ORIGINAL ARTICLE

Antioxidant potential of chili seedlings against anthracnose

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Abstract

Introduction: Diseases and its causative pathogens are the remarkable challenges faced by a plant for its survival, eventually to cause severe food security problems. Host resistance is an eco-friendly approach which requires a depth understanding on host–pathogen interactions. During these interactions, a cascade of defense responsive antioxidants was induced by a pathogen with respect to the type of cultivar while causing a disease. These array of enzymes can be biochemically detected and aid as markers to identify the ability of the host to restrict the pathogen. This study was conducted to detect the *Colletotrichum truncatum* elicited defense responsive antioxidants among the seedlings of G-4, LCA-353, BSS-355, and S-2 chili cultivars. **Materials and Methods:** Chilli seedlings of 25 days old were spray inoculated with pathogen and after 7 days of infection their antioxidant potential was analyzed by estimating few enzymes, *viz.,* phenylalanine ammonia lyase, polyphenol oxidase, superoxide dismutase, catalase, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, reducing power, and total phenol content. **Results:** Biochemical estimations revealed that G-4 cultivar to be a resistant variety showing increased production of defense induced enzymes in all parameters while in contrast BSS-355 remained as susceptible variety. **Conclusion:** Anthracnose induced antioxidative enzymes showed a significant correlation between the enzymes, unveiling G-4as resistant and BSS-355 to be the susceptible chili cultivar with the highest and lowest enzyme activities. Identified resistant cultivar can be used in anthracnose resistant breeding programs and also these enzymes can be employed as biochemical markers to screen the chili genotypes against anthracnose disease.

Key words: Anthracnose, antioxidants, *Capsicum annuum*, defense responsive enzymes

INTRODUCTION

Chilli (*Capsicum annuum* L.) was deliberated to be the fourth most essential vegetable crops cultivated worldwide. It is an indispensable vital element most commonly used in food sector for its spicy taste. Chilli fruits were highly enriched with antioxidants such as vitamin A, vitamin C, flavonoids, β-carotene, lutein, zeaxanthin, and cryptoxanthin which scavenge body from harmful effects of free radicals and develop resistance against several disease conditions. They also possess huge amount of minerals such as iron, potassium, and magnesium which help in boost the immune system and lower the cholesterol levels.[1] Capsaicin an alkaloid compound of chilli was known to possess high medicinal properties such as antibacterial, anticarcinogenic, analgesic, and anti-diabetic properties.[2]

Anthracnose is a prominent devastative disease caused by *Colletotrichum truncatum* with a significant deterioration on crop at both pre- and post-harvest stages majorly attributing to yield loss of chili in India.[3] This fungal disease spreads very rapidly during moist humid conditions leaving very limited chances for farmers to protect the crop. During 2010-2011 India is the largest producer, exporter, and consumer of chilies in the world. Yet, recently, it has been declined to third position due to several biotic and abiotic constrains hampering its production.[4]

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Plants inherently possess immunity which can be triggered by pathogens and induce defense responsive antioxidants which may slow down or restrict the pathogenic nature from spreading a disease. Plants are comprised with lots of natural antioxidants in numerous and diversified forms which are mostly capable of preventing destructive effects of reactive oxygen species. Their defense mechanism varies with respect to type of host. Chilli is rich in biologically active antioxidants such as vitamin A, E, and C involved in synthesis of collagen tissue, metal ion metabolism and antihistamine reactions, also act as immune enhancers and proved to be potent against various types of cancer, coronary heart diseases, advanced age-related macular degeneration and cataracts.[5] Phenolic pungent capsaicin is involved directly in scavenging various free radicals.[6] Flavonoids intake was proven to reduce the risk of cancer. All these antioxidants were reported to possess biochemical and pharmacological effects including anti-inflammation and anti-allergy. The total antioxidant potential is a significant tool for investigating the relationship between dietary antioxidants and pathologies induced by the oxidative stress hence this investigation was aimed at identifying anthracnose resistant or susceptible chilli cultivars by estimating the defense responsive compounds.

