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RESEARCH ARTICLE

Evaluation of biochemical basis of chemical induced basal rot tolerance affecting garlic cultivation in Himachal Pradesh

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Abstract

This study aimed to investigate the biochemical response of chemical inducers for disease resistance in garlic (*Allium sativum* L.) in Himachal Pradesh. Garlic is the second most grown crop in India, which is known for their high nutritional and medicinal properties. The disease has the potential to cause massive losses in the garlic crop, reducing farmers economic profitability; therefore, management may be required. Biochemical evaluations revealed considerable increase in the phenolic activities of the phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) upon treatment with various disease resistance inducers especially ß-aminobutyric acid (BABA), acibenzolar-S-methyl (ASM) and salicylic acid (SA), which were significantly correlated with the basal rot tolerance in the susceptible garlic cultivar.

Keywords: basal rot; biochemical response; disease; garlic

Introduction

Garlic (*Allium sativum* L.) is the second most grown *Allium* species after onion from the Amaryllidaceae family, which originated from Central Asia and is cultivated broadly across the globe in both temperate and subtropical regions due to its significant nutritional value and medicinal benefits (1). It is grown across the world with the total area of 1634 thousand hectares and 307.08 million tonnes productions globally (2). China is the world leader in term of the area and production of garlic followed by India. In India, garlic covers 391 thousand hectares area with the net production of 3185 thousand metric tonnes. It is cultivated all over India. In Himachal Pradesh, it is cultivated on 7.19 thousand hectares, yielding a productivity of 1.61 tonnes per hectare (3).

Garlic is however vulnerable to various plant pathogens including soil borne fungi, which lead to disease such as basal rot resulting significant yield losses (4). The basal rot affecting garlic is attributed to *F. oxysporum*, leading to reduction in high quality of yield in *Allium* spp. globally, both before and after harvest associated with up to 50 % losses in the field and between 30–40 % during storage (5,6).

The control of soil borne pathogens through chemical means is challenging, costly and not advisable due to the risks of groundwater contamination, harm to beneficial non target organisms and the development of fungicide resistant strains of pathogens. Additionally, the growing reliance on chemicals in agriculture continues to be a concern for both environmental safety and public health authorities. As a result, there has been a rise in interest towards new alternative approaches that lessen reliance on agrochemicals, protect the environment and produce lower levels of residues while also being safer for human health in recent years. The present study was aimed to investigate the efficacy of various chemical supplements to induce resistance in garlic against basal rot disease.

Material and Methods

Evaluation of resistance inducing chemicals under field conditions

Seven resistance inducing chemicals with respective concentrations (Table 1, Fig. 1) diluted with distilled sterilized water were evaluated for disease resistance in garlic under field conditions by foliar spray. Artificial inoculation of mass culture of pathogens (4 g m^{-2}) was done one month after the germination of cloves, followed by foliar application of resistance inducing chemicals after one month. The study was carried out using a Randomized Block Design (RBD), with each treatment replicated three times and untreated plants acted as the control. Measurements regarding percent disease incidence and percent disease control were taken.

Table 1. Resistance inducing chemicals with their concentrations and dose used against basal rot pathogen under field conditions

Treatments	Resistance inducing chemicals	Concentrations (mM)	Dose (%)
T_1	SA	1.0	0.015
T_2	Oxalic acid	1.0	0.01
T_3	Potassium oxalate	50.0	0.1
T_4	BABA	1.0	0.01
T_5	ASM	0.05	0.002
T_6	Sodium salicylate	10.0	0.15
T_7	Di-potassium hydrogen phosphate	200.0	2.7
T_8	Control	-	

