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Biochemical defence reactions of black pepper varieties against *Colletotrichum gloeosporioides* incitant of anthracnose disease

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Abstract

Anthracnose caused by *Colletotrichum gloeosporioides* in Black pepper is a severe disease that causes even up to 100 percent crop losses under epidemic conditions. Most of the cultivated varieties of black pepper are highly susceptible to the disease, especially Panniyur 1, the most predominant variety grown in the country. Our study tested 11 black pepper genotypes for their biochemical defense reaction to anthracnose disease. Field observations were initially recorded for two consecutive years in alternate germplasm sites, and the disease incidence ranged between 4.00 and 21.33. The disease incidence under glasshouse conditions upon challenge inoculation was minimal for the variety IISR Girimunda. Upon inoculation with *C. gloeosporioides*, resistant cultivars exhibited significantly higher activity levels of antioxidant enzymes such as peroxidase, polyphenol oxidase, catalase, phenylalanine ammonia-lyase, and superoxide dismutase compared to local check and uninoculated control plants. This suggests that the variety IISR Girimunda's resistance to anthracnose may be attributed to its possession of robust biochemical defense molecules.

Key words: Anthracnose; Peroxidase; Polyphenol oxidase; Catalase; phenylalanine ammonia-lyase; Superoxide dismutase

Introduction

Black pepper (*Piper nigrum* L.), often referred to as the "King of Spices" is the most important and widely used spice in the world, native of moist evergreen forests of the Western Ghats of South India. Several pathogens have been reported to cause diseases in black pepper of which *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. is an important pathogen which causes anthracnose/spike shedding/fungal pollu found in all the black pepper cultivating tracts of India (Biju *et al.,* 2013).

Symptoms of *C. gloeosporioides* are manifested in all the aerial parts, including foliage, stem, spikes and berries. The most devastating effect of anthracnose occurs in misty environments and high-altitude areas during the south-west monsoon period. The severity of the disease varies from 28 to 34 %, causing crop loss between 1.9 to 9.5% (Biju *et al.,* 2013). Presently, the spread of the disease is checked by applications of fungicides, which may negatively affect farmers' income and health; further, it also increases the risk of pesticide contamination and reduces the export value. Thus, cultivating resistant varieties and other capable traits, such as high yield and quality components, would be a viable strategy to curtail this disease.

Several breeding programmes aimed at the incorporation of resistance genes were reported in several crops for the diseases caused by *C. gloeosporioides* (Kim *et al.,* 2008). Still, there are no such reports in black pepper. Resistance to foot rot disease of black pepper is recorded in germplasm and Kottanadan selections (2466, 2471, 2515 and 2433) but not to anthracnose (Bhai *et al.,* 2007). Variation in the resistance level to anthracnose disease in the germplasm and also varieties has been reported by several workers in many crops (Silva *et al.,* 2014; Singh, 2014).

Plants undergo several metabolic functions to defend the invading pathogens by producing enzymes to clear free radicals and reactive oxygen. Several reports focus on changes in enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (P.O.), Polyphenol oxidasae (PPO), catalase (CAT) and superoxide dismutase (SOD). These enzymes serve for the production of bio-resistant substances such as lignin and phytoalexin (Ighodaro and Akinloye, 2018) hence, these enzymes are an alternative method for analyzing the resistance nature indirectly in plants (Liu *et al.,* 2019).

Resistance to anthracnose disease has not been reported so far in black pepper, and the loss caused by *C. gloeosporioides* is always underestimated, further in the era of climate change, which results in heavy rains followed by a large period of mist prevailing in the high altitude regions succumbs black pepper to the disease. Thus, the present study aims to identify resistance sources and explore the resistance mechanism to anthracnose disease in released varieties and local cultivars of black pepper and identify suitable varieties for high altitude and misty regions.