MATERIALS AND METHODS

Isolation and Identification of *C. truncatum*

Isolation was carried out using tissue transplanting technique. Infected portions (1-3 mm in size) were excised from the margins of the pericarp and were surface sterilized with sodium hypochlorite (1%) for 2 min, followed by thorough washing with distilled water and dried on a tissue paper. These dried sterilized portions of infected fruits were transferred onto water agar medium plates and incubated at 25°C for 3 days. Sporulating hyphae were identified under microscope and cultured on potato dextrose agar (PDA) medium plates and incubated at 25 ± 2°C for a week to obtain pure single colonies following the single spore technique.[7] Pure cultures were then stored at 4°C on PDA slants for future experiments. Identification was done by morphological features such as size and shape of conidia, and presence of setae. Cultural characteristic features like colony color and its growth rate, sectoring pattern of surface mycelium were observed and recorded 10 days after inoculation on PDA medium.

Preparation of Spore Suspension

Sterile distilled water (10 ml) was poured onto the surface of 10 days old *C. truncatum* culture to obtain conidial suspension by scraping the conidial mass using a sterile glass slide and suspension was filtered through double layer cheesecloth to remove mycelia and cultural debris. Conidial concentration was adjusted to (10^5 spores/ml) with sterile distilled water using hemocytometer.

Plant Material

In this study most prominent chilli varieties cultivated by farmers from South India, *viz.,* G-4, S-2, LCA-353, and BSS-355 were selected. Seeds were sown in crates filled with a mixture of coco-peat, vermi-compost and black soil, and watered. Chilli seedlings (25 days) were taken for experimental studies.

Spray Inoculation Method

Spore suspension of *C. truncatum* was done by spray inoculation method as described by Susheela[8] onto chilli seedlings and infected seedlings tray were treated as test and uninfected trays were treated as control, both the trays were watered regularly and incubated at 25 ± 2°C. Various biochemical parameters were analyzed in the leaves of control and infected seedlings on the 7th day after inoculation.

Assay of Total Phenol Content (TPC)

Leaves of 1 g from uninfected (control) and infected (test) chilli seedlings were collected and homogenized separately in mortar and pestle using 5 ml of 80% methanol. To 1 ml of extract, 0.5 ml of Folin–Ciocalteu reagent, and 7.5 ml ddH_2O was added. The mixture was set aside at room temperature for 10 min. Then, 1.5 ml of 20% sodium carbonate (w/v) was added to the solution and incubated at 40°C for 20 min in a water bath. Cool the solution in an ice bath. Finally, absorbance was recorded at 755 nm using a spectrophotometer. Total phenolic content was measured as described by Sultana *et al*.[9] The standard curve was prepared using gallic acid and the results were expressed in mg gallic acid equivalent/gram of fresh weight of the sample.

Assay of Phenylalanine Ammonia Lyase (PAL)

Leaf tissue (1 g) of both control and test was homogenized in 5 ml of ice cold Tris buffer (100 mM, pH 8.8) containing 1.2 mM β-mercaptoethanol and the resultant mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was used as enzyme source. To 0.3 ml of enzyme source add 1.2 ml of 25 mM Tris buffer (pH 8.8) and 1.5 ml of substrate 12 mM L-phenylalanine. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Geetha *et al*.[10] with minor modifications. One unit of enzyme activity was expressed as trans-cinnamic acid/min/mg protein.

Assay of Polyphenol Oxidase (PPO)

The enzyme extract was prepared by homogenizing plant tissue (0.5 g) in 2.0 ml of the extraction buffer containing sorbitol, NaCl, and tris-HCl for both control and test leaves separately. The homogenate was centrifuged at 2000 g for 10 min; resultant supernatant was used as enzyme source for the assay. Primarily 2.5 ml of phosphate buffer and

0.3 ml of catechol solution were added in a cuvette and absorbance was read at 495 nm. Then, 0.2 ml of enzyme extract was added into the same cuvette and change in the absorbance was recorded for every 30 s up to 5 min in at 495 nm in spectrophotometer. One unit of catechol oxidase is defined as the amount of enzyme that transforms 1 µmole of dihydrophenol to 1 µmole of quinone per minute. Enzyme units in the sample = K × (ΔA/minute) where, ΔA = change in absorbance. K for catechol oxidase = 0.272. The activity of PPO was estimated as described by Jayachitra and Krithiga[11] with minor modifications and expressed in the terms of change in absorbance min^{-1} mg^{-1} of protein.

