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Stimulation of polyphenol, flavonoids and phenylalanine ammonia lyase (PAL) affected by the infection of wilt disease of *Cuminum cyminum* caused by *fusarium oxysporum*

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Abstract
Plants have developed defense mechanisms to defend themselves against most potential microbial pathogens and diseases. Phenolics (polyphenol, flavonoids) and PAL play an important role in the defense mechanism of the plants. So, this study is carried out to analyze the metabolic modifications in cumin plant after the infection with the pathogen (*Fusarium oxysporum)* by estimating the levels of total phenolic compounds and the activities of phenylalanine ammonia lyase (PAL) in 15 days and one month old cumin plants. The PAL proved high activity in infected plants, edifying the active phase in the synthesis of secondary metabolites in the cumin plant post-infectionally. As a consequence, in infected plants the contents of polyphenols along with flavonoid considerably exceeded in contrast to control plants. Polyphenol, flavonoid content and PAL activity were determined in two varieties viz. GC-4 (resistant) and RZ-209 (susceptible) control and pathogen (*Fusarium oxysporum*; Wilt) inoculated *Cuminumcyminum* (cumin) plants. Polyphenol and flavonoid content were higher in pathogen inoculated plants as compared to control plants with an increase till 168 h. A similar pattern of higher PAL activity was observed after pathogen inoculation with a maximum at 2 h in 15 days and one month old plants of var. GC-4 and in var. RZ-209. The results indicate a role of polyphenol, flavonoid and PAL in the defense response of *Cuminum cyminum*, an economically important spice of arid and semi-arid regions. The obtained results give important information concerning the plant-pathogen interactions, in the defense response for cumin improvement programs seeking the adaptation to diverse range of fungal attack along with adverse environmental factors.

Keywords: *Cuminum cyminum*, fusarium oxysporum, defense response, polyphenols; flavonoids; phenylalanine ammonia lyase

Introduction
Plants responds to pathogen attack or elicitor treatments by activating a wide variety of protective mechanisms designed to prevent pathogen replication and spreading [1]. The defense mechanisms including the rapid production of reactive oxygen species [2]; alterations in the cell wall constitution, accumulation of antimicrobial secondary metabolites known as phytoalexins [3]. Polyphenols are lipophilic in nature having more than one hydroxyl group, various studies proved that accumulation and deposition of phenols to cell wall materials and on cell walls is usually considered as an increase in resistance to fungal hydrolytic enzymes as well as a physical barrier against fungal penetration [4]. Flavonoids, a large subgroup of secondary metabolite and considered as phenolic compounds[5] (Woo *et al*., 2002), protect plants against various biotic and abiotic stresses and play a crucial role in the interaction between the plant and their environment [6]. Phenylalanine ammonia lyase mediates the formation of cinnamic acid from phenylalanine [7] which is a crucial branch point of primary and secondary metabolism and is the first and most important regulatory step in the formation of many phenolic acids.
Cumin (*Cuminum cyminum L*.) is an annual plant belongs to Family *Apiaceae* and it is grown for production of the dry ripe fruits. Cumin is produced in the warm regions of the world, mainly in India, but it has great loss of its yield because it is mainly affected by the soil borne fungus *Fusarium oxysporum* f. sp. *cumini*, causes fusarium wilt disease in this plant. *Fusarium oxysporum* (Wilt causing agent) is one of the most destructive diseases of the crop and is a very common soil-borne fungus [8]. Fayzalla *et al*. (2009) [9] demonstrated that wilt disease caused by *Fusarium oxysporum* was the most important disease of soybean plants. In addition to its common use as spice in our daily life, recent studies have indicated its pharmaceutical and medicinal importance [10].

Sun *et al*. (2013) [11] studied the accumulation of pathogenesis-related proteins, chitinases and beta-1,3-glucanase, as well as total phenols and PAL activity in the treated roots also indicated that MeJA triggered key enzymes of secondary metabolite biosynthetic pathways, suggesting that MeJA was involved in the activation of a disease-related defense systemin banana (*Musa* spp.) root disease. In 2015, the extracts of *in vivo* and *in vitro* grown plants as well as callus tissue of red clover were tested for their antioxidant activities, using different extraction solvent and different antioxidant assays. A significant correlation was found between the antioxidant activity of extracts and their total phenolic and total flavonoid content. According to the findings, the extract of *in vitro* culture of red clover especially the callus tissue possesses a comparable antioxidant activity to the *in vivo* cultured plants' extract [12]. The aim of the present study is to evaluate the defense mechanism of *Cuminum cyminum* in response to the fungal pathogen (*Fusarium oxysporum*), by analysing the occurrence of HR, i.e. changes in the level of total phenolic compounds, flavonoids and PAL activity.