Materials and methods

Field observations: Field observation was recorded at the alternate germplasm maintained at ICAR-Indian Institute of Horticulture Research, Central Horticultural Experiment Station, Chettali, Coorg Dist. Karnataka. The location is situated at 1050 m above mean sea level and average temperature ranged amid 32˚ C and 19˚C. The annual precipitation is 1500 mm with the majority during July and August. The soil is subterranean, dark brown, well drained sandy loam to sandy clay loam. Each replication consists of three plants. The disease incidence was continuously recorded for two years (March and August), 2017-2018; the mean percent disease index was calculated based on the scores as described in

Table 1. From each plant 50 leaves were observed randomly and a score was assigned. The percent disease index was calculated as given below

$$PDI = \frac{(n_0 X 0 + n_1 X 1 + n_2 X 2 ..... n_8 X 8)}{\text{Total number of leaves observed X Maximum grade}} \times 100$$

Where n0 to n8 represent the total number of leaves falling under 0-8 scales, respectively

Based on the disease reaction (PDI), the varieties were classified as highly resistant (<5%), resistant (5.1-10%), moderately resistant (10.1-20 %), moderately susceptible (20.1-30%), susceptible (30.1-40%) and highly susceptible (>50%).

Pathogen: The pathogen *C. gloeosporioides* used in the study was isolated and characterized by ICAR-Indian Institute of Spices Research, Regional Station, Appangala. The sequence of specific regions is already deposited to NCBI carrying the Accession No. KY236319. The culture was maintained in the PDA medium.

***In planta* screening of Black pepper varieties**: Eight varieties released by ICAR-Indian Institute of Spices Research, Calicut, Kerala, India, namely, IISR Sreekara, IISR Subhakara, IISR Pournami, IISR Panchami, IISR Thevam, IISR Sakthi, IISR Malabar Excel and IISR Girimunda, two local cultivars Chumala and Karimunda and one widely spread variety Panniyur 1 was used in the study. Plant varieties at three leaves stage were collected from the nursery and placed in glasshouse conditions for a week. Spore suspension of *C. gloeosporioides* was prepared in a solution containing 5 x 105 spores/mL and sprayed using Ganesh Sprayer. The plants were covered with polythene cover to sustain the humidity for fifteen days. The R.H. was maintained at 95% and temperature 28±2 °C. The percent disease index was calculated up to 45 days at 15 days intervals, as mentioned in Table 1. The experiment was carried out in three replications with ten plants per replication; simultaneously, an uninoculated control was also maintained with Panniyur 1 as local susceptible check.

Tabe 1. The disease rating scale for black pepper anthracnose

Scale	Infection on leaves
0	Nil
1	Isolated spots
2	Sparse spots on young leaves
3	Coalescing spots on young leaves and isolated spots on old leaves
4	Clear coalescing spots on young leaves and old leaves, defoliation of infected young leaves
5	Infection on all aerial parts, distinct crinkling of infected leaves, defoliation of young leaves
6	Infection on all aerial parts, shedding of young leaves and deformity of old leaves
7	Infection on all aerial parts, shedding of young leaves and deformity of old and young leaves
8	Infection on all aerial parts, deformity and defoliation of old as well as young leaves

Biochemical defense characterization: Samples were collected from individual treatments to study the biochemical defense response to pathogen inoculation in rooted black pepper plants under glasshouse conditions. Leaf tissues were collected at the 0^{th}, 1^{st}, 3^{rd}, 5^{th} and 7^{th} day post inoculation. A separate uninoculated control of Panniyur 1 was maintained for comparison.

The leaf tissues collected were incontinently homogenized with liquid nitrogen, and one gram of pulverized sample was extracted with 2 mL of sodium phosphate buffer 0.1 M (pH 7.0).

P.O. activity was assayed as per the procedure described by Hammerschmidt *et al.* (1982). The reaction blend consisted of 2.5 mL of a mixture containing 0.25 percent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide. The activity was expressed as the increase in absorbance at 420 nm $min^{-1}g^{-1}$ of fresh tissue. PPO was estimated using the protocol described by Mayer *et al.* (1965). The response admixture consisted of 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µL of the enzyme extract. To start PPO reaction, 200 mL of 0.01 M catechol was added, and the activity was expressed as a change in absorbance at 490 nm $min^{-1}g^{-1}$ of fresh tissue. PAL activity was assessed as a method explained by Dickerson *et al.* (1984). Enzyme activity was expressed in fresh weight basis as nmol trans-cinnamic acid $min^{-1}g^{-1}$ of fresh tissue.