Assay of Peroxidase (POD)

Leaf tissue (1 g) from control and test samples was homogenized with 4 ml of 0.1 M phosphate buffer (pH 6.5) and centrifuged at 10,000 rpm for 10 min, resultant supernatant was used as enzyme source. To 0.5 ml of enzyme extract add 1.5 ml pyrogallol solution (0.05 M) and 0.5 ml of H_2O_2 was added and mixed thoroughly. The change in absorbance at 430 nm was recorded every 30 s up to 3 min in a spectrophotometer. POD activity was assayed by the method proposed by Abhayashree *et al*.[12] with minor modifications and is expressed in the terms as change in absorbance of reaction mixture min^{-1} mg^{-1} protein.

Assay of Superoxide Dismutase (SOD)

Leaf sample (1 g) of control, the test was homogenized in pre-chilled mortar and pestle using 3.0 ml of potassium phosphate buffer and centrifuged at 2000 rpm for 10 min. Supernatant was used for the assay. Add 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulfate, 0.3 ml of nitro blue tetrazolium (NBT), and water to 0.2 ml of enzyme source in 1.0 ml of water. NADH (0.2 ml) was added to initiate the reaction and incubate at 30°C for 90 s. Then, 1.0 ml of glacial acetic acid was added to stop the reaction. The reaction mixture was then shaken with 4.0 ml of n-butanol, allowed to stand for 10 min and centrifuged. The intensity of chromogen in the butanol layer was read at 560 nm in a spectrophotometer. SOD was evaluated according to the method by Naithani *et al*.[13] with minor modifications. One unit of SOD enzyme activity was defined as the amount of enzyme that gave 50% inhibition of NBT to get reduced in 1 min, and activity was measured in the terms of units/minute/mg protein.

Assay of Catalase (CAT)

The control, test leaf sample (1 g) was homogenized in pre-chilled mortar and pestle using 5 ml of 50 mM potassium phosphate buffer containing 0.1 mM ethylenediaminetetraacetic acid and resultant solution was centrifuged at 12,000 rpm for 10 min at 4°C. Collect the supernatant which is used as enzyme source. To the enzyme extract add 2.5 ml of 50 mM potassium phosphate buffer and 100 mM H_2O_2. CAT was determined by monitoring the decomposition of H_2O_2 at 240 nm for 3 min and its activity was expressed as change in optical density at 240 nm/min/mg protein. CAT was assayed using the method as described by Malekzadeh *et al*.[14] with minor modifications.

Assay of 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

Leaf tissues (1 g) of both control and test samples were homogenized with 5 ml of methanol and incubated overnight at room temperature. Filtrate (1 ml) was added to 3 ml of 0.1 mM DPPH and incubated at dark conditions for 30 min. Absorbance was read at 515 nm using spectrophotometer and percentage of DPPH scavenging activity was determined as per intensity of absorbance and calculated using the formulae,

$$\% \text{ DPPH Activity} = (A_{control} - A_{sample}/A_{contol})*100$$

Standard curve was calibrated using vitamin C and activity was expressed as mg vitamin C equivalent/gram of fresh weight as described by Okoh *et al*.[15]

Assay of Reducing Power

Leaf tissues (1 g) of both control and test samples were homogenized with 5 ml of methanol. To 1 ml of filtrate, 5 ml of phosphate buffer (2 M, pH 6.6) and 5 ml of 1% potassium ferricyanide was added and incubated at 50°C for 20 min. Then, 5 ml of 10% trichloroacetic acid was added to the reaction mixture and centrifuged at 4000 rpm for 10 min. To 5 ml of supernatant, 5 ml of ddH_2O and 1 ml of 0.1% ferric chloride was added and absorbance was read at 700 nm using spectrophotometer. Antioxidant activity was expressed based on intensity of the absorbance as described by Iqbal *et al*.[16]

RESULTS

Morphological Characterization of *C. truncatum*

Topography of isolated pure culture exhibited characteristic features such as white cotton type fluffy mycelial growth, and black color colony with orange colored conidial masses as Figure 1a and b. Colony growth rate per day is 9.2 mm in diameter as reported by Chowdhary *et al*.[17] Microscopical identification was done based on conidia size and shape, presence of setae. Results revealed that conidia is of falcate in shape with 22.3 µm in length and 3.4 µm in width based on the above cultural characteristic features and microscopic observations. The isolated pathogen was of similar to the studies of Srideepthi *et al*.[18] and was confirmed to be *C. truncatum.*

Infection to Seedlings

Raised seedlings were spray inoculated with *C. truncatum* conidial suspension of 5×10^5 conidia/ml, which is suitable concentration for a pathogen to cause a disease in plant at *in*

vitro conditions. After 7th day of inoculation, leaves of both control and infected samples were observed for anthracnose lesions and are used for all the biochemical parameters. Experiments were conducted in triplicates and results were expressed in mean ± standard deviation.