Materials and Methods

Plant Material and Growth Conditions

Two different cultivars of *Cuminum cyminum* viz., var. GC-4 (Resistant) and RZ-209 (Susceptible) were procured from SKN College of Agriculture, Jobner and NRCS (National Research Centre on Seed Spices), Ajmer, Rajasthan, India. Seeds were surface sterilized using 0.1% $HgCl_2$ and grown in pots containing steam-sterilized garden soil in a plant growth chamber with a photoperiod of 14-h light (photo-synthetically active radiation, 35 µmol photons$\cdot m^{-2}\cdot s^{-1}$, provided by cool white Philips fluorescent tubes) and 10-h dark period, 28±2 °C temperature and 60% relative humidity. Two age-groups of plants, i.e. 15 days and one month were taken for experiments after different time intervals (0, 2, 4, 24, 48, 72, 96, 120,144 and 168) of fungal spore inoculation.

Preparation of Spore Suspension and Mode of Infection

The fungal strain of *Fusarium oxysporum* (Microbial Type Culture Collection and Gene Bank No. 284) was procured from IMTECH, Chandigarh, India. The lyophilized fungal strain was activated on potato dextrose broth (PDB) under proper aseptic conditions in the laminar flow. The flasks were incubated in incubator shaker (28±2 °C) for 120 h at 120 rpm. Activated fungal strain was then streaked on potato dextrose agar (PDA) slants.

Preparation of Spore Suspension and Mode of Inoculation

Fungal spore suspension was prepared in sterilized distilled water at a concentration of 10^5 spores/ ml under aseptic conditions and kept in the incubator shaker (28±2 °C) at 120 rpm for 2 h to obtain a uniformly distributed spore suspension. For inoculation, the leaf and stem surfaces of the plants were injured mildly with an abrasive (saw dust paper) to facilitate the entry of spores. The fungal spores on plant surfaces were sprayed with a sprayer. The plants sprayed with autoclaved distilled water without fungal spores but injured mildly with abrasive served as control.

Determination of polyphenols

The determination of polyphenols was done using the method of Mc Donald *et al*. (2001) [13] methods. First, one gramme tissue i.e. whole plant was homogenized in 10 ml of 50% methanol. The supernatant was filtered and then centrifuged at 5000 rpm in a cooling centrifuge for 25 min at 4 °C. Then 0.5 ml of the plant extract was mixed with 5 ml of Folin's reagent and 4 ml (1M) sodium carbonate. The mixture was allowed to stand for 15 min and the total phenols were determined using a spectrophotometer at 765 nm. Polyphenol content was expressed as gallic acid equivalent (mg g^{-1}fw).

Determination of Flavonoids

The determination of flavonoids was done using the method of Chang *et al*. (2002) [14]. One gram tissue i.e. whole plant was homogenized in 25 ml of 95% ethanol. The supernatant was filtered and then centrifuged at 5000 rpm in a cooling centrifuge for 25 min at 4 °C. The flavonoid content was determined by mixing 0.5 ml of extract with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was read at 415 nm. Quercetin was used to prepare the standard curve.

Determination of PAL Activity

Pal activity was determined using the method of Camm and Towers (1973) [15] method. Initially, 1g tissue was homogenized in 15 ml of 0.05 M borate buffer, pH 8.8. The homogenate was filtered and centrifuged at 10,000 rpm for15 min at 4 °C. Then 0.1ml of enzyme extract was mixed with 0.3 ml 50 mM L-phenylalanine. The total volume was adjusted to 3 ml with 0.05 M borate buffer pH 8.8. The reaction mixture was incubated at 30 °C for 15 min and absorbance was taken at 290 nm. The amount of the product formed was calculated using the standard curve of cinnamic acid and calculated as µkatals per gram fresh weight.

Determination of Protein

The protein content in the enzyme extract was determined using the method of Lowry *et al*. (1951) [16].