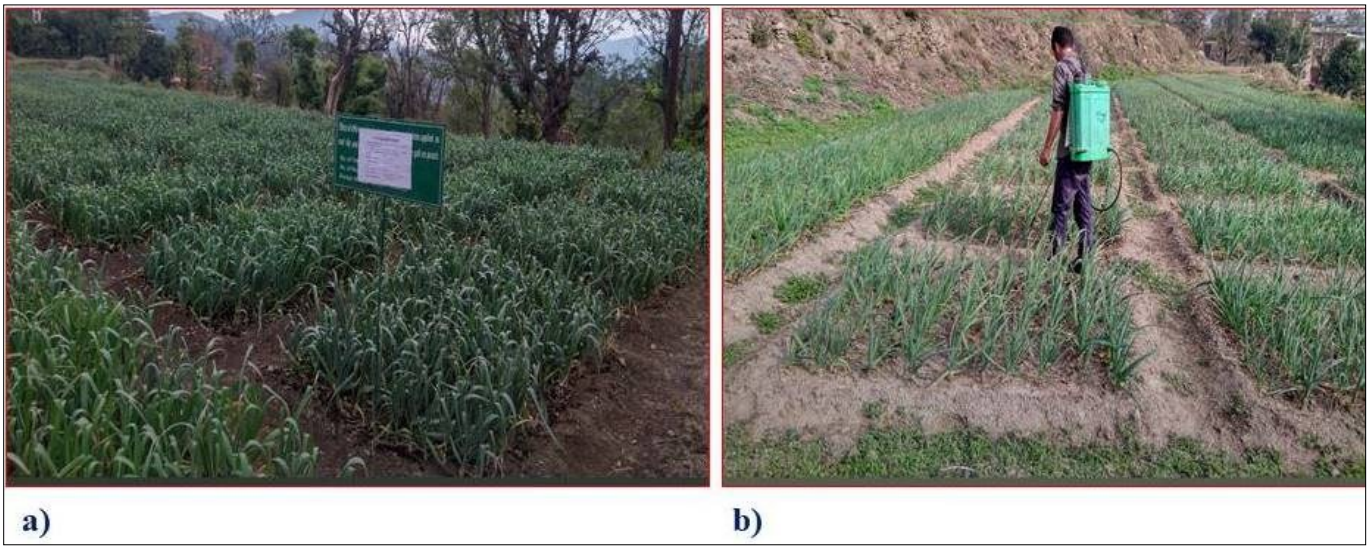

Fig. 1. Foliar spray of resistance inducing chemicals against basal rot of garlic under field conditions.

Estimation of biochemical parameters of plants with induced resistance

Biochemical evaluation of the treated plants was carried out for various parameters including total phenols and defense associated enzymes such as peroxidase, PPO, PAL and SA, which imparted resistance in the host plants by following standard protocols (7). The plants inoculated with the pathogen alone without any chemical treatment served as control. Leaf samples from each treatment were taken at 24, 48, 96 and 120 hr of inoculation for estimating the changes in the total phenolic contents and activity of PPO, PO, PAL and SA.

Total phenol content

Leaf samples from both the resistance induced (or treated plants) and control plants (untreated) were utilized for estimating the total phenolic content using a standardized analytical method (8–10). 1 g of fresh leaf sample dissected and homogenized with 80 % ethanol (4 mL ethanol/g tissue) using mortar and pestle, followed by incubation in the boiling water bath for 10 min and cooled. The extracts were filtered through double layer of muslin cloth and/or Whatman filter paper and raised the final volume to 5 mL by adding 80 % ethanol.

1 mL of Folin-Ciocalteu reagent mixed with alcohol extract in equal volume followed by addition of 2 mL of 20 % sodium carbonate. This mixture was incubated in boiling water bath for a minute before being cooled under running water. The resulting blue solution was diluted to 25 mL with double distilled water, incubated for 1 hr and measured its optical density at 650 nm using a spectrophotometer. A blank that included all the reagents except Folin-Ciocalteu reagent was utilized to set the absorbance to zero and phenolic content determined from a standard curve generated using gallic acid.

Estimation of PPO and PO activity

A leaf sample weighing 0.5 g was crushed in 5 mL of 0.1 M potassium phosphate buffer (pH 7.5) containing 2 % (w/v) polyvinylpolypyrrolidone (PVP) and 0.25 % (v/v) Triton X and the homogenate was centrifuged at 10000 rpm for half an hour (4 °C). The supernatants so obtained served as crude enzyme extracts for the spectrophotometric assessment of enzymatic activities.

