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Use of biotic agents and abiotic compounds against damping off of cauliflower caused by *Pythium aphanidermatum*

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ABSTRACT: Damping off caused by *Pythium aphanidermatum,* is a major disease of vegetables including cauliflower throughout the world. The present study was attempted to understand the effects of abiotic compounds and biotic agents on the damping off disease control, plant vigour index, defense related enzyme activities and other biochemical parameters viz., phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) activities, higher phenol and protein contents of pretreated challenged and untreated challenged cauliflower plants. Disease suppression (85-87%) was observed in *Trichoderma harzianum*, *Aspergillus niger* (Kalisena) and Bion treated plants as compared to chemical treatment. (60-84 %). Maximum plant vigour index (2127-2185) was observed in biological formulations in contrast to abiotic compounds (1461-1767). Enzyme activities (PAL, PO, PPO), total proteins and phenols were significantly higher in Bion, and biotic agent treated plants after third day of challenge inoculation as compared to other treatments. An extra specific protein band of R_f value 3.29 (molecular weight between 26-34 kDa) in SDS-PAGE was observed.

Key words: Abiotic compounds, bioagents, defense related enzymes, cauliflower, *Pythium aphanidermatum*

INTRODUCTION

Damping off caused by *Pythium aphanidermatum* in vegetable crops is economically very important due to its wide host range and worldwide occurrence. It is difficult to control this serious menace by following one line of action. Sanitation using sterile or clean water supplies, application of organic compost and regulation of watering and temperature during seedling growth, soil solarization under hot climatic conditions are some of the practices which contribute to the management of this disease. Existing control measures can become more effective for the management of damping off accompanied by use of bioagents as an alternative.

Soil has enormous untapped potential antagonistic microbes, which are helpful in reducing pathogen inoculum through different mode of action such as competition for nutrients and space, antibiosis, mycoparasitism, production of siderophores and lytic enzymes. The present study aims to understand the effect of biotic agents and abiotic compounds on disease suppression, growth promotion and various biochemical parameters related to induction of disease resistance in the host system.

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MATERIALS AND METHODS

The four chemical compounds (Bion®, Captan®, Bavistin® and Thiram®), and two biotic agents *Trichoderma harzianum*, lab formulation and Kalisena SD (*Aspergillus niger*) (Developed by Biocontrol Lab, IARI, New Delhi, India) were used throughout the experiment.

Isolation and multiplication of the pathogen and cultures. The pathogen *P. aphanidermatum* was isolated from naturally infected plants of cauliflower using potato dextrose agar (PDA) medium and pathogenicity was tested on the seedlings of susceptible variety 'Pusa Synthetic'.

Mass production of the fungal inoculum was on sorghum grains by using standard methods. The sporangia/zoospores produced in flasks were harvested after one week for further use. For mixing in the soil, sorghum grains were crushed and used directly as inoculum.

Seed treatment with biotic agents and abiotic compounds. Seeds of cauliflower were soaked for 24 h in a suspension of Bion® (50mgl^{-1}) made up in sterilized distilled water and air dried on filter papers at room temperature for 24 h prior to sowing. For seed treatment, *Trichoderma* (Th) formulation and Kalisena-SD (*A. niger*) were used (4g/kg^{-1} seeds) fungicides, Captan® (4g kg^{-1}), Thiram® (4 g kg^{-1}) and Bavistin® (2g kg^{-1}) were as per normal seed dressing procedure.

Efficacy of bioagents and chemicals on plant growth. Plant growth-promoting activity of *T. harzianum* and *A. niger* was assessed based on the seedling vigor index by the standard roll towel method (ISTA 1993). The vigor index was calculated by using the formula Vigor Index =(Mean root length + Mean shoot length) x Germination (%) described by Abdul Baki and Anderson (1973).