Results and Discussion

Time course of changes in polyphenolic compound, flavonoid and PAL activity in *Cuminum cyminum* plants inoculated by *Fusarium oxysporum* is shown in figures 1-3. The phenolic compounds, flavonoids and PAL activity in inoculated plants increased significantly than control plants. The healthy cumin plants contained significantly less phenols than the inoculated ones. In 15 days old plants a consistent increase in polyphenol content was observed with a maximum at 120h after pathogen inoculation in both the varieties. Further, the polyphenol content was 2.57 and 2.36 fold higher at 120 h as compared to polyphenol content obtained at 0 h for inoculated cumin plants of var. GC-4 and RZ-209, respectively. On the other hand, in one month old cumin plants the maximum polyphenol content is 3.44 and 2.03 mg.g^{-1}fw at 120 h for var. GC-4 and RZ-209 respectively, which is 119 and 14.04% higher than the control. Further, this is 2.35 and 1.42 fold higher in var. GC-4 and RZ-209, respectively, as compared to 0 h inoculated plants.These results are also supported by Ozgonen *et al*. (2009) [17] who reported the synthesis of twelve different phenolic compounds in pepper plant after treatment with mycorrhizal fungi along with *Phytophthora capsici*. Similarly, Benhamou *et al*. (2000) [18] reported that an endophytic bacterium, *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots after infection by *Pythium ultimum.*

In the 15 days old cumin plants the maximum flavonoid content is 6.39 mg.g^{-1}fw in var. GC-4 and 3.70 mg.g^{-1}fw in

var. RZ-209 at 120h after pathogen inoculation. This is 317.64 and 216.23% lower in control plants than the inoculated plants of var. GC-4 and RZ-209, respectively. The maximum flavonoid content for 15 days old plants varied between 2.13 to 6.39 mg.g^{-1}fw and 1.74 to 3.70 mg.g^{-1}fw from 0 to 120 h in var. GC-4 and RZ-209, respectively, after pathogen inoculation. The flavonoid content for one month old plants varied between 2.12 to 6.60 mg.g^{-1}fw and 1.26 to 3.67 mg.g^{-1}fw from 0 to 120 h in var. GC-4 and RZ-209, respectively, after pathogen inoculation. The maximum flavonoid content was obtained at 120 h which is 3.75 and 2.78 fold higher than their respective control in var. GC-4 and RZ-209, respectively, after pathogen inoculation. Poiatti *et al*. (2009) [19] reported increased activity of phenolic compounds in *Solanum tuberosum* leaves inoculated with *Erwinia carotovora*.

PAL, one of the key enzymes in the phenylpropanoid pathway and the flavonoid pathway, was increased in both incompatible and compatible interactions between plants and pathogens [20] (Harllen *et al*. 2004). In 15 days old plants, the PAL activity in pathogen inoculated plants was 100 and 51.61% higher at 2 h in var. GC-4 and RZ-209, respectively, as compared to control plants. Further, the PAL activity was obtained 7.96 and 4.70 µkat.mg^{-1}protein at 2 h which is 1.85 and 1.59 fold higher as compared to the activity obtained at 0 h for inoculated cumin plants of var. GC-4 and RZ-209, respectively. On the other hand, in one month old plants, the maximum PAL activity was obtained at 2 hrs for both thevarieties viz. GC-4 and RZ-209. Further the PAL activity obtained at 2 hrs is 6.74 and 4.70µkat.mg^{-1}protein which is 2.66 and 2.18 fold higher as compared to 0 h inoculated plants. Gupta *et al*. (2013) [21] also showed that the increased PAL activity was higher in 15 days old plants than the one month old *Erucasativa* plants after pathogen inoculation with *Alternaria brassicicola*.The same results were also reported by Zabala *et al*. (2006) [22] that maximum activity of PAL obtained at 2 hrs after pathogen inoculation. Increased transcript accumulation, 2 and 4 hrs after inoculation and some 8-12 hrs post inoculation. PAL genes can be regulated developmentally, induced by wounding, by low temperatures, by other stress conditions and by pathogen attack [23] (Wu and Lin 2002). The enzyme activity gradually increased in infected leaves in comparison of control ones throughout the experimental period of 168 h. El Modafar *et al*. (2001) [24] observed that there was an increase in PAL in date palm roots in response to inoculation with *Fusarium oxysporum*. sp. *Albedinis*. Same results i.e., increase in PAL activity, were also reported by Umesha (2006) [25] in tomato plant treated with pathogen. This indicates that PAL is involved in the biosynthesis of phenolic compounds through the phenylpropanoid pathway. To further substantiate this, it was reported by Chaman *et al*. (2003) [26] that infestation of plants by aphids causes an increase in the PAL activity. Changes in the activities of PAL in groundnut against *Alternaria alternata* have been reported by Chitra *et al*. (2008) [27]. From the data it can be observed that the PAL activity is higher in one month old plants as compared to 15 days old plants. This may be due to increased resistance in older plants compared to young plants which have been exposed more to stress conditions. Control plants of both resistant and susceptible varieties showed activity in low amount as compared to pathogen inoculated leaves. The results indicate that in all the parameters the values are found higher at every stage of infection in infected plants in comparison of control ones and GC-4 shows greater production of secondary metabolite in comparison of RZ-209 because it may be considered that GC-4 is resistant than that of RZ-209.In particular, the time scale in which they are synthesized could be great importance as a mechanism of quick response to wilt disease, which can lead to a higher degree of resistance. The present study on the defense response of *Cuminum cyminum* plants against the fungal pathogen (*Fusarium oxysporum*) indicates a significant role of polyphenols, flavonoid and PAL which can lead to a high degree of resistance in the plants.