PPO activity

The activity of the PPO (measured as OD/mg protein) was determined using an established method (8). The assay solution included 1.95 mL of a 0.1 M potassium phosphate buffer (pH 7.5), 1 mL of catechol at a concentration of 0.025 M and 50 µL of the diluted crude enzyme extract. The change in absorbance was monitored at 420 nm in 30 sec intervals over a period of 3 min (9).

PO activity

PO activity expressed as OD/mg protein was determined using a standard protocol (10). The reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol prepared with 0.1 M phosphate buffer (pH 6), 0.5 mL of crude enzyme extract and 0.5 mL hydrogen peroxide (1 %). It was incubated at room temperature (28 ± 1 ° C) for 30 min and change in absorbance was measured at 420 nm for 30 sec in the 3 min intervals.

Estimation of PAL activity

A leaf sample weighing 1 g was crushed with 3 mL of ice cold 0.1 M sodium borate buffer (pH 7), containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone. The extract was then filtered using Whatman filter paper and centrifuged at 13000 g for 15 min to isolate the supernatant.

The 0.4 mL of crude enzyme extract added into 0.5 mL of 0.1 M borate buffer, pH 8.8 containing 0.5 mL of 12 mM L-phenylalanine and incubated for 30 min at 30 °C. The trans-cinnamic acid formation from L-phenylalanine was quantified by absorbance at 290 nm and enzyme activity was determined as µg of trans-cinnamic acid per minute per gram of sample derived from the L-phenylalanine through the catalytic activity of PAL.

Estimation of SA

A leaf sample weighing one gram was homogenized in 10 mL of 80 % methanol, followed by centrifugation at 15000 rpm for 30 min at 4 °C to obtain the supernatant. To the tissue homogenate, 0.1 g of ascorbic acid was added and allowed to evaporate at 65 °C. The resulting residues were dissolved in 5 mL of 80 % methanol and 500 µL of this solution was combined with 250 µL of 10 N HCl and 1 mL of methanol, then incubated in a water bath at 80 °C for 2 hr. The reaction was neutralized using 4-5 drops of 1 M $NaHCO_3$ and absorbance was measured at 254 nm for samples collected on days 0, 2, 7, 14 and 21 following pathogen inoculation. The SA content was calculated as µg/g of leaf tissue.

Results

Evaluation of resistance inducing chemicals under field conditions

The efficacy of seven resistance inducing chemicals with different concentrations was determined to induce the basal rot resistance in garlic plants under field conditions through foliar applications (Fig. 1 a-b) and resulted were tabulated in the Table 2. The foliar application of such chemicals had drastically reduced the basal rot incidence in garlic as compared to the untreated control as indicated by 24.65 % disease incidence over control (72.27 %) for foliar application of SA at 0.015 % concentration followed by BABA having 26.32 % disease incidence over control (70.39 %) though statistically at par with each other. Application of di-potassium hydrogen phosphate (2.7 %) resulted basal rot incidence of 45.62 % and 48.69 % (C.D. 1.64; P-value <0.05) (Table 2, Fig. 2).

Biochemical evaluation of plants with induced resistance

Leaf samples from each treatment were collected at 24, 48, 96 and 120 hr post inoculation and used for estimating the changes in the activity of defense related enzymes, phenols and SA. Observations pertaining to activity of these enzymes at different intervals were recorded (Table 3-7).

