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# HPLC Determination of Phenolic Acids in Infected Plants of Chickpea

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Phenolic acids are active antimicrobial compounds and root signaling molecules that play important roles in plant defense responses. The effect of *Macrophomina phaseolina* infection was evaluated in chickpea at different progression periods of infection, in relation to the variation in phenolic compounds within the host. Both *in vivo* and *in vitro* studies were carried out for finding changes in the level of polyphenols in two different aged plants of four cultivars of chickpea. Overall, an increment in the amount of total phenolics within a methanolic extract of diseased leaf tissues as compared to the healthy tissues was observed following infection. Further thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses were used to identify the host's phenolic compounds, which were mostly affected by the host-pathogen interaction. The number of peaks identified were more in infected plants than in control ones. A range of phenolic acids were detected among which 10 were identified by HPLC *viz*., cinnamic acid, *p*-coumaric acid, *o*-coumaric acid, sinapic acid, ferulic acid, gallic acid, chlorogenic acid, kaempferol, myricetin and caffeic acid. The degree of accumulation of these phenolics in infected plants was found to be more than that of untreated plants.

**Key Words: Chickpea, High performance liquid chromatography, *Macrophomina phaseolina*, Phenolic acids, Thin-layer chromatography.**

## INTRODUCTION

Secondary metabolites constitute an important part of both inducible and constitutive plant defenses that target insects, plant pathogens and other competitors, including plants[1,2]. Phenolic acids are a large class of plant secondary metabolite products distributed widely in the plant kingdom. The defensive role of phenolic acids relates to an increase in their content under stressed environmental conditions, such as air pollution, ultraviolet radiation, infection or mechanical damage[3]. The concentration of phenols is always found to be increased after pathogen attack in order to combat infection in the plant, further proving its role in defensive reactions[4]. Results from many studies suggest that esterification of phenols to cell wall materials as well as the accumulation and deposition of phenols in and on cell walls are usually considered as an increase in resistance to fungal hydrolytic enzymes as well as a physical barrier against fungal penetration[5].

Chickpea (*Cicer arietinum* L.) is, after soybean and pea, the third most important grain legume crop in the world. It is one of the most studied legume plants at the biochemical level for phenolics and phytoalexin responses[6]. In developing countries, it is an important source of high-quality protein. Rajasthan state contributes about 20.74 % to the national production of grain and occupies the second position in India. Chickpea occupies more than 10 million hectares of the cultivated areas in the world, with a total production of *ca.* 7 million tons[7]. Low yields are attributed to different factors, among which pathogens and insect attacks are considered the most serious. Nene and Reddy[8] reported many chickpea pests, including 47 pathogens and 50 insects. Despite the high number of chickpea pests, root rot caused by *Macrophomina phaseolina* is considered the most destructive disease of chickpea. Control measures, such as fungicide treatments are expensive besides the problems with regard to environmental pollution, chemical toxicity to humans, animals and fungal resistance, therefore the use of resistant cultivars is probably the best way to manage these diseases. Many breeding and screening programs have been undertaken in order to obtain chickpea varieties with high levels of resistance to the major diseases of chickpea[9]. The aim of the present study was to determine the effect of *M. phaseolina* infection in chickpea plants, by elucidating the effect of fungal colonization on the accumulation of phenolic compounds within infected and healthy leaf tissues. Changes in phenol concentration in *Macrophomina phaseolina* affected chickpea leaves were determined in the present communication with a view to correlate the resistance of the host through elicitation of its defense system.

## EXPERIMENTAL

**Plant material and growth conditions:** Seeds of four varieties of chickpea (*Cicer arietinum* L) *i.e.*, RSG-931, RSG-945, RSG-896, CSJD-884 were procured from Krishi Vigyan Kendra (KVK), Banasthali University, India.

**Preparation of spore suspension:** For infection fungal spore suspension was prepared under aseptic conditions by scrapping the surface of the sporulated mat and inoculating in the conical flask containing autoclaved distilled water. The fungal spore concentration was maintained at $10^5$ spores/mL.

**Mode of plant infection:** Both *in vitro* and *in vivo* experiments were performed in 25 and 35 days old plants of all the four varieties.