On the basis of above results, the synthesis of phenols, flavonoids and PAL and various other biochemical reactions involved in defense response and it can be conclude that enzymes can be used as tools to study the induced defense responses as well as the resistance / susceptibility against a particular pathogen of the Cumin plants. So, these results are the best study to improve this important crop for future.

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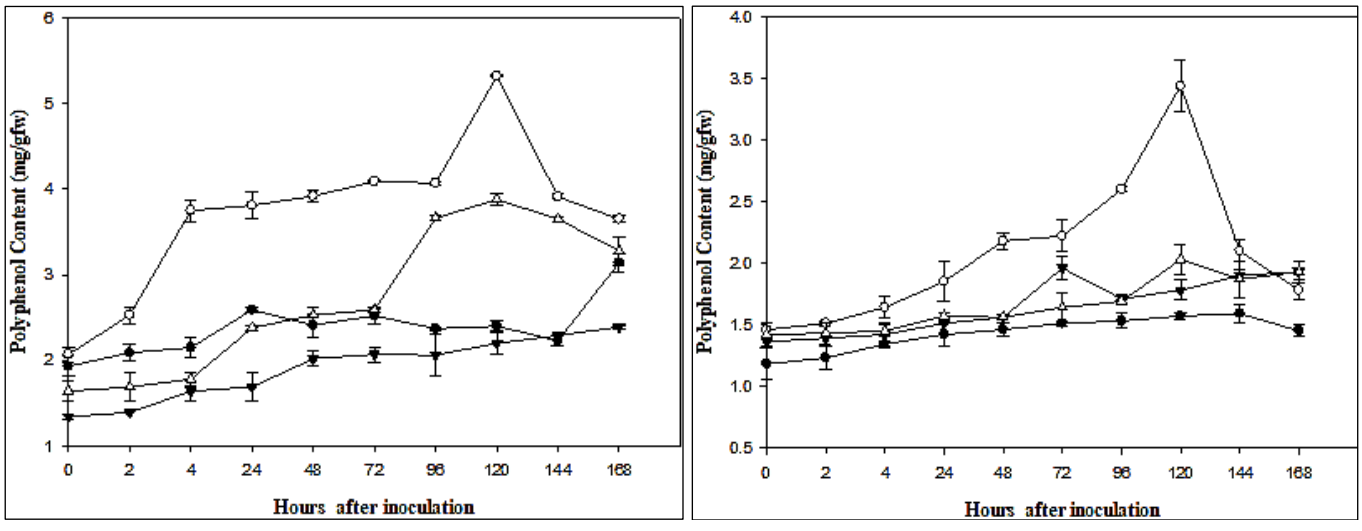

(A) (B)

Fig 1: Polyphenol Content in control and pathogen (*Fusarium oxysporum*) inoculated 15 days (A) and one month (B) old cumin (*Cuminumcyminum*) plants viz. GC-4 and RZ-209. -●- GC-4 control, -○- GC-4 inoculated, -▼- RZ-209 control, -V- RZ-209 inoculated.Each value represents the mean of three replicates with SE determined.