Table 2. Efficacy of resistance inducing chemicals against basal rot of garlic under field conditions

Resistance inducers	Concentration (mM)	Dose (%)	Disease incidence (%)	Disease control (%)
SA	1	0.015	24.65 (29.76)	72.27
Oxalic acid	1	0.01	38.40 (38.29)	56.80
Potassium oxalate	50	0.10	39.77 (39.10)	55.26
BABA	1	0.01	26.32 (30.86)	70.39
ASM	0.05	0.002	42.55(40.71)	52.13
Sodium salicylate	10	0.15	34.67(36.07)	61.00
Di-Potassium hydrogen phosphate	200	2.70	45.62 (42.49)	48.69
Control	-	-	88.90 (70.55)	-
$CD_{0.05}$	-	-	(1.64)	-

Figures in the parentheses are arc sine transformed value

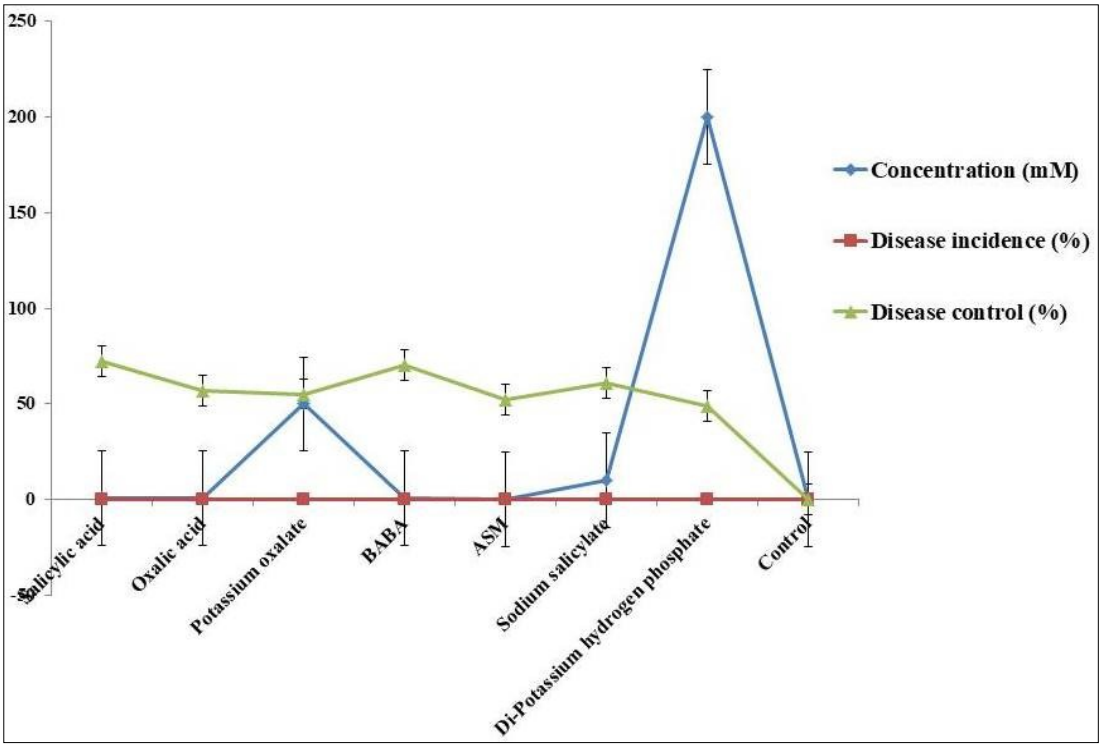

Fig. 2. Variation in the disease incidence of basal rot in garlic plants treated with seven resistance-inducing chemicals and controls.

Table 3. Effect of foliar application of resistance inducing chemicals on total phenol content in garlic leaves

Resistance inducers	Concentration (mM)	Dose (%)	Total phenol (µg/g fresh weight) in leaves					
			Interval (hr)					Mean
			24	48	72	96	120	
SA	1	0.015	91.30	111.00	134.00	138.30	73.00	109.50
Oxalic acid	1	0.01	63.70	94.70	123.30	128.70	23.30	86.70
Potassium oxalate	50	0.1	67.70	97.30	127.70	132.30	15.30	88.10
BABA	1	0.01	92.30	104.00	135.70	141.30	79.70	110.60
ASM	0.05	0.002	72.70	100.70	131.30	135.30	37.70	95.50
Sodium salicylate	10	0.15	77.70	99.70	128.00	134.70	42.70	96.50
Di-Potassium hydrogen phosphate	200	2.7	54.70	83.30	122.70	128.30	41.30	86.10
Control	-	-	11.70	17.00	18.30	12.70	6.00	13.10
Mean	-	-	66.50	88.50	115.10	119.00	39.90	-