***In vitro* experiments:** For these experiments, the plants were removed from the pots and placed in petri plates and then inoculated with the fungal spore suspension of *Macrophomina phaseolina*.

***In vivo* experiments:** In these experiments, the whole plants growing in pots were inoculated spraying with the spore suspension.

After treatment, the experiments were performed in both infected and control plants at interval of 24 h, then analyzed for the changes in phenolics content.

**Assay of phenolic acids:** Phenolic content of guar leaves was estimated by using modified Bray and Thorpe method[10]. The polyphenol extract obtained was mixed Folin-Ciocalteu's reagent. After 3 min of incubation, 25 % sodium carbonate was added. The absorbance was recorded at 725 nm.

**TLC of polyphenols compounds:** Polyphenol compounds were separated by thin-layer chromatography (TLC) using TLC silica gel (20 cm × 20 cm) aluminium sheets from MERCK (Darmstadt, Germany). One dimensional separation of polyphenols was done by using two different solvent mixture *i.e.*, benzene:acetic acid:water (6:7:3) and acetic acid: chloroform (1:9). After development polyphenol compounds were detected by yellow and brown colour spots on TLC plates after fumigation with ammonia and spraying with 1 % ferric chloride solution, respectively.

**HPLC analysis of phenolics:** Extracted phenolics samples were further analyzed by HPLC (Shimadzu LC- 10A) using reverse phase C-18 column with mobile phase comprising orthophosphoric acid as solvent A and orthophosphoric acid:acetic acid:acetonitrile as solvent B for 15 min and monitored at 340 nm. Quantification of individual peaks was achieved by comparison to the sample internal standards. Identification of the chromatographic peaks was performed by comparison to known standards: cinnamic acid, *p*-coumaric acid, *o*-coumaric acid, sinapic acid, ferulic acid, gallic acid, chlorogenic acid, kaempferol, myricetin and caffeic acid.

**Statistical analysis:** The significance of treatments (healthy and infected leaves) was assessed using the one way analysis of variance (ANOVA) using SPSS 16. Differences between treatments were tested at the 0.05 significance level.

## RESULTS AND DISCUSSION

**Polyphenol content using *in vivo* system:** It is evident from Fig. 1 that the polyphenol content was consistently higher in both the 25 and 35 days old pathogen inoculated plants while compared to the controls at different time intervals after inoculation with the pathogen. In both aged plants, the highest value of polyphenol content was recorded at 120 h after pathogen inoculation (Table-1). In 25 days old plants, the polyphenol content in these four varieties *viz.*, RSG-931, RSG-945, RSG-896, CSJD-884 were found to be 44, 36, 49 and 30 % higher in inoculated plants as compared to control ones, respectively at their peak hours. Nearly the same pattern was observed in 35 days old pathogen inoculated and control plants. The observed percentage increase in infected 35 days old plants was 30, 30, 38 and 50 % than the control plants in RSG-931, RSG-945, RSG-896, CSJD-884 varieties at 120 h, respectively.

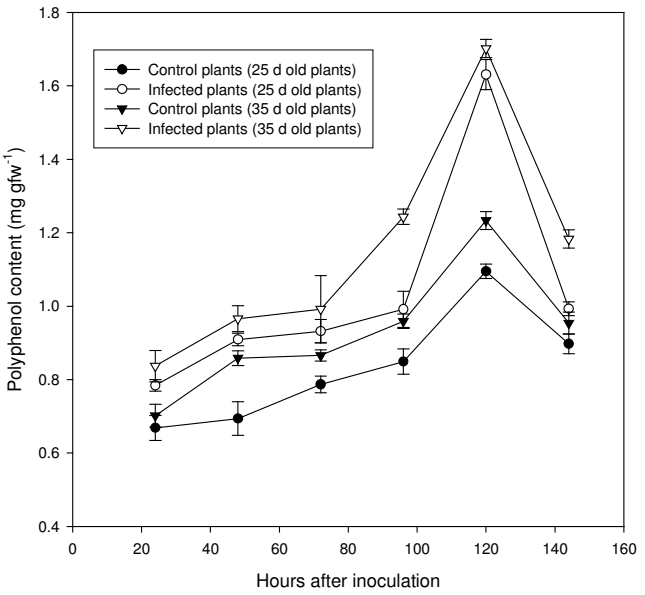


Fig. 1. Polyphenol content (mg gf $w^{-1}$) of control and inoculated plants of RSG 896 with *Macrophomina phaseolina* under *in vivo* conditions

