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Synergistic Effect of Plant Defense Elicitors and Biocontrol Agents on Induction of Defense Enzymes in Pea against Downy Mildew

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ABSTRACT

Background: Plant defense against the pathogens can be induced by using different defense inducers. Plants can be treated with elicitors for fast and more intense mobilization of defense responses which can enhance the resistance against biotic or abiotic stresses.

Methods: The present study has been undertaken to evaluate the synergistic effect of different plant defense inducing chemical (Salicylic acid, Isonicotinic acid, Oxalic acid and Chitosan) and biological (*Trichoderma harzianum* and *Pseudomonas fluorescens*) elicitors. Enzyme activity was expressed as the increase in absorbance using spectrophotometer.

Result: Among all the treatments the maximum PAL activity (35.58 mg/g of fresh weight) was found in case of oxalic acid but after 48 hrs its activity reduced drastically. Next to oxalic acid *Pseudomonas fluorescens* (31.38 mg/g of fresh weight), chitosan + *Trichoderma harzianum* (29.38 mg/g of fresh weight) and chitosan + *Pseudomonas fluorescens* (27.89 mg/g of fresh weight) showed the maximum enzyme activity. The PPO activity reached the highest at 96 hr after challenge inoculation in case of chitosan + *Trichoderma harzianum* (9.74 µmol/min/mg/protein) treated plants followed by *Trichoderma harzianum* (3.53 µmol/min/mg/protein) alone. the maximum PO activity (49.12 µmol/min/mg/protein) was found in case of chitosan + *Pseudomonas fluorescens* treated plants followed by chitosan (42.48 µmol/min/mg/protein) after 72 hrs. the maximum phenolics (27.53 mg/gm of fresh weight) was found in case of chitosan + *Pseudomonas fluorescens* after 48 hrs of treatment.

Key words: Defense enzyme, Downy mildew, Elicitors, Pea.

INTRODUCTION

Pea is affected by a number of diseases caused by fungi (rust, powdery mildew, downy mildew, root rot, *Alternaria* blight, *Aschochyta* blight, wilt, anthracnose, *Cercospora* leaf spot, damping off, seedling rot *etc*.), bacteria (bacterial blight and brown spot), nematodes (cyst nematode, lesion nematode and root-knot nematode) and viruses (cucumber mosaic virus, pea early browning virus, pea enation mosaic, pea mosaic, pea seed borne mosaic, pea streak and pea stunt). These diseases, under favourable conditions, may cause significantly decrease in both yield and quality (Anonymous, 2021). Amongst these, the downy mildew of pea caused by *Peronospora viciae* is a major constraint for pea production (Singh *et al.*, 2020).

Resistance in plants against the various pathogen can induced through application of several natural and synthetic compounds such as salicylic acid (SA), dichloroisonicotinic acid (INA), benzothiadiazole (BTH), β- aminobutyric acid (BABA), chitosan, *etc*. as these compounds can lead to immediate activation of certain defense response genes and rapid induction of chitinase, β-1,2 glucanase, phenylalanine ammonia lyase (PAL) enzymes (Agrios, 2005).

Plants can be treated with elicitors for fast and more intense mobilization of defense responses which can enhance the resistance against biotic or abiotic stresses (Beckers and Conrath, 2007). Different biotic and abiotic factors (pathogen attack and environmental stimuli) may act on plants to initiate systemic acquired resistance (SAR) against subsequent pathogen attack (Barilli *et al*., 2010b; Dann and Deverall, 1995; Kauss *et al*., 1992; Kessmann *et al*., 1994;). SAR has been reported to be effective against different group of pathogens including viruses, fungi, bacteria, nematodes and parasitic weeds (Beckers and Conrath, 2007).

