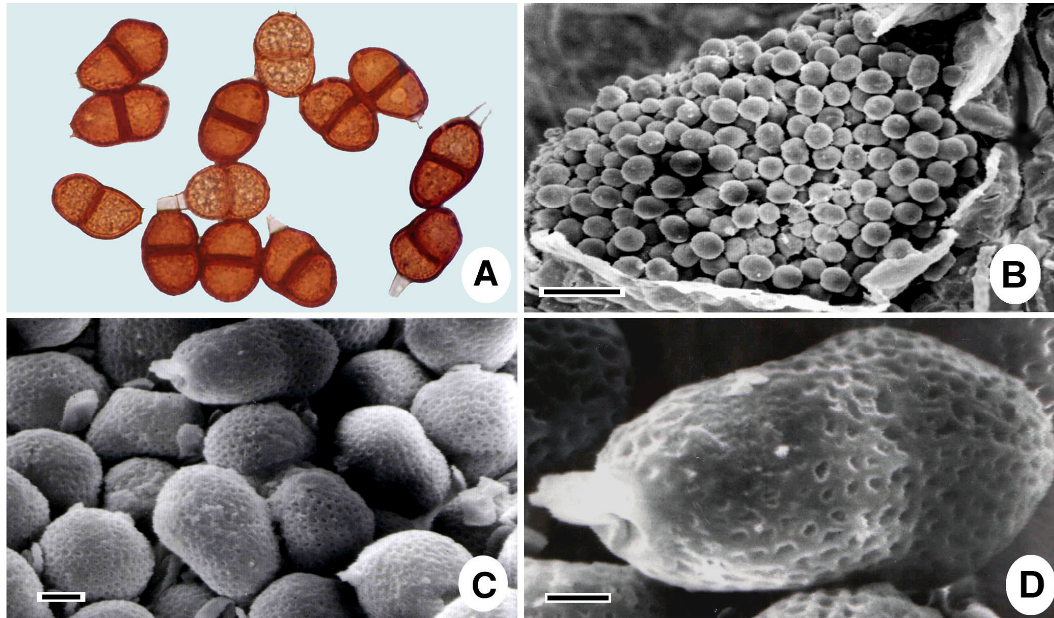

Figure 4. Anise rust *Puccinia pimpinellae*. (A) Teliospores mounted in water (X400), (B) SEM of pustules (telia) showing teliospores breaking through the epidermis and (C and D) SEM surface view of teliospores. Two cells are contained within a smooth surface-spore. Bars: B, 8 µm; C,10 µm; D, 5 µm.

Host range of *P. pimpinellae*

After six days of artificial infection of *Apiaceae* family plants, typical rust symptoms appeared as minute cream flecks on only anise foliage, while no symptoms developed on the other tested plants of *Apiaceae* family. Within three to five days, these flecked areas expanded, erupted and 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 the *Apiaceae* family. The first record of *P. pimpinellae* on anise came from USA in 1960 (USDA, 1960). Recently, Reichling and Bomme (2004) reported *P. pimpinellae* as the rust causative pathogen of anise in the UK.

Chitinolytic activity of the bioagents

Chitin agar plates were used for screening chitinolytic activity of *B. subtilis* strains and *Trichoderma* isolates. Each plate was observed for a chitinase activity as a clearing zone surrounding the colony of the microorganism. Colonies of both bacteria and fungi showing zones of clearance on chitin agar plates were regarded as chitinase-producing. *Trichoderma* Ch4 and *B. subtilis* Bio4 exhibited chitinase activity on chitin agar plates (Figure 5) and CZ/CS ratios of 2.20 and 2.14, respectively, were recorded. According to Ellis (1971), the fungal isolate was identified as *T. harzianum* Ch4. Both *B. subtilis* Bio4 and *T. harzianum* Ch4 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

The obligate parasite *P. pimpinellae* is newly detected in Egypt as rust causative pathogen on anise plants and ordinary fungicides are not recommended for use on medicinal plants, such as anise. Therefore, the following investigation was undertaken as a trial to determine whether biotic (*B. subtilis* Bio4 and *T. harzianum* Ch4) and abiotic elicitors can be used to replace 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) showed 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 the control treatment, reached 68.4 and 81.2 and 71.3 and 82.7% after 8 and 14 weeks of anise sowing during the first and second seasons, respectively. Benzoic acid at 10 mM came next in this respect.