CD $_{0.05}$ **Resistance inducers = 1.28**

Interval = 1.01

Resistance inducers × Interval = 2.86

Table 4. Effect of foliar application of resistance inducing chemicals on PPO activity in garlic leaves

Resistance inducers	Concentration (mM)	Dose (%)	PPO activity (change in absorbance /min/mg fresh weight)					
			Interval (hr)					Mean
			24	48	72	96	120	
SA	1	0.015	0.61	1.18	1.40	1.44	1.07	1.14
Oxalic acid	1	0.01	0.44	0.84	1.04	1.09	0.54	0.79
Potassium oxalate	50	0.10	0.49	0.96	1.13	1.22	0.77	0.91
BABA	1	0.01	0.63	1.20	1.41	1.44	1.08	1.15
ASM	0.05	0.002	0.54	1.05	1.22	1.34	0.92	1.01
Sodium salicylate	10	0.15	0.52	1.04	1.19	1.30	0.87	0.99
Di-Potassium hydrogen phosphate	200	2.70	0.40	0.95	1.02	1.05	0.66	0.82
Control	-	-	0.19	0.23	0.19	0.18	0.13	0.18
Mean	-	-	0.48	0.93	1.08	1.13	0.76	-

CD $_{0.05}$ **Resistance inducers = 0.01**

Interval = 0.01

Resistance inducers × Interval = 0.03

Table 5. Effect of foliar application of resistance inducing chemicals on PAL activity in garlic leaves

Resistance inducers	Concentration (mM)	Dose (%)	PAL activity (µmol trans-cinnamic acid $min^{-1}g^{-1}$)					
			Interval (hr)					Mean
			24	48	72	96	120	
SA	1	0.015	1.06	2.95	3.42	1.65	1.09	2.03
Oxalic acid	1	0.01	0.77	2.54	2.88	1.10	0.66	1.59
Potassium oxalate	50	0.1	0.81	2.64	2.96	1.24	0.89	1.71
BABA	1	0.01	0.97	2.94	3.42	1.66	1.25	2.05
ASM	0.05	0.002	0.92	2.73	3.13	1.39	1.01	1.84
Sodium salicylate	10	0.15	0.88	2.73	2.99	1.32	0.95	1.78
Di-Potassium hydrogen phosphate	200	2.7	0.74	2.48	2.74	1.06	0.79	1.56
Control	-	-	0.50	0.42	0.22	0.17	0.12	0.29
Mean	-	-	0.83	2.43	2.72	1.20	0.84	-

CD $_{0.05}$ **Resistance inducers = 0.02**

Interval = 0.02

Resistance inducers × Interval = 0.05

Table 6. Effect of foliar application of resistance inducing chemicals on PO activity in garlic leaves

Resistance inducers	Concentration (mM)	Dose (%)	PO activity (change in absorbance /min/mg fresh wt.)					
			Interval (hr)					Mean
			24	48	72	96	120	
SA	1	0.015	2.30	3.35	3.40	3.82	3.42	3.26
Oxalic acid	1	0.01	1.42	2.29	1.82	1.46	0.88	1.57
Potassium oxalate	50	0.10	1.22	1.99	1.52	1.16	0.75	1.31
BABA	1	0.01	2.82	3.69	3.22	2.86	2.28	2.97
ASM	0.05	0.002	2.32	3.19	2.72	2.36	1.78	2.47
Sodium salicylate	10	0.15	1.82	2.69	2.22	1.86	1.28	1.97
Di-Potassium hydrogen phosphate	200	2.70	1.62	2.03	1.41	1.42	0.65	1.42
Control	-	-	1.12	1.53	1.21	1.12	0.50	1.11
Mean	-	-	1.83	2.59	2.19	2.00	1.49	-