Pea (*Pisum sativum* L.) plants when treated with SA and 4-aminobutyric acid showed increased activities of phenol inducing enzymes used in plant defense. The

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different enzymes *viz.*, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and superoxide dismutase along with phenolic content responded to various treatment showing variation in their actions (Katoch *et al*., 2005). SA was the first synthetic compound that showed the enhanced activation of a variety of defense responses against major plant pathogens on various crops (Kauss *et al*., 1992; Kessmann *et al*., 1994). Peroxidase activity in cucumber (*Cucumis sativa* L.) and tobacco (*Nicotina tabaccum* L.) after treatment with SA have been reported to increase (Schneider and Ullrich, 1994). Exogenous applications of salicylic acid and benzothiadiazole solutions is commonly used in faba bean to induce systemic acquired resistance (SAR) against rust (*Uromyces viciae-fabae*), ascochyta blight (*Ascochyta fabae*) and broomrape (*Orobanche crenata*) Sillero *et al*. (2012). Surekha *et al*. (2014). The role of *Trichoderma viride* in inducing defense enzymes (Peroxidase, Polyphenol Oxidase and Phenylalanine ammonia Lyase) and total phenolic content in black gram exposed to pathogens *Fusarium oxysporum* and *Alternaria alternate* have been studied and it reported that the biocontrol agent, *T. viride* induces higher levels of defense enzyme in black gram during pathogenesis by *F. oxysporum* and *A. alteranata*. It has been reported that plant-mediated systemic resistance against the *M. javanica* in tomato cv. CALJN3 was induced after applying salicylic acid (SA) and *Pseudomonas fluorescens* CHAO as elicitors Nikoo *et al*. (2014). *Trichoderma* spp. is effective biocontrol agents against different pathogens and some isolates are also known for their ability to induce systemic resistance in plants (Harman *et al*., 2004).

Chitosan and its derivatives display antibiotic activity against microorganisms including bacteria (Liu *et al*., 2007) and fungi (Trotel-Aziz *et al*., 2006). It has been confirmed that treatments based on *Trichoderma harzianum* alone or in combination with chitosan led to an increase in the total phenols and to an enhancement of chitinase and β,1-3-glucanase activities in leaves of treated tomato plants compared with the untreated ones El-Mohamedy *et al*., 2014. Induction of systemic resistance is associated with gene induction which leads to the activation of a wide range of resistance mechanisms induction of defense enzymes, increase peroxidase activity, free oxygen radicals, hypersensitive response *etc*. The study on induction of host defense through biotic and abiotic elicitors can be one of the effective sustainable approaches in disease management.

MATERIALS AND METHODS

Soil was collected from Norman E. Borlaug Crop Research Centre, G.B Pant University of Agriculture Technology, Pantnagar and was autoclaved at 21lb (121.6°C) for one hour for three consecutive days. The sterilized soil was filled in plastic pots (5 kg) and kept in glasshouse. Pots were watered and left for two days for maintaining appropriate moisture for proper seed germination. Seeds of highly susceptible pea cultivar 'Arkel' were washed and sterilized with sodium hypochlorite for 60-90 seconds followed by 2-3 times of consecutive washing with sterilized distilled water under aseptic condition. Fifteen seeds were sowed in each pot and after germination five healthy seedlings were maintained in each pot. Pots were watered regularly as and when reuired for maintaining optimum moisture. Experiments were conducted in a completely randomized design with three replications. Different concentrations of biotic and abiotic elicitors alone and/or in combination (Table 1) were prepared in sterilized distil water and were applied with the help of an atomizer on 30 days old pea plants. After 24 hours of spray, the top five leaves were collected from all the treatments followed by challenge inoculation with spore suspension of *Peronospora pisi* at the rate of 1.5 x 10^6 spores/ml. Sampling (5 leaves per replication) was done at 24, 48, 72 and 96 hr post challenge inoculation. Fresh leaves were weighed and used for determining the activity of enzymes. The experimental results were analyzed statistically using Duncan's multiple range test (DMRT) at P< 0.5. The following enzymatic analyses were done:

a) Peroxidase (PO) activity

Assay of PO activity was carried out as per the standard protocol. Hammerschmidt *et al*. (1982). Enzyme extract was prepared by homogenizing 1 g of leaf samples in 0.1M sodium phosphate buffer (pH 6.0). It was than centrifuged at 10,000 rpm for 20 min. The reaction mixture consisted of 2.5 ml of a mixture containing 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide. The enzyme extract (0.1 ml) was added to initiate the reaction, which was followed calorimetrically at 470 nm. The boiled enzyme preparation served as blank. Enzyme activity was expressed as the increase in absorbance at 480 nm min^{-1} mg^{-1} leaf sample.

b) Polyphenol oxidase (PPO) acivity

PPO activity was determined as per the standard procedure Mayer *et al*. (1965). Leaf samples (1 g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 10, 000 rpm for 20 min at 4°C. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 µL of the enzyme extract and 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µL of 0.01M catechol was added and the activity was expressed as changes in absorbance at 495 nm min^{-1} mg^{-1} leaf sample.

c) Phenylalanine Ammonia Lyase (PAL) activit

Enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.0) was used as per the standard method Ross and Sederoff (1992). About 1 g of leaf sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged for 2.0 min at 10,000 rpm. The supernatant was used as a crude extract for enzyme activity. The assay mixture containing 100 µl of enzyme, 500 µl of 50 mM Tris HCl (pH 8.8) and 600 µl of 1 mM

L- phenylalanine was incubated for 60 min and the reaction was arrested by adding, 2 N HCl. Later 1.5 ml of toluene was added, vortexed for 30 sec, centrifuged (1000 rpm, 5 min) and the toluene fraction containing trans- cinnamic acid was separated. The toluene phase was measured at 290 nm against the blank of toluene. Standard curve was drawn with graded amounts of cinnamic acid in toluene. The enzyme activity was expressed in µ moles of cinnamic acid min^{-1} mg^{-1} of protein.

d) Total phenolics

Total phenolics content was determined by following the standard protocol demonstrated by Swain and Hills (1959). One gram leaf sample was homogenized in 10 ml of 80% methanol followed by 15 minutes of agitation at 70°C. One ml of homogenized methanolic extract was added to 5 ml of distilled water containing 250 µl of Folin-ciocalteu reagent, after this the solution was incubated at 25°C. After 3 min, 1ml of a saturated solution of Na_2CO_3 and 1 ml of distilled water were added in the solution and the reaction mixture prepared was incubated for 1h at 25°C. The absorption of the developed color was measured using spectrophotometer at 725 nm. The total soluble phenolic content was calculated by comparison using a standard curve obtained from Folin-Ciocalteu reaction with catechol. Results were expressed as phenol equivalent in µg g^1 of fresh weight.

RESULTS AND DISCUSSION

Effect of elicitors on Phenylalanine ammonia lyase (PAL) activity

A significant increase in PAL activity was spectro photometrically determine in all the treated plants. Foliar application followed by challenge inoculation lead to increase in PAL activity. In most of the treatments the PAL activity reached the highest level at 48 hr after challenge inoculation and then slowly declined (Fig 1). Among all the treatments the maximum PAL activity (35.58 mg/g of fresh weight) was found in case of oxalic acid but after 48 hrs its activity reduced drastically. Next to oxalic acid *Pseudomonas fluorescens* (31.38 mg/g of fresh weight), chitosan + *Trichoderma harzianum* (29.38 mg/g of fresh weight) and chitosan + *Pseudomonas fluorescens* (27.89 mg/g of fresh weight) showed the maximum enzyme activity. This finding is well supported by the study in which high induction of PAL in cucumber plants treated with *T. asperellum* have been reported Shoresh *et al*., (2005).