Assay of superoxide dismutase (SOD) and CAT: Enzyme extraction was carried out by homogenizing 1 g leaf in 2 mL of 0.2 M citrate phosphate buffer (pH 6.5) at 4 °C and centrifuged at 15,000 g at 4 °C for 30 min. The supernatant was used as the enzyme source, and the ability of SOD activity to inhibit the photochemical reduction of NBT was measured. The SOD activity was expressed in SOD units g^{-1} tissue (50% NBT inhibition = 1 unit). To determine catalase activity, we followed the spectrophotometric method described by Chaparro-Giraldo *et al.* (2000), which involved preparing a 3 mL assay mixture containing 100 µL enzyme extract, 100 mM potassium phosphate buffer (pH 7.5), and 2.5 mM H_2O_2 (prepared immediately before use).

Estimation of total phenols: Phenol content was estimated per the procedure by Zieslin and Ben-Zaken (1993). One gram of root tissue was homogenized in 10 mL of 80 percent methanol with a pestle and mortar and agitated for 15 min at 70 °C. To prepare the sample, 1 mL of the methanolic extract was mixed with 5 mL of distilled water and 250 µL of Folin Cicalteau reagent (1N), and the resulting solution was kept at 25°C. After 3 minutes, 1 mL of a saturated solution of sodium carbonate and 1 mL of distilled water were added, and the reaction mixture was incubated for 1 hour at 25°C. The absorption of the blue colour was measured using UV-visible spectrophotometer (Model - Varian Cary 50, Victoria, Australia) at 726 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Cicalteau reagent with a phenol solution (C_6H_6O) and expressed as catechol equivalents g^{-1} tissue weight.

Native gel electrophoresis

Peroxidase (P.O.): To analyze the expression pattern of various peroxidase isoforms, we used native gel electrophoresis, which involved polyacrylamide gel electrophoresis with an 8% resolving gel and a 4% stacking gel. The isoform of P.O. was carried out by the method described by Sindhu *et al.* (1984), and PPO was analyzed by the method Jayaraman *et al.* (1987).

Statistical analysis: The *in vitro* bioassay experiments were laid out in a completely randomized design (CRD), the percent data was transformed using arc sine transformation and statistical analysis was carried out using the online software package WASP 2.0 developed by ICAR-CCARI Goa.

Results

Field Experiments: Observations on the incidence of anthracnose disease in pepper germplasm revealed that, IISR Girimunda plants recorded lesser disease severity of 4.66, 5.33, 4.00 and 5.00 percent, respectively, for the consecutive two years, whereas Chumala, Panniyur 1 and Karimunda recorded maximum percent disease index of 21.33, 19.66 and 21.33 respectively. In general, the disease index was maximum in all the varieties and cultivars during post-monsoon (August) and less during March (Table 2) and diseases' reactions ranged from highly resistance to moderately susceptible.

***In planta* screening of varieties:** *In planta,* screening of varieties under glasshouse conditions showed varied disease symptoms, which were recorded up to 45 days. Maximum disease incidence was noticed in IISR Subhakara (12.00 %) followed by Panniyur 1, IISR Sreekara and Karimunda (10.33 %). The lowest disease incidence was noticed in IISR Girimunda (1.00 %), and this study shows resistance (Table 3).

Induction of Biochemical defense: An increase in the P.O. activities was observed in the black pepper variety up to the fifth day after a decrease in the total activity. Maximum P.O. activity was recorded in the variety IISR Malabar Excel followed by IISR Girimunda (Table 4). The activity of PPO showed variation in their expression pattern. The maximum change in the absorbance value was noticed in the variety IISR Malabar Excel (Table 5). The activity of the PAL enzyme differed among the varieties. Contrastingly to P.O. and PPO, the activity of PAL was maximum in the variety IISR Girimunda (27.85) (Table 6).