CHI has been reported to inhibit germination and

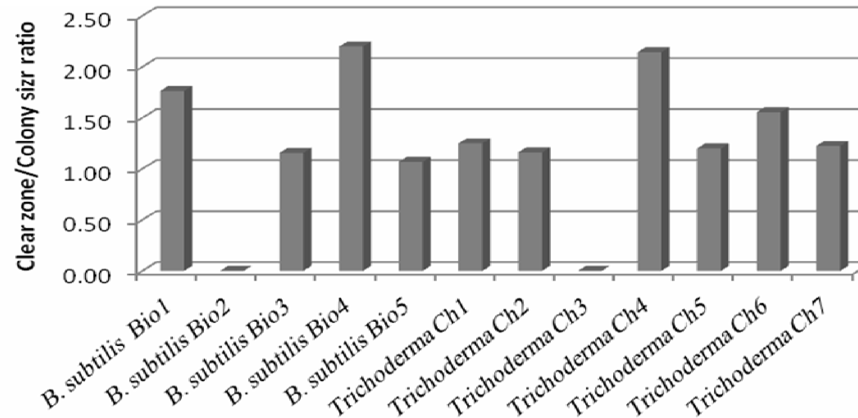

Figure 5. Chitinase activity as a ratio of clear zone to colony size after three days for bacteria and five days for fungi.

Table 1. Efficacy of biotic and abiotic elicitors applied to anise leaves on rust disease development under natural infection.

Treatment	Conc.	1st season				2nd season			
		8 week		14 week		8 week		14 week	
		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
Fungicide	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	5g L^{-1}	19.72	30.05	31.87	34.27	12.79	35.45	27.00	31.42
	15g 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	$6x10^7$cfu ml^{-1}	17.55	23.66	29.64	49.40	14.74	26.84	25.42	30.69
T. harzianum	$5x10^6$ spore ml^{-1}	19.93	35.06	46.00	59.16	17.24	35.74	35.34	58.07
LSD at *P*<=0.05		5.35	13.00	8.31	18.31	3.83	12.71	8.56	17.41

*Mean is the average of five replicates.

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 to as a hydrophobic material, 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. harzianum* Ch4, especially in the long term (14 weeks) of both seasons. *B. subtilis* Bio4 was

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

Treatment	Conc.	Plant height (cm)		Leaves No. $plant^{-1}$		Shoot dry weight(g $plant^{-1}$)	
		1st season	2nd season	1st season	2nd season	1st season	2nd season
Check		52.2*	55.9	39.2	35.2	8.89	9.27
Fungicide	0.175 ml L^{-1}	61.2	64.5	51.4	56.0	11.19	11.73
	0.35 ml L^{-1}	60.0	62.9	49.8	54.2	11.23	11.46
Kaolin	5g L^{-1}	62.2	60.7	49.6	53.0	10.75	10.84
	15g L^{-1}	61.6	65.9	53.4	59.4	11.65	12.01
Chitosan	500 ppm	60.2	60.0	46.2	50.0	11.25	11.56
	1000 ppm	65.8	70.0	59.4	64.0	12.12	12.67
Hydroquinone	10 mM	60.0	66.3	56.8	63.0	11.60	12.04
	15 mM	61.2	65.4	58.8	63.2	11.89	12.12
Benzoic acid	5 mM	59.0	62.2	53.2	57.8	11.32	11.80
	10 mM	62.0	66.0	55.0	59.4	12.03	12.36
Tri-Sod. Orthrophosphate	10 mM	58.0	59.2	49.4	49.4	11.50	10.82
	15 mM	62.0	59.8	62.2	60.2	11.80	11.43
Pot. Sod. (+)-tartrate	10 mM	64.8	66.7	56.4	61.8	12.39	12.86
	15 mM	58.6	60.2	51.0	54.0	11.67	11.95
B. subtilis	$6x10^7$cfu ml^{-1}	57.6	59.1	52.2	55.0	10.24	11.32
T. harzianum	$5x10^6$ spore ml^{-1}	55.9	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

found to be more effective than *T. harzianum* Ch4. *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* 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

At 14 weeks after 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 second season. Finally, PST recorded the highest significant increment of shoot dry weight (12.39 and 12.86 g) in both seasons. However, neither *B. subtilis* Bio4 nor *T. harzianum* Ch4 exerted any significant impact on anise growth, except increasing in number of leaves per plant during the second season. These increases may be attributed to the action 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 with 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) and 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 revealed non-significant differences among treatments in carotenoids. This increment may be due to stimulating pigment formation and enhancing the efficacy of the photosynthetic apparatus with a better potential for resistance and decrease in photophosphorylation rate usually occurring after infection (Amaresh and Bhatt, 1998). On the other hand, none of the tested microbes recorded any significant variation in photosynthetic pigments by comparison.