Effect of elicitors on Polyphenol oxidase (PPO) activity

A significant increase in PPO activity was spectro photometrically determined in all the treated plants. The PPO activity reached the highest at 96 hr after challenge inoculation in case of chitosan + *Trichoderma harzianum* (9.74 µmol/min/mg/protein) treated plants followed by *Trichoderma harzianum* (3.53 µmol/min/mg/protein) alone. But in other treatments maximum enzymatic activity was observed in the treatments *viz*. SA (6.2 µmol/min/mg/protein) followed by, oxalic acid (5.9 µmol/min/mg/protein) and chitosan + *Pseudomonas fluorescens* (5.36 µmol/min/mg/protein) after 72 hrs of challenge inoculation. It can be concluded that maximum enzymatic activity is seen 72 hrs after foliar application followed by challenge inoculation (Fig 2). The results are in affirmation with the study wherein a gradual increase in polyphenol content was observed when treated with *T. harzianum* T10 or *T. harzianum* T61 (Nawrocka *et al*., 2011; Sriram *et al*., 2009).

Effect of elicitors on Peroxidase (PO) activity

Among all the treatments, the maximum PO activity (49.12 µmol/min/mg/protein) was found in case of chitosan + *Pseudomonas fluorescens* treated plants followed by chitosan (42.48 µmol/min/mg/protein) after 72 hrs (Fig 3). Among other treatments *Pseudomonas fluorescens* (45.46 µmol/min/mg/protein) followed by chitosan (43.14 µmol/min/mg/protein) induced maximum enzymatic activity after 24 hrs of treatment. Peroxidase and polyphenol oxidases catalyse the formation lignin and other oxidative phenols that contribute to the formation of defense barriers for reinforcing the cell structure (Bhashan *et al*., 1985).

Effect of elicitors on total phenolics

A significant increase in total phenolics was noticed in all the treated plants. Among all the treatments, the maximum phenolics (27.53 mg/gm of fresh weight) was found in case of chitosan + *Pseudomonas fluorescens* after 48 hrs of treatment. Whereas *Pseudomonas fluorescens* alone showed maximum enzyme activity (18.94 mg/gm of fresh weight) after 24 hrs of treatment (Fig 4). It has been reported that the phenolics compound have microbial activity Mandal *et al*., 2010.

Table 1: List of elicitors and biocontrol agents used in the present study.

Treatments	Name (source)	Concentration
T1	Salicylic acid (Sigma-Aldrich)	0.72 mM
T2	Isonicotinic acid (Sigma-Aldrich)	0.5 mM
T3	Oxalic acid (Sigma-Aldrich)	0.5 mM
T4	Chitosan (Sigma-Aldrich)	1g/100ml (1%)
T5	Pant bioagent 1 (*Trichoderma harzianum*)	10g/litre
T6	Pant bioagent 2 (*Pseudomonas fluorescens*)	10g/litre
T7	Chitosan + *Trichoderma harzianum*	0.5g/100ml+ 5g/litre
T8	Chitosan + *Pseudomonas fluorescens*	0.5g/100ml+ 5g/litre
T9	Water	-

Several workers have reported the potential of chemical treatments to activate and increase natural plant disease resistance (Kuc, 1995; Ryals *et al*., 1994). It has been reported that inoculation of unifoliate leaves of nine days old green bean (*Phaseolus vulgaris*) with spore suspension of *Colletotrichum lindemuthianum* (10^4 conidia/ml), that cause local lesions, or spraying with 2-6 dichloroisonicotinic acid (20µg/ml) induces resistance in the upper leaves against challenge inoculation of *U. appendiculatus* afterwards (Dann and Deverall, 1995). Rauscher *et al*. (1999) reported broad bean leaves treated with salicylic acid or 2, 6, dichloroisonicotinic acid induces resistance against the rust fungus *Uromyces viciae-fabae* resulting in reduced rust pustules density.

T. harzianum and chitosan activates the host defense genes leading to physical and biochemical responses in plant cells involved in disease suppression. These changes included increase in accumulation of phenol compounds and increase in activity of host defense enzymes (Taheri and Tarighi 2012; Sukand and Kulkarni, 2006).