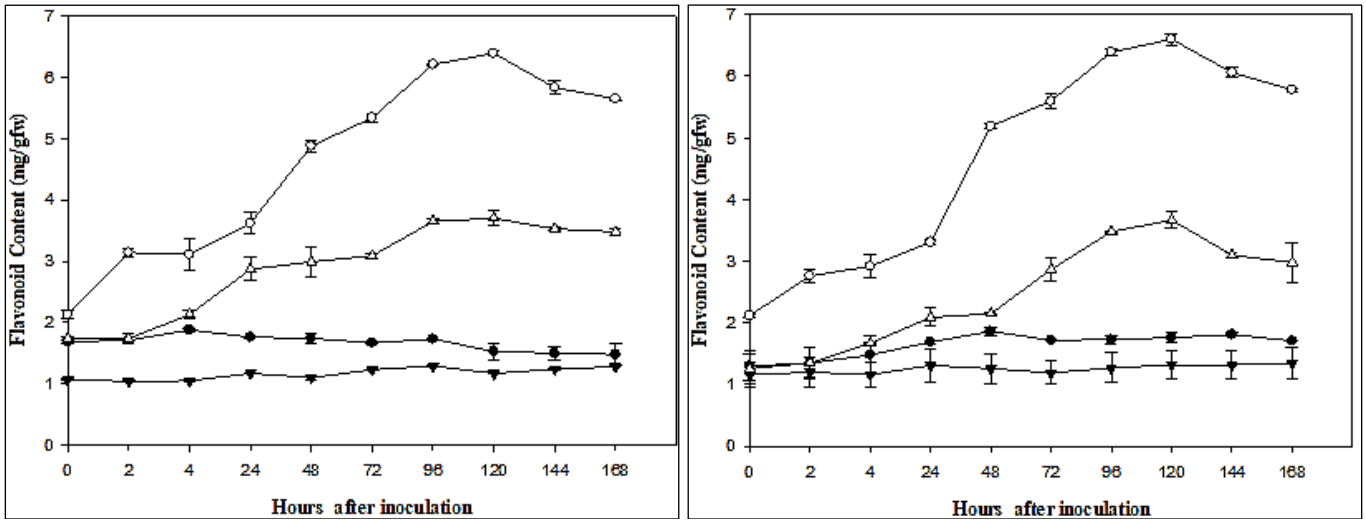

Fig 2: Flavonoid Content in control and pathogen (*Fusarium oxysporum*) inoculated 15 days (A) and one month (B) old cumin (*Cuminum cyminum*) plants viz. GC-4 and RZ-209-●- GC-4 control, -○- GC-4 inoculated, -▼- RZ-209 control, -∇- RZ-209 inoculated. Each value represents the mean of three replicates with SE determined.

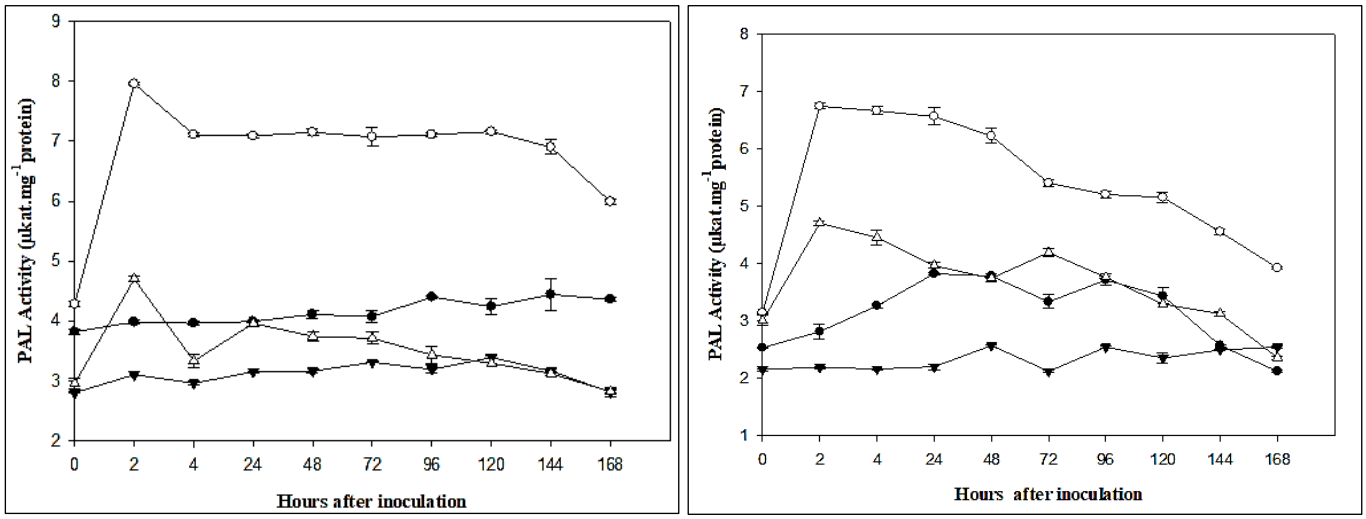

Fig 3: PAL activity in control and pathogen (*Fusarium oxysporum*) inoculated 15 days (A) and one month (B) old cumin (*Cuminumcyminum*) plants viz. GC-4 and RZ-209. -●- GC-4 control, -○- GC-4 inoculated, -▼- RZ-209 control, -∇- RZ-209 inoculated. Each value represents the mean of three replicates with SE determined.

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