Similarly, an increase in activity of catalase activity was noticed in all the varieties with significant variations. Maximum activity was noticed during the 5th day in the variety IISR Girimunda (Table 7). Significant variation was not noticed in the SOD

Table 2. Anthracnose disease incidence under field conditions

Treatment	Varieties	Mean percent disease index March, 2017	Mean per cent disease index Aug, 2017	Mean percent disease index March, 2018	Mean percent disease index Aug, 2018	Disease reactions
T1	Chomala	6.66^{d}	16.66^{a}	8.33^{cd}	21.33^{a}	M.S.
T2	Panniyur 1	9.33^{a}	17.33^{a}	10.66^{a}	19.66^{ab}	MR
T3	Karimunda	8.66^{b}	16.66^{a}	7.33^{ef}	21.33^{a}	MS
T4	Sreekara	7.33^{c}	16.33^{a}	8.66^{bc}	16.33^{cde}	MR
T5	Subhakara	7.33^{c}	16.33^{a}	9.33^{b}	18.66^{bc}	M.R.
T6	Pournami	6.66^{d}	12.66^{b}	9.33^{bc}	16.66^{cde}	MR
T7	Panchami	6.66^{d}	12.66^{a}	8.66^{de}	15.66^{e}	MR
T8	Thevan	5.66^{e}	16.66^{a}	6.66^{de}	16.66^{cde}	M.R.
T9	Sakthi	6.33^{d}	16.66^{a}	7.66^{bc}	18.33^{bcd}	MR
T10	Malabar Excel	6.33^{f}	8.33^{c}	8.66^{g}	12.33^{f}	MR
T11	Girimunda	4.66	5.33^{d}	4.00	5.00^{g}	H.R.
	Mean	6.87	14.15	8.12	16.54	
	CV(%)	3.76	5.67	4.29	5.61	
	SE (d)	0.211	0.655	0.284	0.757	
	LSD @ 1%	0.6012	1.839	0.808	2.158	

MS- Moderately Susceptible, MR- Moderately Resistance, H.R.- Highly Resistance

Table 3. Anthracnose disease incidence under glasshouse conditions

Treatments	Varieties	Mean per cent disease index on 15th day	Mean per cent disease index on 25th day	Mean per cent disease index on 45th day
T1	Chomala	3.33	6.66	6.66
T2	Panniyur 1	2.33	8.33	10.33
T3	Karimunda	1.66	6.66	10.33
T4	Sreekara	2.00	6.00	10.33
T5	Subhakara	1.66	6.66	12.00
T6	Pournami	0.00	1.33	2.66
T7	Panchami	0.00	1.33	2.00
T8	Thevam	1.66	2.33	2.00
T9	Sakthi	1.66	1.66	1.66
T10	Malabar Excel	1.00	1.66	1.66
T11	Girimunda	0.00	1.00	1.00
T12	Uninoculated Panniyur 1	1.66	2.33	2.33
	Mean	1.41	3.83	5.24
	SE(d)	0.072	0.209	0.299
	LSD @ 1%	0.2008	0.5805	1.2918

Table 4. Changes in peroxidase (P.O.) activity in black pepper plants upon challenged with *C. gloeosporioides* under glasshouse conditions

Treatments	Days after inoculation				
	0	1	3	5	7
T1	1.75^{g}	2.27^{cd}	2.07^{de}	2.09^{e}	2.16^{d}
T2	2.26^{efg}	2.11^{de}	3.07^{ab}	3.32^{ab}	3.03^{bc}
T3	1.78^{fg}	1.68^{e}	1.78^{e}	2.39^{de}	2.13^{d}
T4	2.21^{de}	2.7^{bc}	2.96^{b}	3.24^{bc}	2.41^{d}
T5	2.4^{cde}	2.67^{bc}	2.33^{d}	2.72^{cd}	2.39^{d}
T6	2.17^{def}	2.33^{cd}	2.9b^{e}	2.48^{de}	2.59^{cd}
T7	2.57^{cd}	2.98^{ab}	2.45^{cd}	3.38^{ab}	3.11^{b}
T8	2.45^{cde}	2.96^{ab}	3.19^{ab}	3.59^{ab}	3.27^{ab}
T9	2.98^{ab}	3.06^{ab}	3.32^{ab}	3.63^{ab}	3.36^{ab}
T10	3.^{23a}	3.32^{a}	3.48^{a}	3.79^{a}	3.46^{ab}
T11	2.74^{bc}	2.8^{b}	3.19^{ab}	3.68^{ab}	3.67^{a}
T12	2.15^{efg}	2.19^{d}	2.26^{d}	2.15^{e}	2.15^{d}