Efficacy of *T. harzianum, A. niger* and chemicals against damping off disease under greenhouse conditions. The mass multiplied virulent strain of *P. aphanidermatum* was mixed with the sterilized potting medium (soil: sand: farm yard manure at 1:1:1 w/w/w) at the ratio of 19:1 w/w and placed in 15 x 30 cm pots. Treated and untreated seeds of cauliflower were sown in the pots @ 25 seeds per pot and watered regularly. There were three replication having four pots in each replication. The disease incidence, root length and plant height were recorded at 25 days after sowing by randomly selecting five plants in each pot.

Induction of defense mechanism and challenge inoculation*. A. niger and T. harzianum* were used to study the induction of defense mechanism in cauliflower against *P. aphanidermatum*. The following treatments were included (1) *T. harzianum* and *A. niger* (Biotic) used as seeds treatment and seedling dip and plants were challenge inoculated with *P. aphanidermatum,* 15 days after planting (50 g sorghum grain-medium containing 10^3cfu g^{-1} medium in each pot); (2) seeds treated with chemicals and elicitor (abiotic) and challenge inoculated with the pathogen 15 days after sowing and (3) untreated plants inoculated with the pathogen 15 days after sowing (control). Seeds were sown in earthen pots filled with sterilized potting soil at 25 seeds per pot. Three replications were maintained in each treatment, each consisting of eight pots. The experiments were conducted using randomized block design in a greenhouse. The humidity in the green house was maintained around 80% and the temperature at 26°C (day)/20°C (night).

Plants were carefully uprooted without causing any damage to root tissues at different time intervals (0, 1, 2, 3, 4, 5, 7 and 10 days after the pathogen inoculation). Four plants were sampled from each replication, separately for biochemical analysis. Fresh roots were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized root tissues were stored at –70°C.

Estimation of phenylalanine ammonia lyase (PAL) activity. Root samples (1g) were homogenized in 3 ml of ice-cold 0.1 M sodium borate buffer pH 7.0, containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrolidone. The extract was filtered through cheesecloth and the filtrate was centrifuged at 16,000g for 15 min. The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson, *et.al.*1984). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The amount of trans-cinnamic acid synthesized was calculated (Dickerson *et.al.*,1984). Enzyme activity was expressed as nmol trans-cinnamic acid min $^{-1}$ g^{-1} fresh weight.

Assay of peroxidase (PO). Root samples (1g) were homogenized in 2 ml of 0.1 M phosphate buffer, pH 7.0 at 4°C. The homogenate was centrifuged at 16,000 g at 4 °C for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 0.5ml root extract, 1.5 ml of 0.05M pyrogallol and 0.5ml of 0.1% hydrogen peroxide was incubated at room temperature (28±2 °C). The changes in absorbance

at 420 nm were recorded at 30-second intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min $^{-1}$ g^{-1} fresh weight (Hammerschmidt,*et.al.,* 1982).

Assay of polyphenol oxidase (PPO). Root samples (1g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000g for 15 min at 4 °C. The supernatant was used as enzyme source. The reaction mixture consisted of 200 ml of the enzyme extract and 1.5ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 ml of 0.01-M catechol was added and the activity was expressed as changes in absorbance at 495-nm, min^{-1} g^{-1} fresh weight (Mayer *et.al.,*1965). For testing of significance of all the results general statistical procedure were adopted.

Estimation of total phenol. Root samples (1g) were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70 °C (Zieslin and Ben-Zaken, 1993). One milliliter of the methanol extract was added to 5 ml of distilled water and 250 ml of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25 °C. The absorbance of the developed blue colour was measured using a spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics were expressed as µg catechol g^{-1} fresh weight.