CD $_{0.05}$ **Resistance inducers = 0.03**

Interval = 0.02

Resistance inducers × Interval = 0.07

Table 7. Effect of foliar application of resistance inducing chemicals on SA content in garlic leaves

Resistance inducers	Concentration (mM)	Dose (%)	SA content (µg/g fresh weight)					
			Interval (hr)					Mean
			24	48	72	96	120	
SA	1	0.015	34.33	52.00	53.33	60.67	52.33	50.53
Oxalic acid	1	0.01	20.67	36.67	42.67	42.67	34.67	35.47
Potassium oxalate	50	0.10	24.33	41.00	47.00	46.67	35.00	38.80
BABA	1	0.01	36.87	46.36	55.07	59.33	53.33	50.19
ASM	0.05	0.002	31.33	44.33	52.67	53.33	41.33	44.60
Sodium salicylate	10	0.15	27.00	42.33	52.00	51.33	36.67	41.87
Di-Potassium hydrogen phosphate	200	2.70	21.67	32.67	38.67	43.67	33.67	34.07
Control	-	-	17.67	28.00	25.33	20.33	15.67	21.40
Mean	-	-	26.73	40.42	45.84	47.25	37.83	-

$CD_{0.05}$ **Resistance inducers = 1.00**
Interval = 0.79
Resistance inducers × Interval = 2.24

Total phenol content

The foliar application of resistance inducing had considerably increased phenolic content such as 66.50 µg/g fresh weight after 24 hr to 119.0 µg/g fresh weight after 96 hr, which were statistically different from each other (Table 3). The phenolic content started to decline thereafter and was minimum after 120 hr (39.90 µg/g fresh weight). Phenols were highest for the BABA (110.60 µg/g tissue) followed by SA (109.50 µg/g tissue) and sodium salicylate (96.50 µg/g tissue) over untreated control (13.10 µg/g tissue). Interaction studies showed maximum phenol content after 96 hr of inoculation of BABA (141.30 µg/g tissue) followed by SA (138.30 µg/g tissue) and ASM (135.30 µg/g tissue) (Fig. 3a, Table 3).

Estimation of PPO activity

The applications of resistance inducing chemicals had significantly increased PO activity in garlic with increasing pattern over time as maximum activity recorded after 96 hr of inoculation (1.44) for BABA followed by ASM (1.34) and sodium salicylate (1.30). The minimum PO activity observed in untreated control (0.18) followed by di-potassium hydrogen phosphate (1.05) and oxalic acid (1.09). It was statistically at par for the treatments BABA and SA (Fig. 3b, Table 4).

Estimation of PAL

PAL activity increased slowly in all the chemical treatments and continued to increase till 72 hr and started to decline thereafter. The highest activity observed in BABA and SA (3.42 µmol trans-cinnamic acid $min^{-1}g^{-1}$) followed by sodium salicylate (2.99 µmol trans-cinnamic acid $min^{-1}g^{-1}$) and potassium oxalate (2.96 µmol trans-cinnamic acid $min^{-1}g^{-1}$). The minimum activity found in untreated control (0.22 µmol trans-cinnamic acid $min^{-1}g^{-1}$) followed di-potassium hydrogen phosphate (2.74 µmol trans-cinnamic acid $min^{-1}g^{-1}$) and oxalic acid (2.88 µmol trans-cinnamic acid $min^{-1}g^{-1}$) (Fig. 3c, Table 5).

Estimation of PO activity

The highest PO activity was recorded in the SA treatment (3.26 change in absorbance/min/mg fresh weight) followed by BABA giving 2.97 change in absorbance/min/mg fresh weight though statistically different to each other. The increasing pattern was observed from 24 hr (1.83) to 48 hr post inoculation (2.59), which was statistically differed from each other. The PO activity started decreasing after 72 hr up to 120 hr from 2.19 to 1.49. Combined effects were also statistically different and highest for BABA after 48 hr (3.69) followed by SA (3.35) (Fig. 3d, Table 6).