Table 5. Changes in ploypheonl oxidase (PPO) activity in black pepper plants upon challenged with *C. gloeosporioides* under glasshouse conditions

Treatments	Days after inoculation				
	0	1	3	5	7
T1	0.72^{f}	1.05^{d}	1.13^{b}	1.37^{c}	1.30^{abc}
T2	0.91^{d}	1.31^{b}	1.36^{a}	1.40^{bc}	1.36^{a}
T3	1.00^{c}	1.06^{d}	1.09^{b}	1.21^{de}	1.16^{de}
T4	1.19^{a}	1.42^{a}	1.46^{a}	1.51^{a}	1.30^{abc}
T5	0.66^{f}	0.71f^{g}	0.83^{d}	0.93^{g}	0.94^{g}
T6	0.67^{f}	0.93^{e}	1.07^{bc}	1.26^{d}	1.12^{def}
T7	0.67^{f}	0.94^{e}	0.97^{c}	1.13^{ef}	1.10^{ef}
T8	0.71^{f}	0.78^{f}	1.06^{bc}	1.09^{f}	1.06^{f}
T9	0.81^{f}	1.21^{c}	1.46^{a}	1.50^{ab}	1.32^{a}
T10	1.07^{b}	1.21^{c}	1.42^{a}	1.57^{a}	1.20^{cd}
T11	0.71^{f}	1.31^{b}	1.45^{a}	1.46^{abc}	1.21^{bcd}
T12	0.66^{f}	0.67^{g}	0.71^{e}	0.72^{h}	0.72^{h}

Table 6. Changes in phenylalanine ammonia-lyase (PAL) activity in Black pepper plants upon challenged with *C. gloeosporioides* under glasshouse conditions

Treatments	Days after inoculation				
	0	1	3	5	7
T1	12.07^{de}	12.04^{f}	14.02^{de}	12.68^{e}	10.11^{e}
T2	14^{cd}	16.4^{cde}	19.21^{abc}	20.26^{bc}	21.20^{abc}
T3	11.28^{ef}	14.26^{ef}	16.32^{cd}	13.85^{de}	9.5^{e}
T4	13.73^{de}	14.74^{def}	15.88^{cd}	19.16^{c}	18.18^{cd}
T5	14.39^{cd}	17.16^{bcd}	18.21^{bcd}	21.03^{bc}	20.03^{bcd}
T6	17.78^{b}	17.31^{bcd}	18.85^{bc}	22.3^{bc}	19.66^{bcd}
T7	17.5^{b}	18.98^{c}	19.57^{abc}	23.21^{abc}	21.56^{abc}
T8	14.3^{cd}	16.56^{cde}	17.18^{cd}	20.41^{bc}	18.6^{cd}
T9	16.35^{bc}	19.63^{b}	21.82^{ab}	24.81^{ab}	23.86^{ab}
T10	13.53^{de}	14.23^{ef}	17.26^{cd}	18.23^{cd}	16.21^{d}
T11	20.89^{a}	22.78^{a}	23.48^{a}	27.85^{a}	25.87^{a}
T12	9.14^{f}	7.3^{g}	11.21^{e}	9.88^{e}	6.86^{e}

Table 7. Changes in catalase (CAT) activity in black pepper plants upon treatment with *C. gloeosporioides* under glasshouse conditions