SDS-PAGE analysis. The protein profiles of cauliflower root extract samples (priorly seed treatment) were estimated by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Root samples were collected on the third day after pathogen inoculation, at which time the activity of proteins was maximum. The protein extract was prepared by homogenizing 1g of root samples in 2 ml of 0.1 M sodium phosphate buffer pH 7.0 and centrifuged at 16,000 g for 20 min at 4 °C. The protein content of the sample was determined (Bradford, 1976). Samples (50 mg protein) were loaded onto 2.5 % staking and 12 % resolving SDS-PAGE gels. After electrophoresis, protein profile was visualized by soaking the gels in staining solution containing 0.1% coomassie brilliant blue R250 and 50% methanol and 10% acetate buffer (20 mM, pH 4.2) The gel were destained for 30 min in 0.1% acetic acid solution and the comparison was made on the visual basis or by photograph with the control and treatments itself Medium range protein molecular weight marker (15kDa to 118kDa) was used along with the samples. The R_f value of individual protein bands was calculated by the formula: R_f value=(distance migrated by protein band/ distance migrated by dye) (Hames and Richwood, 1990).

RESULTS

Effect of seed treatment with biotic agents and biotic elicitors on the reduction of damping off. The lower values of disease incidence were found in Bion® (50 mg l $^{-1}$) treatment 11.05% followed by 12.12%, 12.75% and 13.20% in Th formulation, *A.niger* and Bavistin®, respectively and there was increase in the plant vigor index (Table-1). The efficacy of Th formulation and *A.niger* in plant vigor index was higher than that of other chemicals. The maximum plant vigor index was

Table 1. Efficacy of chemicals and bioagents against *Pythium aphanidermatum* damping off of cauliflower under green house conditions.

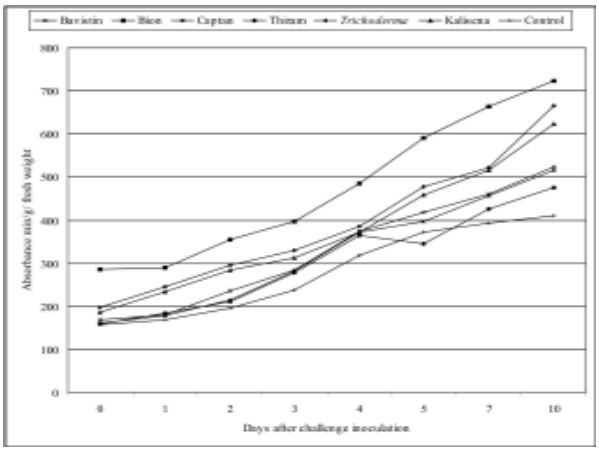
Treatment	Disease Incidence (%)*	Disease Control (%)	Plant vigor index
Bavistin®	13.20 (21.30)	84.17	1742.42
Captan	33.25 (35.21)	60.11	1414.26
Thiram®	22.65 (28.41)	72.84	1461.12
Bion®	11.05 (19.41)	86.75	1767.32
T.harzianum	12.12 (20.38)	85.46	2185.08
A.niger (Kalisena)	12.75 (20.92)	84.76	2127.00
Control	83.40 (65.96)	00.00	313.04
CD p=0.05	4.265		

*The data in parenthesis are arsine transformed prior to the analysis.

found to be 2185.08 in *T.harzianum* followed by *A.niger* 2127 and Bion® 1767.32 and fungicidal treatments with lower values of plant vigour index (1414—1762).

Induction of defense-related enzymes and phenolic compounds.