Estimation of TPC

In this investigation, average phenol content among four cultivars studied at control and treated conditions was 61.03 mg/g FWt and 66.45mg/g FWt, respectively. Cultivar G-4 showed highest (74.90 mg/g FWt) content of phenolic compounds while BSS-355 found to poses the lowest amount (62.17 mg/g FWt) [Figure 2].

Estimation of PAL Activity

Enzyme activity of PAL was maximum in G-4 (0.85 nmol cinnamic acid/min/mg protein) while minimum in BSS-355 (0.24 nmol cinnamic acid/min/mg protein). Average activity of PAL enzyme among cultivars in control and infected conditions was 0.34 and 0.52 nmol cinnamic acid/min/mg protein, respectively [Figure 3].

Estimation of PPO Activity

Average PPO enzyme activity among control and treated plants was 57.07 units/mg protein and 68.59 units/mg protein, respectively [Figure 4]. In this work, cultivar G-4 (74.66 units/mg protein) revealed to displayed highest PPO enzyme activity, followed by S-2 (71.66 units/mg protein), LCA-353 (67.53 units/mg protein), and least being BSS-355 (60.53 units/mg protein).

Estimation of POD Activity

Significant difference was observed in POD enzyme activity between control plants and infected plants. Average POD enzyme activity was found to be 10.88 units/mg protein and 17.63 units/mg protein in control and infected seedlings, respectively [Figure 5]. Among four cultivars, G-4 (19.88 units/mg) exhibited higher POD enzyme activity while LCA-353 (15.83 units/mg) exhibited lower enzyme activity.

Estimation of SOD Activity

SOD enzyme activity was recorded high in G-4 (0.85 units/g FWt), followed by BSS- 355 (0.8 units/g FWt), S-2 (0.76 units/g FWt), and LCA-353 (0.73 units/g FWt) in treated seedlings. In control seedlings, average SOD enzyme activity among cultivars was 0.35 units/g FWt [Figure 6].

Estimation of CAT Activity

Average CAT enzyme activity was observed to be 1.34 units/mg protein among the cultivars at control conditions while it was 3.38 units/mg in infected cultivars [Figure 7]. Among infected cultivars, highest CAT activity was exhibited by G-4 (4.41 units/mg protein) and BSS-355 (2.34 units/mg protein).

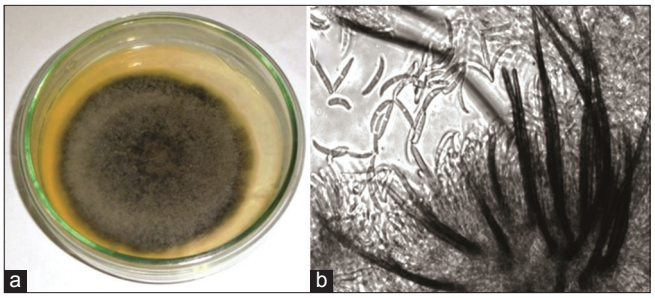

Figure 1: (a) *Colletotrichum truncatu*m on potato dextrose agar medium, (b) microview of conidia with setae

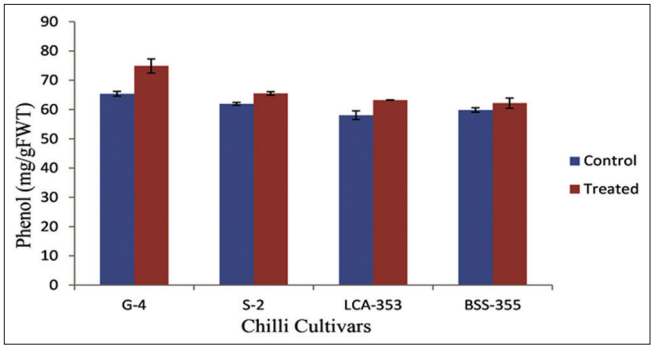

Figure 2: Total phenol content among four cultivars under control and stress conditions