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

Treatment		Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids
Check		1.26*	1.19	2.55	0.116
Fungicide	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	5g L^{-1}	1.30	1.09	2.47	0.114
	15g 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	$6x10^7$cfu ml^{-1}	1.52	1.32	2.81	0.112
T. harzianum	$5x10^6$ spore ml^{-1}	1.43	0.78	2.71	0.198
LSD at *P*<=0.05		0.51	0.40	0.65	NS

*Mean is the average of three replicates.

Table 4. Inflorescence number, 1000-fr0uit weight and anise yield as influenced by biotic and abiotic elicitors under natural infection by *P. pimpinellae*

Treatment	Conc.	*Inflorescence No. $plant^{-1}$		1000-Fruit weight(g)		Fruits yields (kg $Fed.^{-1}$)	
		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
Fungicide	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	5g L^{-1}	53.4	59.2	2.15	2.39	400.0	380.2
	15g 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	$6x10^7$cfu ml^{-1}	51.8	57.0	2.59	2.22	494.4	514.4
T. harzianum	$5x10^6$ 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

*Number of Inflorescence were recorded after 16 week from sowing
**Mean is the average of five replicates

Anise yield

Data in Table 4 showed that the tested inducers and bio-agents that were applied to control *P. pimpinellae* had a 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. harzianum* in all tested parameters. The role of CHI in increasing anise yield may 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 photo assimilate. This, in turn, may lead 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.

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

REFERENCES

Abdullah MT, Ali NY, Suleman P (2008). Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary with Trichoderma *harzianum* and Bacillus amyloliquefaciens. Crop Protection, 27: 1354-1359.

Agrios GN (2005). Plant Pathology. 5th Edition. Academic Press, San Diego, USA.

Amaresh C, Bhatt RK (1998). Biochemical and physiological response to salicylic acid in reaction to systemic acquired resistance. Photosynthetica, 35: 255-258.

Amin AA, Rashad EL-Sh M, EL-Abagy HMH (2007). Physiological effect of indole-3-butyric acid and salicylic acid on growth, yield and chemical constituents of onion plants. J App. Sci. Res., 3: 1554-1563.

Castro MS, Fontes W (2005). Plant defense and antimicrobial peptides. Protein and Peptide Letters., 12: 11-16.

Chevallier A (1996). The Encyclopedia of Medicinal Plants. A DK Publishing Book. New York p. 250.

Cody RM (1989). Distribution of Chitinase and Chitobiase in Bacillus. Current Microbiol, 19: 201-205.

De Gara L, de Pinto MC, Tommasi F (2003). The antioxidant systems vis-à-vis reactive oxygen species during plant pathogen interaction. Plant Physiol. Biochem., 41: 863-870.

Ellis MB (1971). Dematiaceous Hyphomycetes. Commonwealth Res. Mycological Institute, Kew, England.

Ghoneem KMI (2003). Seed-borne diseases of common medicinal plants in Egypt. PhD dissertation, Fac. Agric. University of Mansoura, Egypt.

Govindasamy V, Balasubramanian R (1989). Biological control of groundnut rust, *Puccinia arachidis* by *Trichoderma harzianum*. J. Plant Dis. Prot., 96: 337-345.

Hadwiger LA, Kendra DF, Fristensky BW, Wagoner N (1986). Chitosan both activates genes in plants and inhibits RNA synthesis in fungi. In Chitin in Nature and Technology. eds R. A. Muzzarelli, C. Jeuniaux and G. W. Gooday, Plenum Press, New York. pp. 209-214.

Hamiduzzaman MM, Jakab G, Barnavon L, Neuhaus JM, Mauch-Mani B (2005). β-Aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. Molecular Plant-Microbe Interactions, 18: 819–829.