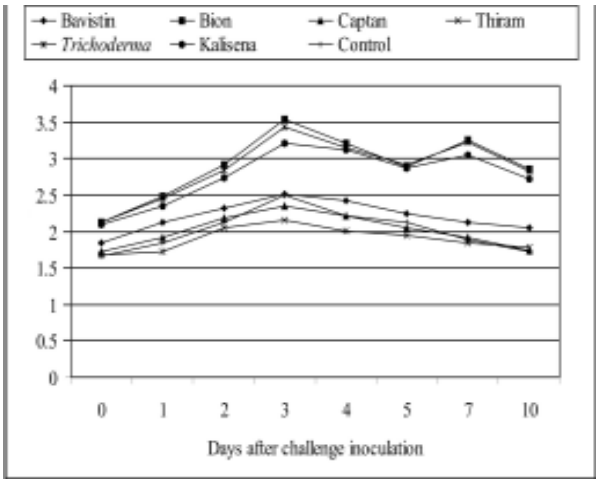
On PAL activity. Cauliflower, seeds treated with chemical and bioagents induced the plants to synthesize PAL, whereas an additional increase in the synthesis was observed in Bion® chemical pretreated plants challenge inoculated with *P. aphanidermatum* (Fig.1). The activity (nmol trans-cinnamic acid min $^{-1}$ 100 g $^{-1}$ fresh weight) increased from first day after challenge inoculation but the maximum and highest percent increase was found on the fourth day after pathogen inoculation and thereafter the activity remained increasing at higher levels throughout the experiment period of 10 days. In plants treated with the pathogen alone, drastic increase of PAL activity was observed for a period of 2-5 days and thereafter the increase rate was lower.



Line indicates standard deviations of three replications.

Fig. 1. Influence of seed treatment with chemicals and bioagents on PAL activity in cauliflower challenged with *Pythium aphanidermatum*.

On peroxidase (PO) activity. Levels of peroxidase activity in biotic and abiotic inducerd plants showed increasing trend is all the treatments. The highest increased activity of PO was in Bion® treated plants challenge inoculated with the pathogen and it remained at higher level throughout the experimental period (Fig.2). The PO activity of Bion® treated plants was followed

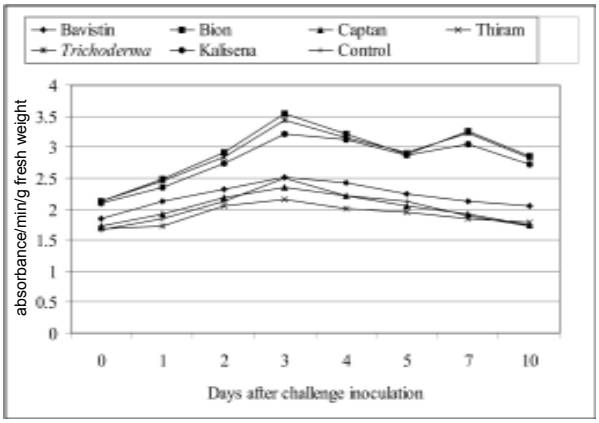


Line indicates standard deviations of three replications.

Fig. 2. Influence of seed treatment with chemicals and bioagents on peroxidase activity in cauliflower

by Th formulation and *A.niger* treated plants and was comparable to other chemicals and control treatments.

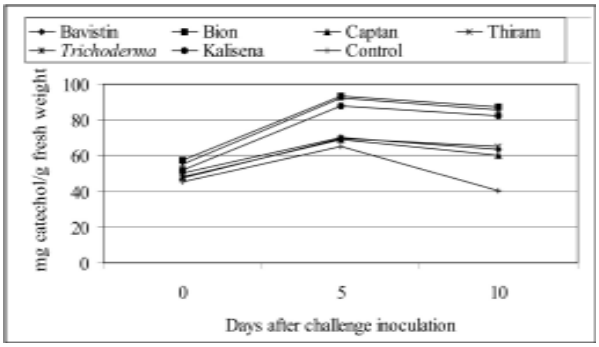
On polyphenol oxidase (PPO) activity. The maximum polyphenol oxidase activity was observed in Bion® followed by Th formulation and *A.niger* treated plants. The PPO activity reached its maximum at fourth day after challenge inoculation thereafter, lowered but was higher than 0 days of challenge inoculation (Fig.3). However, in the untreated plants (control) reducing trend of PPO was observed.



Line indicates standard deviations of three replications.