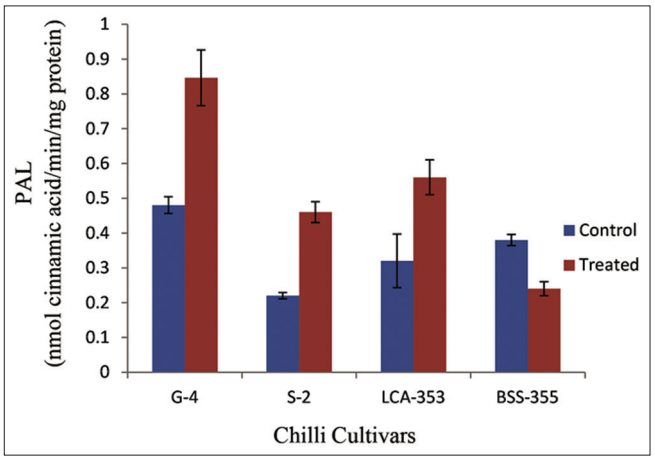

Figure 3: Phenylalanine ammonia lyase enzyme activities in uninfected and infected seedlings of chili

Estimation of DPPH Radical Scavenging Activity

Among the four cultivars investigated, G-4 showed highest percentage of DPPH radical scavenging activity (75.75%) while BSS-355 appeared to have the lowest (48.52%) and cultivars S-2 and LCA-353 exhibited average DPPH scavenging activity [Figure 8].

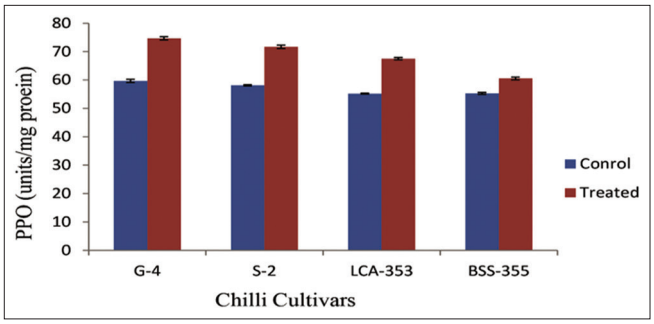

Figure 4: Polyphenol oxidase enzyme activities among control and test seedlings of chili cultivars

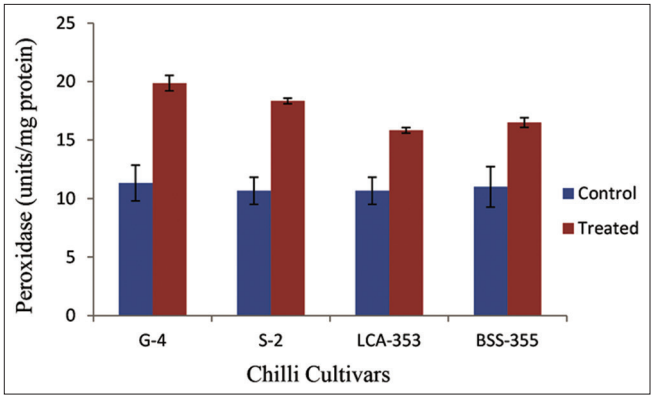

Figure 5: Peroxidase enzyme activities before and after *Colletotrichum truncatum* infection in chili

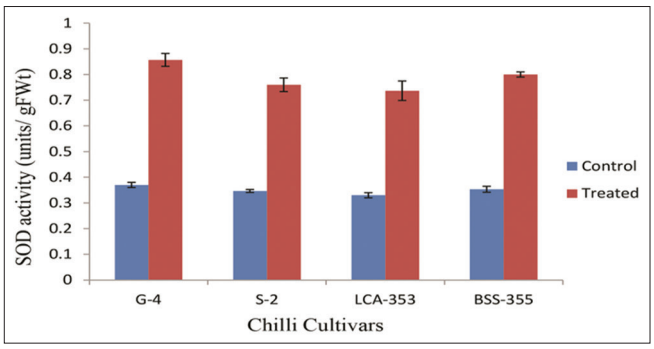

Figure 6: Superoxide dismutase enzyme activities among control and treated chili cultivars

Estimation of Reducing Power

In this work, average reducing power across cultivars was 67.65%. Reducing power was highest in G-4 (76.97%), followed by S-2 (68.23%), BSS-355 (63.05%), and lowest was found in LCA-353 (62.36%) [Figure 9].