Heath M (2000). Hypersensitive response related death. Plant Mol. Biol., 44: 321-334.

Hsieh TF, Huang JW (1999). Effect of film-forming polymers on control of lily leaf blight caused by *Botrytis elliptica*. Eur. J. Plant Pathol., 105: 501-508.

Hsu SC, Lockwood JL (1975). Powdered chitin agar as a selective medium for enumeration of Actinomycetes in water and soil. Appl. Microbiol., 29: 422-426.

James C (1971). An illustrated series of assessment keys for plant diseases, their preparation and usage. Can. Pl. Dis. Surv., 51: 39-65.

Lee TS, Shaner G (1985). Oligogenic inheritance of length of latent period in six slow leaf-rusting wheat cultivars. Phytopathol., 75: 636–643.

Mackinney G (1941). Absorption of light by chlorophyll solution. J. Biol. Chem., 140: 315-322.

Malolepsza U, Rózalaska S (2005). Nitric oxide and hydrogen peroxide in tomato resistance. Nitric oxide modulates hydrogen peroxide level in O.hydroxyethylorutin-induced resistance to *Botrytis cinerea* in tomato. Plant Physiol Biochem., 43: 623-635.

Monreal J, Reese ET (1969). The chitinase of *Serratia marcescens*. Canadian J. Microbiol., 15: 689-696.

Nampoothiri KM, Baiju TV, Sandhya C, Sabu A, Szakacs G, Pandey A (2004). Process optimization for antifungal chitinase production by *Trichoderma* harzianum. Process. Biochem., 38: 1583-1590.

Reichling J, Bomme U (2004). Cultivation and plant material of the genus pimpinella. In: Jodral, M.M (ed.): Illicium, Pimpinella and Foeniculum. Vol. 40. Medicinal and Aromatic Plants-Industrial Profiles Series, CRC Press, UK. p. 118.

Robinson JM, Britz SJ (2000). Tolerance of a field grown soybean cultivars to elevated ozon level is concurrent with higher leaflet ascorbic acid level, higher ascorbate-dehydroascorbate redox status and long term photosynthetic productivity. Photosynthetic Research, 64: 77-87.

Rytter JL, Lukezic FL, Craig R, Moorman GW (1989). Biological control of geranium rust by *Bacillus subtilis*. Phytopathol., 79: 367-370.

Sathiyabama M, Balasubramanian R (1998). Chitosan induces resistance components in *Arachis hypogaea* against leaf rust caused by *Puccinia arachidis* Speg. Crop protection, 17: 307-313.

Shakirova FM, Sakhabutdinova AR, Bezrukova MV, Fatkhutdinova RA, Fatkhutdinova DR (2003). Changes in the hormonal status of wheat seedlings induced by salicylic acid and salinity. Plant Sci., 164: 317-322.

Sharathchandra RG, Niranjan RS, Shetty NP, Amruthesh KN, Shekar SH (2004). A chitosan formulation Elexa induces downy mildew disease resistance and growth promotion in pearl millet. Crop Prot., 23: 881-888.

Shipton WA, Brown JF (1962). A whole leaf clearing and staining technique to demonstrate host-pathogen relationships of wheat stem rust. Phytopathology, 52: 1313.

Sridevi M, Mallaiah KV (2008). Factors effecting chitinase activity of *Rhizobium* sp.from *Sesbania sesban*. Biologia, 63: 307-312.

Ulhoa CJ, Peberdy JF (1991). Regulation of chitinases in *Trichoderma harzianum*. J General Microbiol., 137: 2163-2169.

Umar S, Bansal SK (1995). Potassium requirement of mustard (*Brassica juncea* L.) under moisture stress conditions. Plant Physiol. and Biochem., New Delhi, 22(2): 130-135.

USDA (United States Department of Agriculture) (1960). Index of plant diseases in the United States. Agricultural Research Service. Agric. Handbook. 165. USGPO. Washington, DC.

Utkhede RS (1984). Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum.* Can. J. Bot., 62: 1032-1035.

Walters D, Walsh D, Newton A, Lyon G (2005). Induced Resistance for Plant Disease Control: Maximizing the Efficacy of Resistance Elicitors. Phytopathol., 95: 1368-1373.