Fig. 3. Influence of seed treatment with chemicals and bioagents on poly phenol oxidase activity in cauliflower

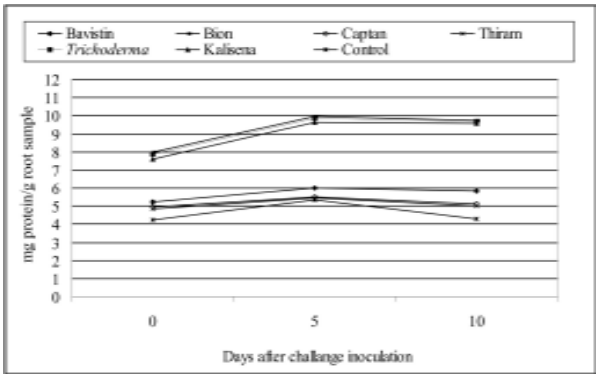
Effect of seed treatment on total phenols. The maximum phenolic content was observed in Bion® treated plants challenge inoculated with the pathogen and the higher amount of total phenolics were noticed even on the 10th day after challenge inoculation. (Fig.4). The phenolic contents in Th formulation, Bion® and *A.niger* treated plants was maximum at 5th day after challenge inoculation thereafter, it decline but remain higher than that of control and other treatments.



Line indicates standard deviations of three replications.

Fig. 4. Influence of seed treatment with chemicals and bioagents on phenol in cauliflower

On protein contents. The highest amount of protein was shownd by Bion®, *T.harzianum* (Th) formulation and *A.niger* treated plants, respectively, challenge inoculated with the pathogen (Fig.5). Maximum level was observed on the fifth day after pathogen challenge and thereafter the activity slightly decreased but remained at higher levels throughout the experimental period. The protein content in control was significantly lower than that of Bion®, Th formulation, and *A.niger* treated plants.



Line indicates standard deviations of three replications.

Fig. 5. Estimations of total protein in cauliflower roots challenged with *Pythium aphanidermatum,* mg protein g root samples $^{-1}$

SDS-PAGE-protein profile of cauliflower plants, treated with biotic agents and abiotic compounds. A comparative study of proteins obtained from cauliflower plants treated with biotic agents and abiotic compounds and challenge inoculated with *P. aphanidermatum,* lysed protoplasts and homogenized tissues were carried out to detect the variation in the corresponding profiles. The expression of protein profile pattern was quite similar in number (total 16 bands), relative intensity and position of bands along the profile except one more extra protein band of R_f value 3.29 (molecular weight between 26-34 kDa) in three treatments was observed (Fig. 6). The extra protein bands were observed in pathogen challenged and biotic agents treated plants.

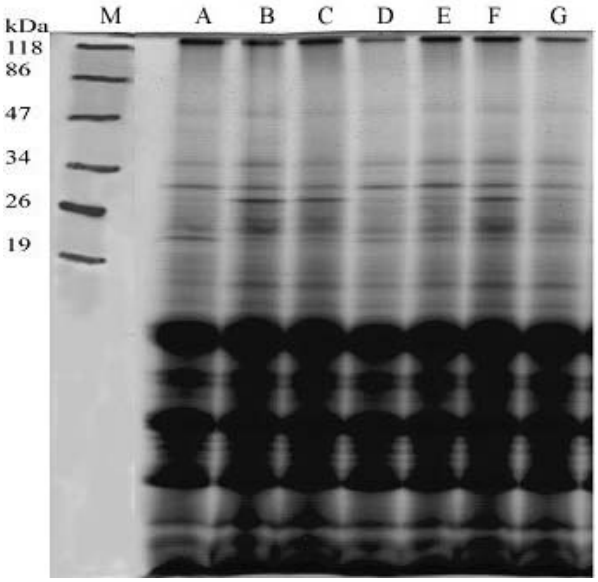


Fig. 6. SDS-PAGE protein profile of (A) untreated control plant, plant treated with (B) *T. harzianum,* (C) *A. niger*, (D) captan, (E) thiram, (F) bion (CGA 245704), (G) bavistin and (M) medium range molecular weight protein marker.