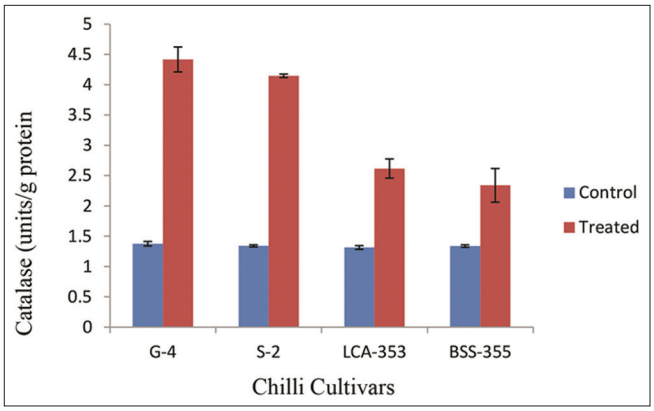

Figure 7: Catalase activity among chili cultivars under control and treated conditions

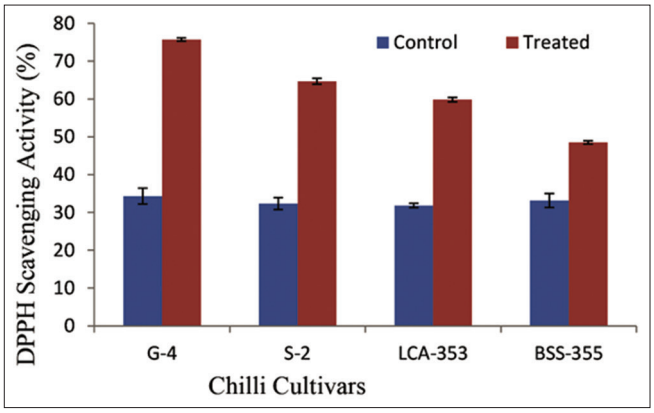

Figure 8: 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity of chili at both control and stressed conditions

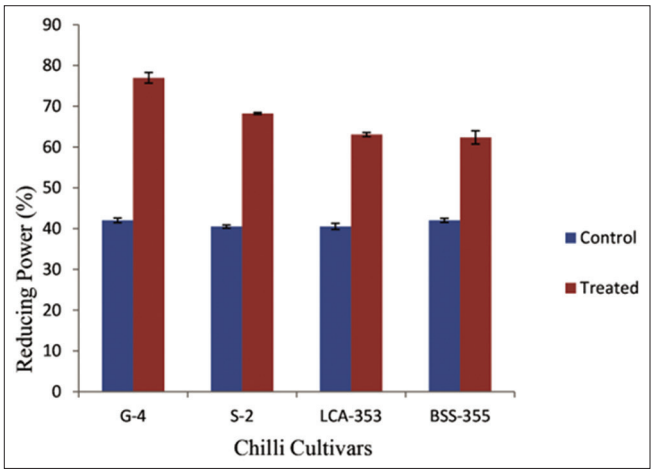

Figure 9: Reducing power of chili cultivars at control and treated conditions

DISCUSSION

Antioxidant activity of plant is mainly ascribed to the occurrence of phenolic compounds.[19] Phenolic compounds are the primary defense-related enzymes normally released or induced in plant to adapt tolerance from various stressful conditions. Phenolic compounds have an ability to modify gene expression.[20] In this work all the cultivars had exhibited relatively higher amounts of phenolic compounds when infected than in normal conditions. Under biotic stress conditions cultivar G-4 was known to be resistant among them as it had exhibited high levels of phenols which consequently enable the production of ROS to scavenge effectively against toxic free radicals and enhance the

survival ability of the plant. Phenols are potential phytoalexins or phytoanticipins mainly involved in signaling resistance mechanism of a plant to fight against pathogen attack.[21]