It was observed that treatment with *T. harzianum* followed by challenge inoculation of *M. phaseolina* enhanced induction of defense enzymes such as peroxidase (PO) and polyphenol oxidase (PPO) and defense related compounds like total phenol and ortho-dihydric phenol Sreedevi *et al*. (2011).

Numerous findings in other plant pathogen system such as Puccinia helianthi/sunflower (Prats *et al*., 2002), Uromyces appendiculatus/common bean (Iriti and Faoro, 2003), or Uromyces pisi/pea (Barilli *et al*., 2010a, 2010b) based on SAR that leads to reduction of infection frequency has also been reported.

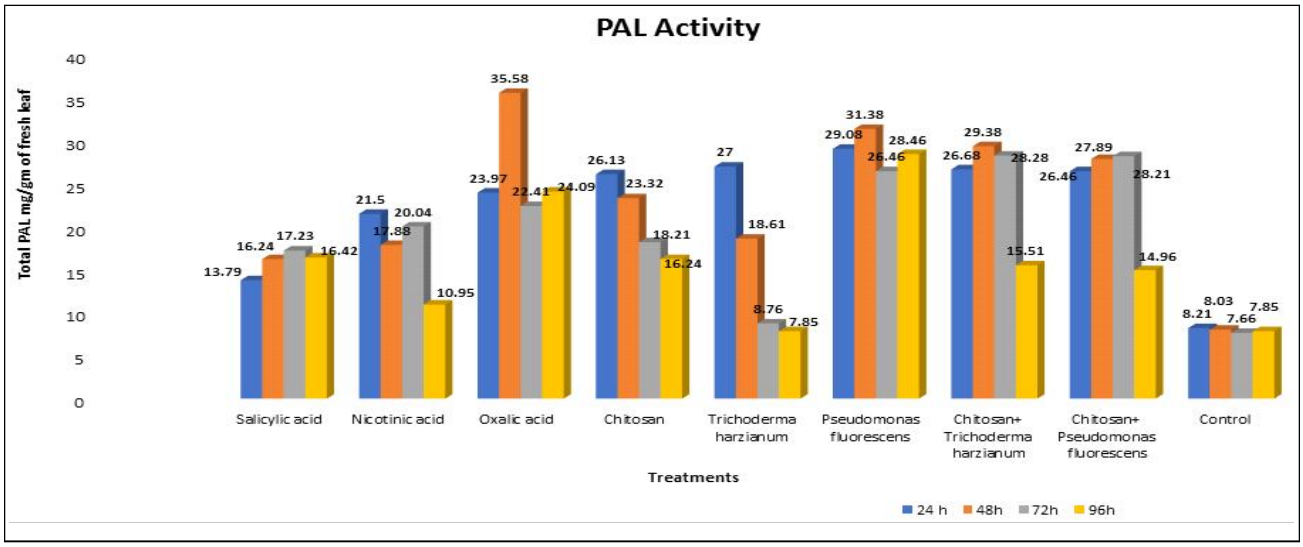

Fig 1: Effect of elicitors on Phenylalanine ammonia lyase (PAL) activity in pea under controlled condition.

