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Effect of GI chitosan on biochemical parameters of pea leaves infected by *Erysiphe pisi*

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Abstract
Powdery mildew caused by *Erysiphe pisi* is one of the major devastating disease affecting the pea crop. In order to bring residue free peas, there is need to manage these diseases through eco-friendly measures such as by using the chitosan which is antifungal as well as induces resistance in plants. GI chitosan is a deacetylated derivative of chitin obtained by gamma irradiation of chitosan. Present experiment was conducted to assess the effect of GI chitosan on biochemical parameter i.e. Phenols, β-1,3-glucanase and chitinase present in powdery mildew infected pea leaves. The highest content of phenols, β-1,3-glucanase, and chitinase were observed when two sprays of GI chitosan @75 ppm were given at 30 and 40 DAS. The results indicate that there were highest contents of phenols (1.56 and 2.85 mg /g respectively), β-1,3-glucanase (1.97 and 2.76 n moles of D glucose min^{-1} mg^{-1} protein respectively), and chitinase (2.01 and 2.89 mg N- acetylglucosamine released g^{-1} soluble protein h^{-1} respectively) as compared to control. Phenols, β-1,3-glucanase and chitinase was found increased in the GI chitosan treatment which are the defence activators of the pathogen. Thus, preventive sprays of GI chitosan reduce the powdery mildew intensity in pea.

Keywords: pea, phenols, β-1,3-glucanase, chitinase, gamma irradiation, chitosan, *Pisum sativum*, *Erysiphe pisi*

Introduction
Pea (*Pisum sativum* L.) is a valuable vegetable crop all over the world. It belongs to family Leguminoceae (Trebuchet *et al.*, 1953) [27]. Pea represents a wide range of agricultural and horticultural uses. In India, it is cultivated for dry seeds as well as for green pods as vegetables. Production and productivity of pea has been very low because of various reasons *viz*. poor germination, inadequate use of fertilizers, incidence of diseases and pests, etc. Powdery mildew of pea is one of the severe diseases of pea. The loss is proportionate to the disease intensity and varies considerably depending on the stage of plant growth. In 100 % infected crop the reduction in pod numbers is estimated to about 21-31 % and reduction in pod weight about 24-27 % (Singh, 1987) [23].

For managing these diseases farmers are spraying fungicides, which are toxic to human health. Since the green peas are directly consumed, it leads to many health problems. In order to bring residue free peas there is need to manage these diseases through eco-friendly measures by using the chitosan which is antifungal as well as induces resistance in plants. This has no residual effect on any beneficial organism.

Chitosan is an organic natural biopolymer modified from chitin, which is the main structural component of squid pens, cell walls of some fungi and shrimp and crab shells (Suchada *et al*., 2010) [25]. Chitin is the second most abundant polymer in nature after cellulose (Cohen-Kupiec and Chet, 1998) [7]. Chitosan is comprised of 2-acetamido-2-deoxy-b-D-glucose (N-acetyl-D-glucosamine) and 2-amino-2-deoxy-b-D-glucan (D-glucosamine) attached via b-(1, 4) linkages (Austin *et al*., 1981; Tsigos *et al*., 2000) [1, 28] to form a high molecular weight (MW) biopolymer that is non-toxic and biodegradable. The amine group derived positive charges on chitosan at pH of greater than 6.0 is largely responsible for conferring the diverse and unique physiological and biological properties of chitosan (Fukuda, 1980) [10]. It has been shown to modulate plant disease (Rodriguez *et al*., 2007; Falcon-Rodriguez *et al*., 2011) [19, 9], phytoalexin production and reactive oxygen species (ROS) generation (Lee *et al*., 1999) [13], induce cell wall lignification (Pospieszny and Zielinska 1997; Vander *et al*., 1998) [17, 29]. Low molecular weight chitosan obtained by irradiation treatment, *viz*. microwave, UV, gamma rays etc.

tissues and which induces a hypersensitive reaction as a consequence of oxidative microburst and phenolic compound.