Treatments	Days after inoculation				
	0	1	3	5	7
T1	0.43^{fg}	0.72^{ef}	0.93^{de}	1.15^{c}	1.00^{ef}
T2	0.49^{ef}	0.74^{ef}	1.2^{cd}	1.39^{bc}	1.66^{cd}
T3	0.38^{fg}	0.54^{f}	0.78^{e}	0.68^{de}	0.82^{f}
T4	0.48^{ef}	0.78^{def}	1.21^{cd}	1.01^{c}	1.01^{ef}
T5	0.65^{bcd}	0.82^{de}	0.75^{e}	1.28^{c}	1.28^{de}
T6	0.6^{cde}	0.79^{def}	1.37^{bc}	1.74^{b}	1.69^{bcd}
T7	0.69^{bc}	1.04^{cd}	1.6^{b}	2.2^{a}	2.10^{ab}
T8	0.52^{def}	1.26^{bc}	1.69^{b}	1.76^{b}	1.75^{bc}
T9	0.33^{gh}	1.44^{b}	2.23^{a}	2.37^{a}	2.53^{a}
T10	0.79^{ab}	1.44^{b}	2.24^{a}	2.31^{a}	2.53^{a}
T11	0.86^{a}	1.8^{a}	2.27^{a}	2.57^{a}	2.10^{ab}
T12	0.23^{h}	0.25^{g}	0.3^{f}	0.25^{e}	0.25^{g}

Table 8. Changes in superoxide dismutase (SOD) activity in black pepper plants upon challenged with *C. gloeosporioides* under glasshouse conditions

Treatments	Days after inoculation				
	0	1	3	5	7
T1	1.50	1.80^{a}	2.13^{a}	2.03^{a}	1.96^{a}
T2	1.50	1.81^{a}	2.14^{a}	2.05^{a}	1.97^{a}
T3	1.50	1.81^{a}	2.16^{a}	2.07^{a}	1.96^{a}
T4	1.51	1.81^{a}	2.19^{a}	2.07^{a}	1.95^{a}
T5	1.53	1.81^{a}	2.15^{a}	2.08^{a}	1.97^{a}
T6	1.63	1.81^{a}	2.16^{a}	2.07^{a}	1.99^{a}
T7	1.53	1.81^{a}	2.11^{a}	2.05^{a}	2.00^{a}
T8	1.53	1.81^{a}	2.12^{a}	2.06^{a}	1.99^{a}
T9	1.55	1.81^{a}	2.14^{a}	2.05^{a}	1.95^{a}
T10	1.52	1.81^{a}	2.20^{a}	2.04^{a}	1.96^{a}
T11	1.50	1.83^{a}	2.14^{a}	1.96^{a}	1.98^{a}
T12	1.51	1.51^{b}	1.52^{b}	1.50^{b}	1.50^{b}

activity (Table 8). Variations in the activity of phenol were also observed in all the treatments, the variety IISR Girimunda showed the maximum level of total phenol on 5th-day post inoculation (Table 9).

Table 9. Changes in phenol activity in black pepper plants upon challenged with *C. gloeosporioides* under glasshouse conditions

Treatments	Days after inoculation				
	0	1	3	5	7
T1	8.11^{g}	9.61^{f}	10.64^{f}	11.10^{d}	8.50^{g}
T2	11.00^{cde}	12.6^{cd}	13.12^{e}	10.05^{d}	15.21^{c}
T3	9.82^{f}	10.45^{ef}	8.76^{g}	13.35^{c}	8.96^{g}
T4	12.00^{b}	12.09^{d}	14.66^{bcd}	13.4^{c}	13.39^{de}
T5	10.61^{def}	13.57^{bc}	15.09^{bc}	12.60^{c}	13.27^{de}
T6	11.58^{bcd}	12.19^{d}	13.88^{cde}	13.14^{c}	12.32^{e}
T7	12.22^{b}	13.48^{bc}	15.08^{bc}	15.57^{b}	13.69^{d}
T8	12.21^{b}	13.96^{b}	15.11^{b}	15.00^{b}	13.37^{de}
T9	11.65^{bc}	11.81^{d}	13.8^{de}	16.03^{b}	16.07^{c}
T10	15.31^{a}	17.41^{a}	19.11^{a}	19.40^{a}	18.73^{b}
T11	14.88^{a}	17.29^{a}	18.61^{a}	20.22^{a}	20.24^{a}
T12	10.02^{ef}	11.51^{de}	11.00^{f}	11.24^{d}	11.06^{f}