DISCUSSION

In the present investigation, seed treatment with Bion®, *T. harzianum* and *A.niger* formulation was found most effective in reducing the damping off disease incidence per cent in cauliflower plants. The maximum disease control percentage was recorded in plants treated with Bion® followed by *T. harzianum* and *A.niger* formulation, Bavistin, Thiram®, Captan®. Plant vigour index was maximum

in bioformulation treatments as compared to Bion and chemically treated plants. Similar reports have been made by Pietr *et. al.*, (2002) who have found 40-118% increase in dry weight of young vegetable plants (cabbage, tomato, leek and cucumber) grown in potting compost amended with *Trichoderma* isolates (10^6 cfu/dcm^3) than plants grown in untreated control compost. The increase in plant dry weight during the early stage of development was observed to be correlated significantly with the increase of PAL activity in plant tissue (Pietr *et. al.*, 2002). In the present investigation *T. harzianum, A.niger* and Bion pretreated plants also showed higher increase in PAL, PO, and PPO activity, total protein and phenol contents at 4, 5, 7 and 10 days after *P. aphanidermatum* inoculation. Similarly, Nzojiyobiri *et. al.*, (2003) have also reported higher activities of PAL and PO susceptible rice cv. Yuanfengzao seedlings pretreated with the *T. harzianum* strain NF9 at 16, 96 and 120 h after pathogen (*M. grisea, X. oryzae* pv. *oryzae*) inoculation than that in untreated ones in greenhouse experiments. Early and increased synthesis of PAL, PO, PPO [catechol oxidase] enzymes and total phenolics were also observed in the *T. viride*, *Pseudomonas fluorescens* and *Bacillus subtilis* pretreated peppermint plants challenged with *R. solani* by Kamalakannan *et. al.* (2003). Yedidia *et. al.* (1999) have also observed that *T. harzianum* strain T-203 inoculation into the roots of cucumber cv. Delila seedlings, initiated increased peroxidase and chitinase activities within 48 and 72 h, respectively.

Bion® (CGA245704) has been reported to have its significant effects on disease control by many workers. (Sharma, 2002 and Gawande and Sharma, 2003) which may be due to its well-known property of inducing resistance and reducing the disease severity by triggering various defense related pathways and systemic spread of resistance factors from seed to cotyledons.

T.harzianum and *T. hamatum* which are capable of antagonizing sensitive pathogenic fungi by producing antibiotics and lytic enzymes, have been reported to induce systemic resistance in tomato, lettuce, pepper, bean, and tobacco against gray mold, caused by *Botrytis cinerea* (Meyer *et. al.*, 1998; Sharma and Sain, 2004). Tuzun (2001) described that constitutive accumulation of defense related gene products were an integral part of both multigenic resistance and ISR. Several defense related genes encoding proteins are synthesized in ISR by *T. harzianum*, it has been hypothesized that induced resistance by *Trichoderma* and *Aspergillus* isolates is related to multigenic/polygenic (horizontal) resistance in plants, which is effective against multiple pathogen/races of pathogens. The findings of this study indicate a positive correlation among plant growth promotion and induced accumulation of PAL, PO, PPO, phenolic substances and PR-proteins in response to infection by the *P. aphanidermatum* and the sensitization of the plants for resistance.

It is inferred that *T. harzianum* and *A. niger* formulation consistently reduced the incidence of *Pythium* damping off of cauliflower and increased plant vigor index. Prior treatment of cauliflower seeds with these bioagents triggered the plant-mediated defense mechanism in addition to reducing disease by direct antagonism, mycoparasitism, and competition in response to infection by *P. aphanidermatum* as induced by CGA 245704 (Bion) a plant sensitizer and defense related proteins inducer. Thus it has been found that *T. harzianum* and *A. niger* shows induction of defense related enzymes and growth promotion in cauliflower against nursery pathogens and could be utilized as an ecofriendly, inexpensive, effective and integrated pest management strategy.

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