Materials and Methods

Source of isolate

Powdery mildew infected leaves sample of pea plants treated with GI chitosan were collected from field of Vegetable Improvement Project, National Agriculture Research Project, Ganeshkhind, Pune-67.

Chitosan

The Gamma irradiated chitosan was kindly provided by Vasantdada Sugar Institute, Manjari, Pune which was prepared by irradiating normal chitosan with electron Beam 100 KGy dose at BRIT, BARC, Mumbai.

Estimation of phenols, β-1,3-glucanase and chitinase by following methods

Phenols

Total phenol estimation can be carried out with the Folin-Ciocalteau reagent. The assay of Phenols was carried out as per the method described by Sadasivam and Manickam (2008) [20].

Principle: Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

Materials: 1. Ethanol 80 %, 2. Folin-Ciocalteau Reagent, 3. Na_2CO_3, 20 %, 4. Standard (100 mg Catechol in 100 ml Water), 5. Dilute 10 times for a working standard

Procedure

1. Weigh exactly 0.5-1.0 g of the sample and grind it with a pestle and mortar in 10-times volume of 80 % ethanol. 2. Centrifuge the homogenate at 10,000 rpm for 20 mins. Save the supernatant. Re-extract the residue with five times the volume of 80 % ethanol, centrifuge and pool the supernatants. 3. Evaporate the supernatant to dryness. 4.

Dissolve the residue in a known volume of distilled water (5 ml). 5. Pipette out different aliquots (0.2-2 ml) into test tubes. 6. Make up the volume in each tube to 3 ml with water. 7. Add 0.5 ml of Folin-Ciocalteau reagent. 8. After 3 min, add 2 ml of 20 % Na_2CO_3 solution to each tube. 9. Mix thoroughly. Place the tubes in a boiling water for exactly one min, cool and measure the absorbance at 650 nm against reagent blank. 10. Prepare a standard curve using different concentrations of catechol.

Calculations

From the standard curve find out the concentration of phenols in the test sample and express as mg phenols/100 g material.

2. β-1,3-glucanase activity

The assay of β-1,3-glucanase was carried out as per the method described by Rakshit *et al*. (2000) [18].

Reagents

1. 0.2 M Tris-HCl buffer (pH 7.5): 50 ml of 0.4 M Tris solution was mixed with 40 ml of 0.4 M HCl and the volume made to 100 ml with distilled water. 2. 0.1 M potassium acetate buffer (pH 5.2): 21 ml of 0.1 M acetic acid was mixed with 79 ml of 0.1 M potassium acetate (-9.81 g in 1000 ml). 3. Laminarin (0.1 % w/v): 0.1 % in 0.1 M potassium acetate buffer (pH 5.2)

Enzyme Extraction

The fresh leaf samples 0.2 g were washed in sterile distilled water and blotted with filter paper and macerated with chilled 2 ml of 0.2 M Tris-HCl buffer (7.5) in pre-chilled morter and pestle. The homogenate was centrifuged at 11,000X g at 4^0 C for 30 min and the supernatant was used as the crude enzyme extract.

Protein estimation

The protein contained in the crude enzyme extract was estimated according to the method of Bradford (1976) [4].

Enzyme assay

One ml reaction mixture contained 950 µl of Laminarin and 50 µl of crude enzyme extract was incubated at 37^0 C for 30 min and the reducing sugar released in to the solution at the end of reaction was estimated by Nelson-Somogyi method and the absorbance was read at 520 nm. The β-1,3-glucanase activity was expressed as n-moles of glucose released mg^{-1} protein min^{-1}.

Chitinase activity

The chitinases activity was estimated according to the methods described by Thimmaiah (1999) [26] and Giri *et al*. (1998) [11].