In this study, cultivar S-2 showing high percent DPPH scavenging activity also showed high phenol content and BSS-355 having lowest DPPH scavenging activity has low phenol content. Correlation studies between percent DPPH radical scavenging activity and phenolic compounds revealed a correlation of 0.98. Observed correlation demonstrates strong relation between presence of phenolic compounds and radical scavenging effect. Wangcharoen and Morasuk[22] also reported significant correlation between percent DPPH radical scavenging assay and TPC. DPPH scavenging activity varied across the cultivars ranging from 48 to 75%. This variation may have been resulted as a consequence of dissimilar profiles of reducing substances among the cultivars. Pulido *et al*.[23] described that these differences in concentration of reducing substances, will result in the changes of antioxidant activities among different cultivars of same species. Furthermore, cultivars having good reducing powers showed relatively high phenol contents which in turn facilitates for better radical scavenging. Antioxidant activity of a compound has been reported to correlate with its reducing power.[24]

Metabolic changes in plants resulting from wounding or pathogen invasion have been reported in numerous host–pathogen systems.[25] Expression of several defense-related enzymes (such as PAL, POD, PPO, and SOD, and CAT) is known to play a major role in determining degree of resistant a plant can possess against phytopathogens. These enzymes are either directly or indirectly involved in Host Resistance development.[26] Hence, this investigation looked on the levels of these enzymes resulting with host resistance.

PAL is a key enzyme of phenylpropanoid pathway, involved in the synthesis of phytoalexins and salicylic acid possessing antifungal activity to stimulate plant defense responses. PAL activity got increased in G-4 variety after 7 days of inoculation when compared to that of S-2 and LCA-353. While significant decrease was observed in BSS-355 cultivar. In plants, if pre-existing antifungal phenols are not sufficient to halt the development and infection processes of pathogen then plant select PAL mediated phenolic pathways to defend the pathogen attack. Interestingly, G-4 cultivar showed higher phenol and PAL contents confirming their resistance mode. Ghosal *et al*.[27] also reported that triggering of PAL and a successive increase in phenol contents were considered to be the key enzymes associated with resistance mechanism in plants.

PPOs are oxidoreductases involved in metabolizing plant mono or diphenolic compounds into quinones leading to the generation of reactive oxygen species and protein complexes in response to pathogen attack. POD is involved in the oxidative polymerization of phenolic compounds involved in synthesis of lignin and cross-linking of isodityrosine bridges in cell walls. These compounds act as barriers against pathogen invasion and hence constitute part of host resistance mechanisms. Hence, both POD and PPO were considered to be key enzymes in plant defense system. Among four cultivars G-4 variety was considered to be a resistant cultivar as it showed accumulated levels of both PPO and POD against the pathogens survival than S-2, BSS-355, and LCA-353 cultivars. Results were similar to that of Meena *et al*.[28] who reported increased activity of POD and PPO in geminivirus inoculated leaves of capsicum.

Increased activity of SOD is often correlated with increased tolerance of the plant against environmental stresses. This analysis also revealed a significant increase in SOD enzyme activity when plants were infected with pathogen. This increase would probably indicate a mechanism adapted by plant to overcome stress. In this work, SOD enzyme was more in G-4 which is considered to be tolerant as it has accumulated relatively higher amounts of antioxidant enzymes which would help the plant to resist pathogen invasion. It was suggested that SOD can be used as an indirect selection criterion for screening drought-resistant plant materials.[29]

Under stress conditions, there is an accumulation of higher amounts of H_2O_2. When cells are stressed for energy and are rapidly generating H_2O_2 through catabolic processes, H_2O_2 is degraded by CAT in an energy efficient manner.[30] There was a significant difference in the amount of CAT activity among the cultivars between control and treated conditions. This difference is due to the difference in H_2O_2 accumulation in the cultivars. Interestingly cultivars which showed higher PPO and PAL activity exhibited high CAT activity. Recently, increased activity of PAL, POD, PPO, and also antioxidant enzyme CAT was observed in ripe and green chili fruits on infection with *Colletotrichum capsici*.[31] In this analysis, a strong correlation was found among different antioxidant enzymes PPO, POD, and PAL whose activity has been increased significantly in infected plants. Okey *et al*.[25] also reported that a significant increase in these enzyme activities was observed in inoculated and wounded cocoa clones. The researchers also reported that clones with higher enzyme activities exhibited resistance to *Phytophthora palmivora* infection. From the results of this study, G4 variety can be considered as a resistant cultivar. Hence, this variety can be used in anthracnose resistance development breeding program.

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