**Polyphenol content using *in vitro* system:** *In vitro* investigation using excised plants were also carried out in both 25 as well as 35 days old plants. The trend of polyphenol content in both pathogen inoculated and control plants is clearly shown in Fig. 2 by taking an example of one variety

TABLE-1
POLYPHENOL CONTENT (mg gf $w^{-1}$) IN CONTROL AND INFECTED PLANTS OF CHICKPEA UNDER *IN VIVO* CONDITIONS AT PEAK HOUR (120 h)

| Varieties | 25 days old | | 35 days old | |
|---|---|---|---|---|
| | Control | Infected | Control | Infected |
| RSG-931 | 0.893 ± 0.023 | 1.281 ± 0.031 | 0.935 ± 0.017 | 1.216 ± 0.035* |
| RSG-945 | 1.042 ± 0.035 | 1.410 ± 0.036* | 1.173 ± 0.016 | 1.525 ± 0.041 |
| RSG-896 | 1.095 ± 0.019 | 1.631 ± 0.041 | 1.233 ± 0.025 | 1.702 ± 0.026* |
| CSJD-884 | 0.904 ± 0.019 | 1.170 ± 0.030* | 0.998 ± 0.018 | 1.497 ± 0.032 |

Values are given as mean ± SD of three replication in each group. *Shows the significant value which is carried out at $p \leq 0.05$ significant level.

*i.e.*, RSG-896. The phenolic content was always found to be higher in infected plants as compared to control even at different intervals of infection in both aged plants. In 25 and 35 days old plants, the level of polyphenols was found to be maximum after 48 h of infection in all varieties (Table-2). The percentage rise in infected plants of RSG-931, RSG-945, RSG-896, CSJD-884 varieties were found to be 29, 31, 29 and 32 % in 25 days old and 36, 30, 49 and 36% in 35 days old as compared to control plants, respectively.

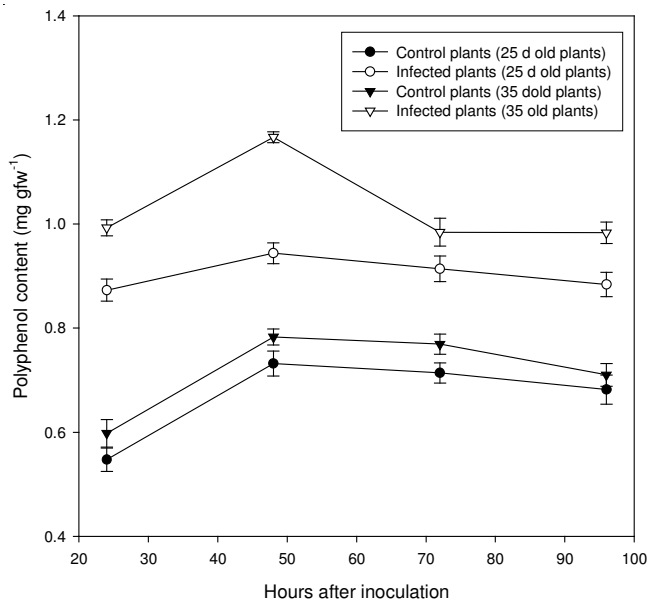


Fig. 2. Polyphenol content (mg $gfw^{-1}$) of control and inoculated plants of RSG 896 with *Macrophomina phaseolina* under *in vitro* conditions

**Thin layer chromatography:** The comparison of TLC plates of control with the pathogen inoculated plant extract shows that the mobility of spot in both of them was nearly identical. Fig. 3 shows the TLC results of polyphenol compounds when benzene: acetic acid: water used as solvent and fumigated with ammonia. With this solvent system after fumigation, the sample developed two major spots. The calculated $R_f$ value of infected was found to be little higher than the