Reagents

1. 0.1 M sodium citrate buffer (pH 5.0): 70 ml of 0.1 M citric acid and 130 ml of 0.1M sodium citrate were mixed together. 2. Colloidal chitin: Chitin (1.83 mg) was dissolved in 1 ml of sodium acetate buffer (pH 5.2). 3. 0.1 M sodium acetate buffer (pH 5.2): 2.1 ml of 0.1 M acetic acid and 7.9 ml of 0.1 M sodium acetate were mixed together. 4. Standard 0.4 M N-acetyl glucosamine solution- 100 mg of N-acetyl glucosamine. Dissolved in 100 ml water which acts as a stock solution. From this stock solution10 ml was diluted to 100 ml with distilled water. 1 ml of this working solution contains 100 µg of N-acetyl glucosamine.

Procedure

Fresh pea leaf samples (0.25 g) were macerated with 6 ml of 0.1 M sodium citrate buffer in pre-chilled mortar and pestle. The homogenate was centrifuged at 10,000X g for 10 min at 10^0C and the supernatant was used as crude source of chitinase.

For the assay, 1 ml of supernatant, 4 ml of chitin suspension containing 15 mg of BSA were boiled in water bath at 37 ^{0}C for 3 hr. One ml water and one ml reaction mixture were boiled in centrifuge tube for 10 min and was centrifuged. An aliquot of 0.5 ml was taken for the estimation of N-acetylglucosamine as per the method of Nelson-Somogyi's (Somogyi1952). The protein content in the crude enzyme extract was estimated according to the method of Lowry *et al.* (1951) [14]. The chitinase activity was expressed as mg of N-acetylglucosamine released g^{-1} soluble protein min^{-1}.

Table 1: Influence on phenol, β-1,3-glucanase and chitinase enzymes, due to application of GI chitosan at 30 and 40 DAS

Sr. No.	Treatments	Phenols (mg/g)				β-1,3-glucanase (n moles of D glucose min^{-1} mg^{-1} protein)				Chitinase (mg N-acetylglucosamine released g^{-1} soluble protein h^{-1})			
		35 DAS	(%) Increase over control	45 DAS	(%) Increase over control	35 DAS	(%) Increase over control	45 DAS	(%) Increase over control	35 DAS	(%) Increase over control	45 DAS	(%) Increase over control
1	Foliar spray of GI chitosan @ 50 ppm at 30 DAS	1.39	57.95	1.60	68.42	1.50	67.67	1.78	79.80	1.79	101.12	2.00	104.08
2	Foliar spray of GI chitosan @ 75 ppm at 30 DAS	1.55	76.14	1.79	88.42	1.79	98.89	1.99	101.01	2.00	124.72	2.11	115.31
3	Foliar spray of GI chitosan @ 50 ppm at 40 DAS	0.89	1.14	1.42	49.47	0.98	8.89	1.48	49.00	0.88	-1.12	1.56	59.18
4	Foliar spray of GI chitosan @ 75 ppm at 40 DAS	0.90	2.27	1.57	65.26	0.97	7.78	1.68	69.70	0.90	1.12	1.78	81.63
5	Foliar sprays of GI chitosan @ 50 ppm at 30 and 40DAS	1.40	59.09	2.21	132.63	1.89	110.00	2.11	113.13	1.79	101.12	2.57	162.24
6	Foliar sprays of GI chitosan @ 75 ppm at 30 and 40DAS	1.56	77.27	2.85	200.00	1.97	118.89	2.76	178.79	2.01	125.84	2.89	191.00
7	Seed treatment GI chitosan @ 50 ppm	1.19	35.23	1.37	44.21	1.18	31.11	1.33	34.34	1.35	51.69	1.44	46.94
8	Seed treatment GI chitosan @ 75 ppm	1.22	38.64	1.55	63.16	1.33	47.78	1.47	48.48	1.53	71.91	1.63	66.33
9	Foliar sprays of wettable sulphur @ 0.2% at 30 and 40 DAS	1.37	55.68	1.67	75.79	1.48	64.44	1.79	80.81	1.77	98.88	2.04	108.16
10	Control	0.88	0.00	0.95	0.00	0.90	0.00	0.99	0.00	0.89	0.00	0.98	0.00
SE (m) ±		0.01		0.02		0.01		0.01		0.01		0.01	
CD (5%)		0.02		0.05		0.02		0.03		0.03		0.03	