Estimation of SA activity

The foliar application of SA (0.015 %) had significantly increased the salicylic quantity in garlic leaves (50.53 µg/g fresh weight) followed by BABA (50.19 µg/g), which was however statistically like each other. The increasing pattern was recorded from 24 hr (26.73 µg/g fresh weight) to 96 hr post inoculation (47.25 µg/g fresh weight), which was statistically significant. After 96 hr, salicylic content started to decline up to 120 hr giving 37.83 µg/g fresh weight (Fig. 3e, Table 7).

Discussion

In the present study, foliar application of resistance inducing chemicals had considerably increased the phenolic content such as 66.50 µg/g fresh weight after 24 hr to 119.0 µg/g fresh weight after 96 hr, which is statistically significant (Table 3). The phenolic content started to decline thereafter and was minimum after 120 hr (39.90 µg/g fresh weight). Phenols were highest for BABA (110.60 µg/g tissue) followed by SA (109.50 µg/g tissue) and sodium salicylate (96.50 µg/g tissue) over untreated control (13.10 µg/g tissue). Interaction studies showed maximum phenol content after 96 hr of inoculation of BABA (141.30 µg/g tissue) followed by SA (138.30 µg/g tissue) and ASM (135.30 µg/g tissue).

A significant rise in phenolic content during interaction between incompatible host and pathogen, enhances resistance through a hypersensitive response. Phenolic compounds possess fungitoxic properties; their buildup either reinforces the mechanical strength of the host cell wall or forms a barrier to hinder pathogen entry (11). The applications of resistance inducing chemicals had significant increased PO activity in garlic with increasing pattern over time and maximum activity recorded after 96 hr of inoculation (1.44) for BABA followed by ASM (1.34) and sodium salicylate (1.30). The minimum PO activity observed in untreated control (0.18) followed by di-potassium hydrogen phosphate (1.05) and oxalic acid (1.09), respectively