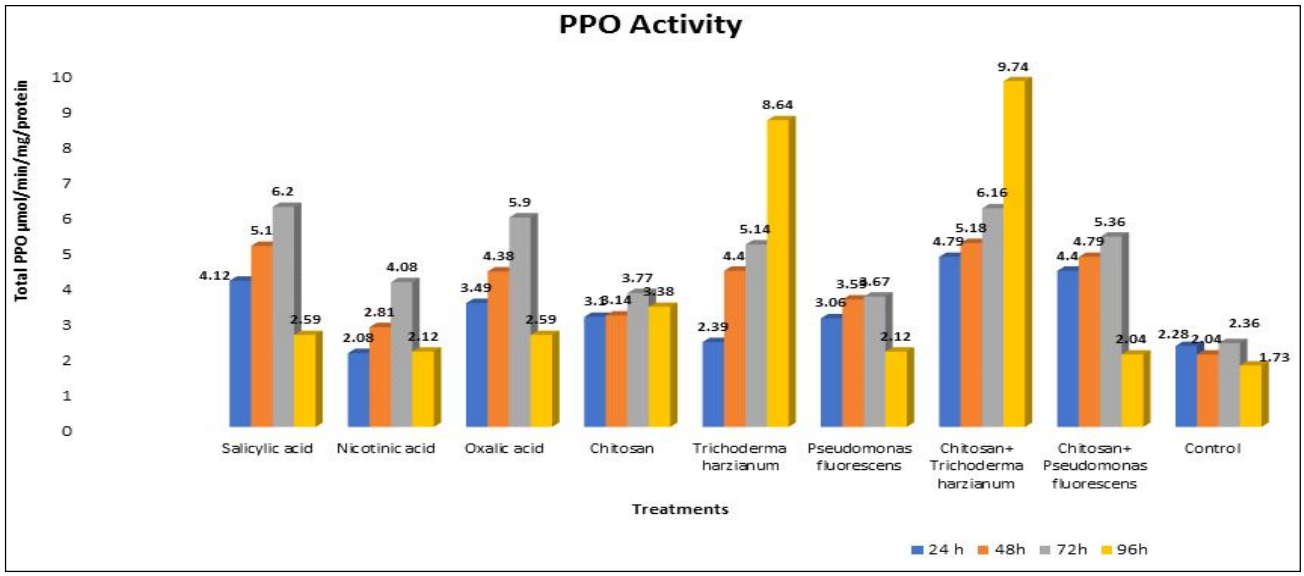

Fig 2: Effect of elicitors on Polyphenol oxidase (PPO) activity in pea under controlled condition.