Results and Discussion

Phenols, β-1,3-glucanase and chitinase activities in pea leaves after application of GI chitosan at 30 and 40 DAS

The phenols, β-1,3-glucanase and chitinase enzyme's activity was estimated twice after application of different GI chitosan treatments at 30 and 40 DAS. The data pertaining to enzymatic activities of these enzymes are presented in Table 1

Phenols

The maximum amount of phenols was present in GI chitosan @ 75 ppm sprayed at 30 and 40 DAS (1.56 and 2.85 mg/g, respectively) and was 77.2 per cent and 200.00 per cent, respectively increased over control. The amount of phenols was more in seed treatment of GI chitosan @ 75 ppm for 1 hr (1.55 mg/g) and was 63.16 per cent increased over control. The minimum amount of phenols was present in control (0.95 mg/g). Sprayings of different GI chitosan concentrations improves the phenols content after 35 and 45 DAS in pea, the results are in conformity with the work of Singh (2016) who reported that improvement in phytochemical content in spinach plant, where he reported that the foliar spray of chitosan at a concentration 0.01 mg/ml able to cause an increase in enzymatic (peroxidase, catalase and phenylalanine ammonium lyase) and non-enzymatic (total phenolics, flavonoids and proteins) defensive metabolites.

β-1,3-glucanase

The maximum amount of β-1,3-glucanase was present in GI chitosan @ 75 ppm sprayed at 30 and 40 DAS (1.97 and 2.76 n moles of D glucose min^{-1} mg^{-1} protein, respectively) it was 118.89 and 178.79 per cent, respectively increased over control. The amount of β-1,3-glucanase was present in seed treatment of GI chitosan @ 75 PPM for 1 h (1.47 n moles of D glucose min^{-1} mg^{-1} protein), it was 48.48 per cent increased over control. The minimum amount of β-1,3-glucanase was present in treatment control (0.99 n moles of D glucose min^{-1} mg^{-1} protein). The treatment differences were statistically significant over absolute control. The results are matching with the earlier findings of Simmons (1994) [21] and Buchner *et al.* (2002) [5] where they reported that β-1,3-glucanase is generally induced in response to pathogen attack. The results are also in agreement with reports of earlier workers Katoch *et al.* (2004) [15] where they reported that maximum enhancement of β-1,3-glucanase activity in pea resistant cultivar than susceptible cultivars. Here in present investigation sprayings of GI chitosan induced the resistance in pea.

Chitinase

The maximum amount of chitinase was present in GI chitosan @ 75 ppm sprayed at 30 and 40 DAS (2.01 and 2.89 mg N-acetylglucosamine released g^{-1} soluble protein h^{-1} respectively) it was 125.84 and 191.00 per cent, respectively increased over control. The amount of chitinase was found increased in seed treatment of GI chitosan @ 75 PPM for 1 h (1.63 mg N- acetylglucosamine released g^{-1} soluble protein h^{-1}) and it was 66.33 per cent increased over control. The minimum amount of chitinase was present in control (0.98 mg N- acetylglucosamine released g^{-1} soluble protein h^{-1}). Earlier scientists Aziz *et al*. (2006) [2] studied the effect of chitosan on grapevine leaves and found that application of chitosan leads to marked induction of chitinase. The results are also in agreement with the earlier workers Zhang *et al.* (1998) [31], Boon-Ek (2013) [3], Wang *et al.* (2013) [30] and Landi (2014) [12] where they reported that in pepper, raspberry, and strawberry plants chitosan was found to be effective in inducing plant defense mechanisms due to the higher level of expression of chitinase and β-1,3 glucanase genes.

Conclusion

Phenols, β-1,3-glucanase and chitinase activities were found increased in the GI chitosan treatment which are the defense activators of the pathogen. Thus, preventive sprays of GI chitosan reduces the powdery mildew intensity in pea.

Application of GI chitosan during active crop growth stage was helpful in building of defense mechanism in pea plant and reducing the powdery mildew intensity.

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