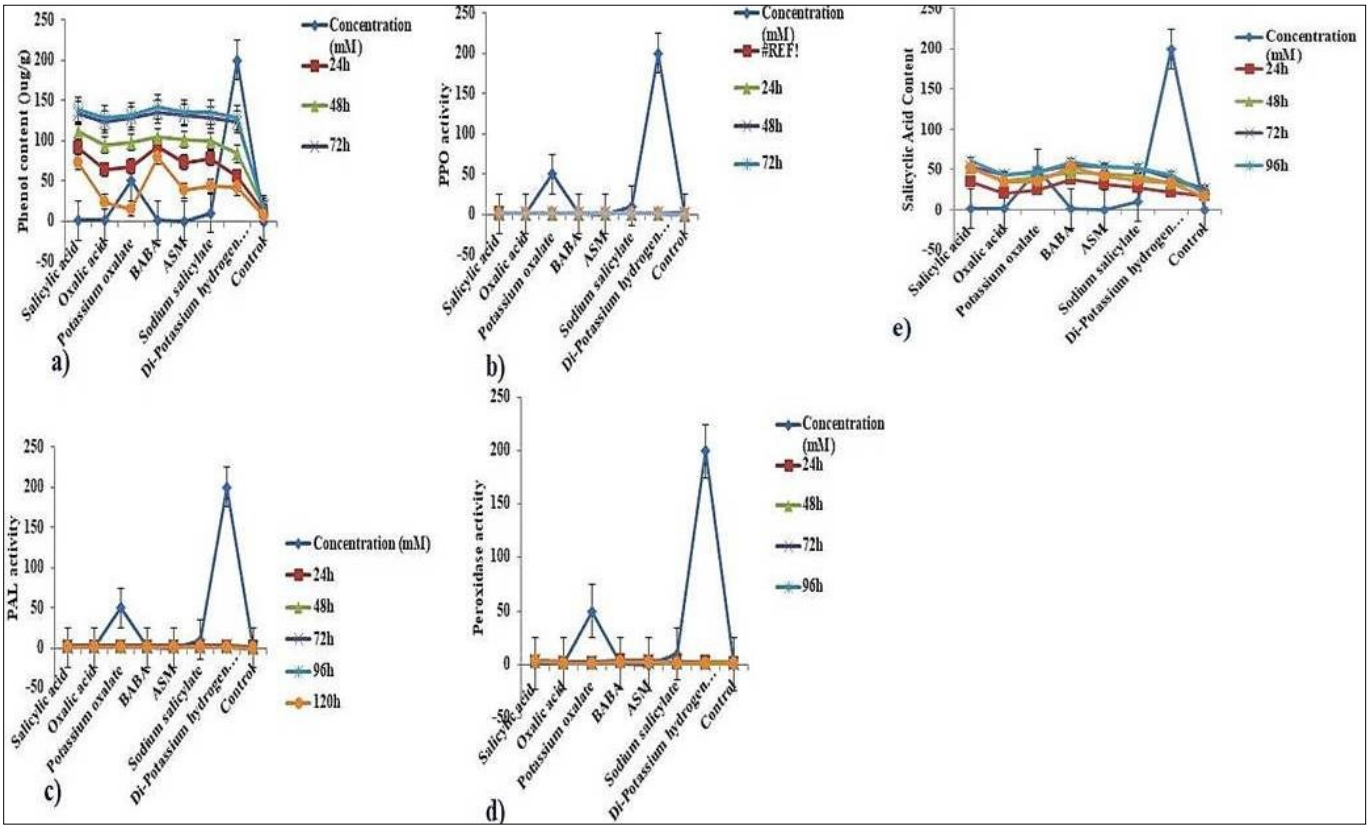

Fig. 3. Variation in different biochemical contents. a) phenolic content; b) PPO; c) PAL d) peroxidase enzyme activities; e) SA level in garlic plants treated with seven resistance-inducing chemicals.

(12). It was statistically at par for treatments BABA and SA. Previous studies have shown an increase in PPO activity on 5th and 9th day post inoculation in tomato and roots and shoots of susceptible chickpea cultivars treated with SA (13,14).

Research depicted significantly increased in the SA level seven days after treatment with chitosan and BTH in grapevine plants, which was considerably low in the non-treated plants. SA inhibits catalase formation linked to more hydrogen peroxide (H_2O_2) or reactive oxygen species production associated with hypersensitive response to pathogens. Thus, it is an intermediary in the signalling pathway that triggers the expression of defense related genes (15). Utilization of the potassium silicate to enhance resistance against white rot disease in onions had increased yield. Potassium dihydrogen phosphate was also used to manage powdery mildew in grapevines, achieving complete inhibition with a 2.5 % KH_2PO_4 application 24 hr prior to inoculation and has significantly reduced disease incidence (16). Additionally, research revealed effectiveness of BABA in controlling downy mildew in lettuce through soil drenching with 1.25 mg of BABA, leading to the fall in disease incidence of over 90 % (17). The foliar application of BABA had significantly controlled the *Alternaria alternata* infection in jujube (*Zizyphus jujube* Mill.) (18).

Conclusion

The present study emphasized on foliar applications of the SA found more effective in reducing the basal rot tolerance followed by BABA, which increased the quantities of phenol and SA and activities of the PAL, PPO and PO enzymes several folds in the treated plants.

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Authors' contributions

AK led the writing of the original draft, developed the methodology, performed formal analysis and investigation, contributed to data curation and visualization and secured funding for the study. MG conceptualized the research, provided supervision, contributed to data curation, and participated in reviewing and editing the manuscript. SKS and SK co-drafted the manuscript, contributed to methodology and visualization, and assisted in data curation and editing. AS contributed to the conceptual framework, supported methodology development, and was involved in data curation. SD participated in data collection and was actively involved in the experimental investigation. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest.

Ethical issues: None

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