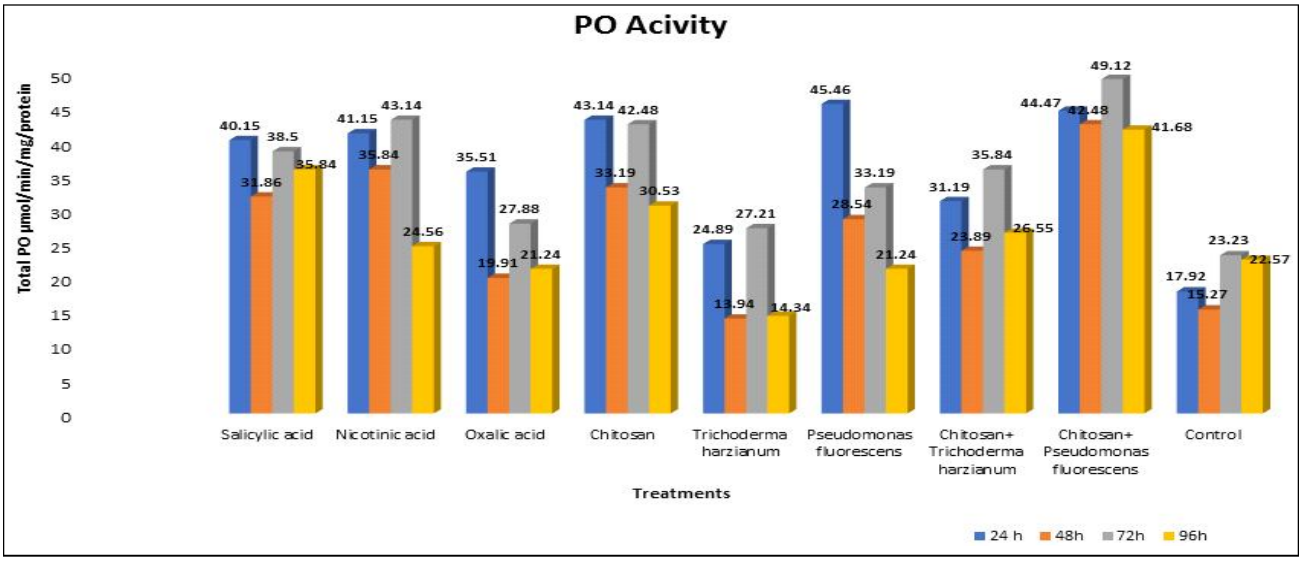

Fig 3: Effect of elicitors application on Peroxidase (PO) activity in pea under controlled condition.

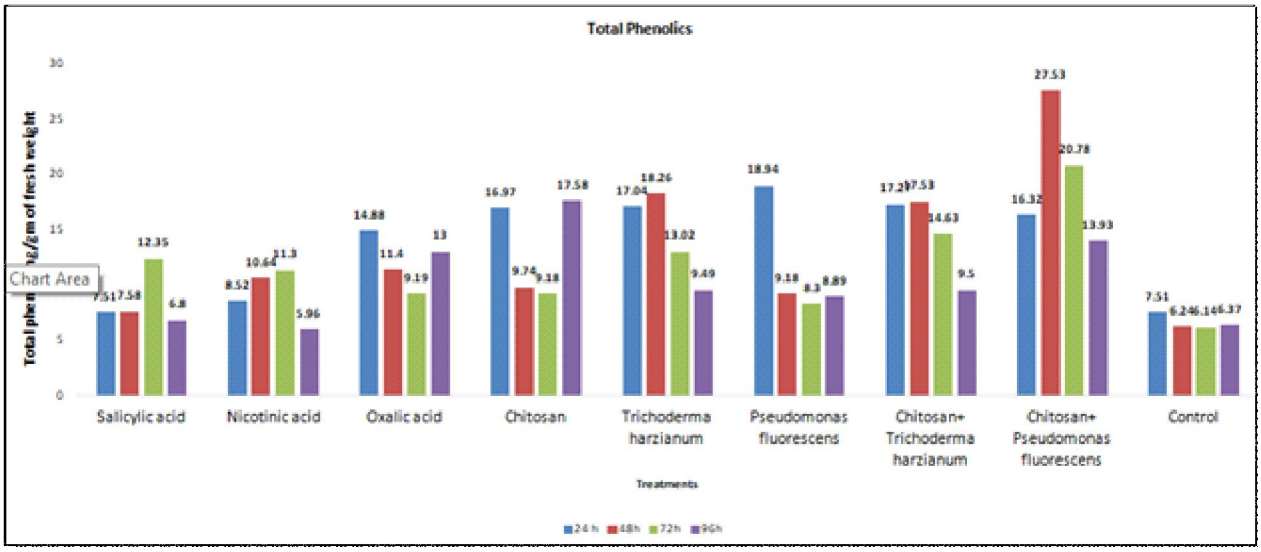

Fig 4: Effect of elicitors on total phenolics in pea under controlled condition.

Upadhyay *et al*., 2016 reported significant increase in defense related enzymes *viz*., peroxidase, polyphenol oxidase and phenylalanine ammonia lyase and total phenols in pea against *Uromyces viciae–fabae* (Pers.) J. Schrot after the application of different elicitors applied.

CONCLUSION

Among all the elicitors chitosan, chitosan in combination with *Trichoderma harzianum*, chitosan in combination with *Pseudomonas fluorescens* and oxalic acid were found to be effective in induction of total phenols, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase as compare to control. The increase in defense enzymes and phenolics content were found 48 hrs after the challenge inoculation in almost all the elicitors tested. The current study revealed that inducing plant defense mechanisms by applying *T. harzianum* and *Pseudomonas fluorescens* particularly in combination with chitosan could provide protection of pea plants against downy mildew disease.

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