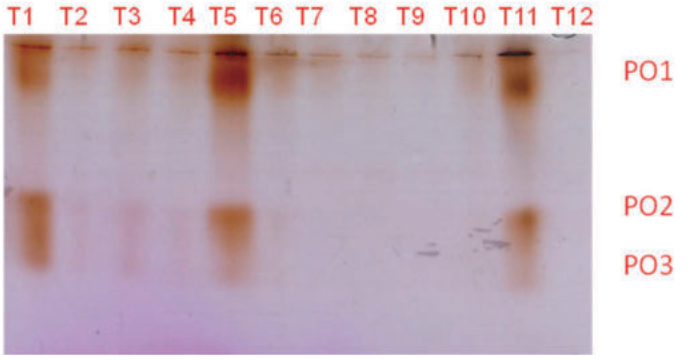

Fig. 1. Expression of PO isoforms upon challenge inoculation with *C. gloeosporioides*

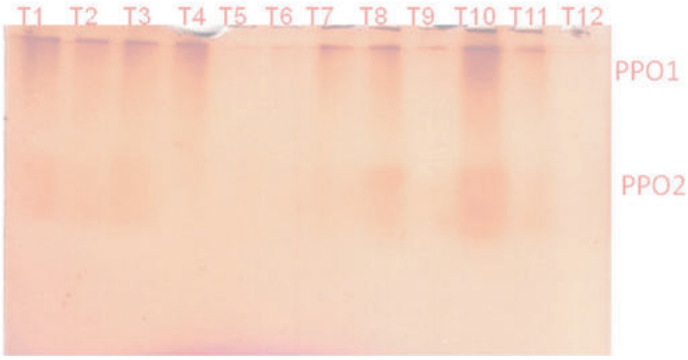

Fig. 2. Expression of PPO isoforms upon challenge inoculation with *C. gloeosporioides*

Native gel electrophoresis: The results of native gel electrophoresis did not show much difference in P.O. and PPO expression levels. Most of the treatments showed expression of three peroxidase isoforms; among them, T1, T5 and T11 showed a higher level of expression (Fig 1). Two isoforms, *viz.,* PPO1 and PPO2, were observed in IISR Girimunda and IISR Thevam (Fig 2).

Discussion

Anthracnose of black pepper is one of the most devastating diseases since it infects all the aerial parts of the plants; spike shedding is a serious issue that causes heavy loss to growers. The characterization of varieties for resistance reaction studies was

done with the initial field observations recorded during March 2017 in an alternate germplasm site. More than thirty varieties and local cultivars are maintained; each showed a variable reaction. IISR-released varieties and dominant cultivars grown in the region were selected for glasshouse studies. Natural disease incidence was recorded under field conditions in the germplasm consecutively for two years during (March and August), 2017 and 2018. For the two years, IISR Girimunda plants recorded lesser disease severity of 4.66, 5.33, 4.00 and 5.00 percent, respectively. In contrast, Chumala, Panniyur 1 and Karimunda recorded maximum disease index. Under glasshouse conditions, Maximum disease incidence was noticed in IISR Subhakara (12.00 %), followed by Panniyur 1, IISR Sreekara and Karimunda (10.33 %). The least disease incidence was noticed in IISR Girimunda (1.00 %), and this study shows the presence of resistance.

The presence of biochemical defence molecules in the plant hinders infection and disease formation. Peroxidase is a key enzyme in lignin biosynthesis (Almagro *et al.,* 2009), which has been implicated in several physiological functions that may contribute to resistance. In our research, we noted enhanced P.O. activity in the IISR Malabar Excel variety, with IISR Girimunda (3.68) exhibiting the subsequent highest activity. The connection between elevated P.O. activity and the resistance of *Hibiscus trionum* to *Verticillium dahliae* was observed, showing a positive correlation with resistance or susceptibility levels in wild Malvaceae species, as demonstrated by Golubenko *et al.* (2007). Accumulation of hydrogen peroxide at the site of pathogen infection is one of the first detectable symptoms of plant and pathogen defense interaction. Plant peroxidases are highly responsible for Reactive oxygen species (ROS) accretion in response to pathogen infection (Trujillo *et al.,* 2004). Increased activity of peroxidase was noticed in black pepper cultivar (04-P24) upon infection with *Phytophthora capsici* and suggested to be used as a biochemical marker for identification of resistance (Bhai *et al.,* 2021)

Polyphenol oxidases (PPO) are enzymes that use molecular oxygen to catalyze the oxidation of mono phenolic and ortho diphenolic compounds. The covalent modification and cross-linking of nucleophilic substituents of amino acids and proteins by PPO-derived quinones are thought to exert an anti-nutritive defense against plant pathogens and insects (Beecher *et al.,* 2012; Tran *et al.,* 2012). During incompatible (resistant) plant–pathogen interactions, PPOs are induced as recorded in tomato Fusarium wilt, late blight of potato and bacterial wilt (Poiatti *et al.,* 2009). In this study, IISR Malabar Excel expressed major activity of PPO followed by IISR Sreekara.

Phenylalanine ammonia-lyase (PAL) plays an important role in the biosynthesis of various defense chemicals and it is the first enzyme in phenyl propanoid metabolism (Pandey *et al.,* 2017; Yu *et al.,* 2018). PAL activity could be induced during plant-pathogen interactions for initiation of the salicylic acid (S.A.) pathway (Duan *et al.,* 2014) PAL (*CaPAL1*) gene, which was induced in pepper leaves by avirulent *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) infection (Kim *et al.,* 2014). Phenylpropanoid-derived compounds have been documented to play a vital role in plant defense to microbial pathogens (Kim and Hwang, 2014). In the present investigation, the maximum activity of PAL is recorded in the black pepper variety IISR Girimunda, suggesting the initiation of the phenylpropanoid pathway and S.A. pathways.

In reply to the creation of reactive oxygen molecules in the plant pathogen interface to defend the adjacent cell, certain defensive enzymes are involved, and the ROS generated by pathogens can be scavenged by defending enzymes, which can result in the efficient diminution of oxidative burst (Gechev *et al.,* 2003). Among these protective enzymes, superoxide dismutase (SOD) serves as the first line of defence against ROS (Apel and Hirt, 2004) and its enzymatic battle results in the formation of H_2O_2. CAT then detoxifies the disintegration of H_2O_2 into water and oxygen. An increase in activity of catalase activity was noticed in all the varieties with significant variations. Maximum activity was noticed in the variety IISR Girimunda (2.57). Significant variation was not noticed in the SOD activity, but increased activity of SOD was observed in all the treatments up to 3rd day, and thereafter, it decreased. Enhanced activities of the cell-protective enzymes SOD and CAT is essential elements of defense responses in pea seedling leaves to oxidative stress triggered by pea herbivores and plant pathogens (Bednarski *et al.,* 2013).

The presence of phenolic compounds enhances the mechanical strength of the host cell wall and also inhibits the invading pathogenic organisms. Pathogenic hyphae bordered by phenolic substances made substantial morphological variation, including cytoplasmic ineptitude and loss of protoplasmic content. Significant variation in the activity of phenol was also observed in all the varieties, in the present study, IISR Girmunda showed maximum phenol content, and it is one of the major reasons for resistance to *C. gloeosporioides*. Induction of enzymes such as PAL and P.O. leading to the accumulation of phenolics and lignin can occur in response to insect and pathogen attacks (Tahsili *et al.,* 2014).

The use of resistant varieties provides the most effective method of managing the disease. Hence, an approach towards integrated disease management and the use of resistant varieties is critical to reducing pesticide use and cultivating eco-safe agriculture. Further, under high altitudes and misty climatic conditions, fungicidal sprays are not very effective, and the cultivation of resistant variety is the only solution to increase the production of black pepper.

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Compliance with ethical standards

The co-authors have no conflicts of interest, and no human participants or animals were used in the current research. All the authors provided informed consent for the submission of the manuscript.

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