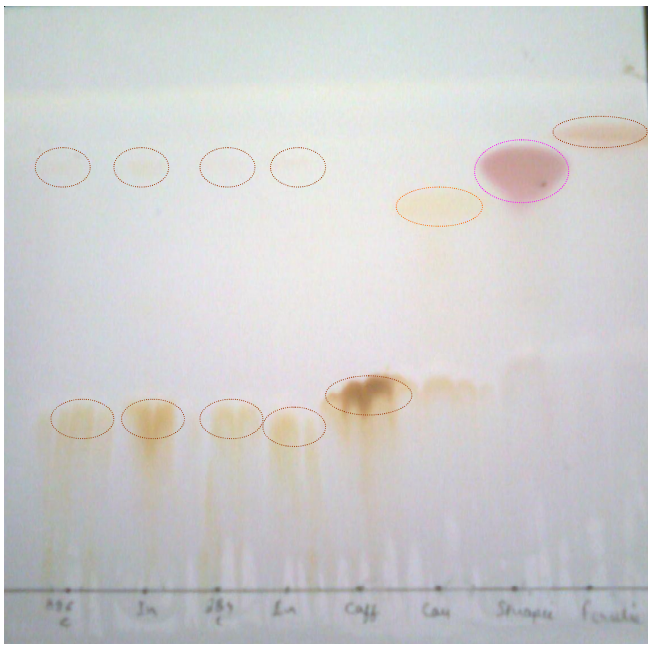


Fig. 3. TLC of polyphenol compounds with benzene:acetic acid:water and then fumed with ammonia A-896 C, B-896 I, C-884C, D-884I, E: caffeic acid, F: caumaric acid, G: sinapic acid, H: ferulic acid

control plant's extract. Table-3 represents the $R_f$ value of the spots obtained by both solvents and spraying system. A comparison of $R_f$ values of sample with that of co-chromatographed authentic compounds confirmed the presence of four hydroxycinnamic acids *viz.*, caffeic acid, ferulic acid, sinapic acid and coumaric acid in chickpea.

**High performance liquid chromatography:** The HPLC chromatograms of 25 days old control and pathogen inoculated plants of RSG-896 were given in Fig. 4. From the study of HPLC chromatogram of this variety, it was evident that while there were a total of 18 distinct peaks in the control plants, this number was increased to 20 in the plants exposed to pathogen. Further, it can also be observed that the total integration areas of these peaks were 18244388 units in the control plants while compared to the infected where this value

TABLE-2
POLYPHENOL CONTENT (mg $gfw^{-1}$) IN CONTROL AND INFECTED PLANTS OF CHICKPEA UNDER *IN VITRO* CONDITIONS AT PEAK HOUR (48 h)

| Varieties | 25 days old | | 35 days old | |
|---|---|---|---|---|
| | Control | Infected | Control | Infected |
| RSG-931 | 0.687 ± 0.038 | 0.886 ± 0.026* | 0.712 ± 0.015 | 0.968 ± 0.025* |
| RSG-945 | 0.695 ± 0.021 | 0.911 ± 0.026 | 0.808 ± 0.023 | 1.050 ± 0.027* |
| RSG-896 | 0.732 ± 0.024 | 0.944 ± 0.019* | 0.783 ± 0.015 | 1.167 ± 0.011 |
| CSJD-884 | 0.658 ± 0.010 | 0.868 ± 0.020* | 0.758 ± 0.021 | 1.031 ± 0.011 |

Values are given as mean ± SD of three replication in each group. *Shows the significant value which is carried out at $p \leq 0.05$ significant level.

TABLE-3
ONE-DIMENSIONAL THIN LAYER CHROMATOGRAPHY OF POLYPHENOLS EXTRACTED FROM CHICKPEA

| Solvent mixtures | Spraying solution | Colour appeared | $R_f$ Values | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 896C | 896I | 884C | 884I | CA | FA | SA | CU |
| Acetic acid:chloroform (9:1) | 1 % Ferric chloride | Brown spots | 0.84 | 0.85 | 0.084 | 0.85 | 0.28 | 0.86 | 0.88 | 0.69 |
| Benzene:acetic acid:water (6:7:3) | Fuming with ammonia | Yellow spots | 0.33<br>0.83 | 0.35<br>0.84 | 0.33<br>0.83 | 0.34<br>0.84 | 0.38 | 0.87 | 0.82 | 0.39 |

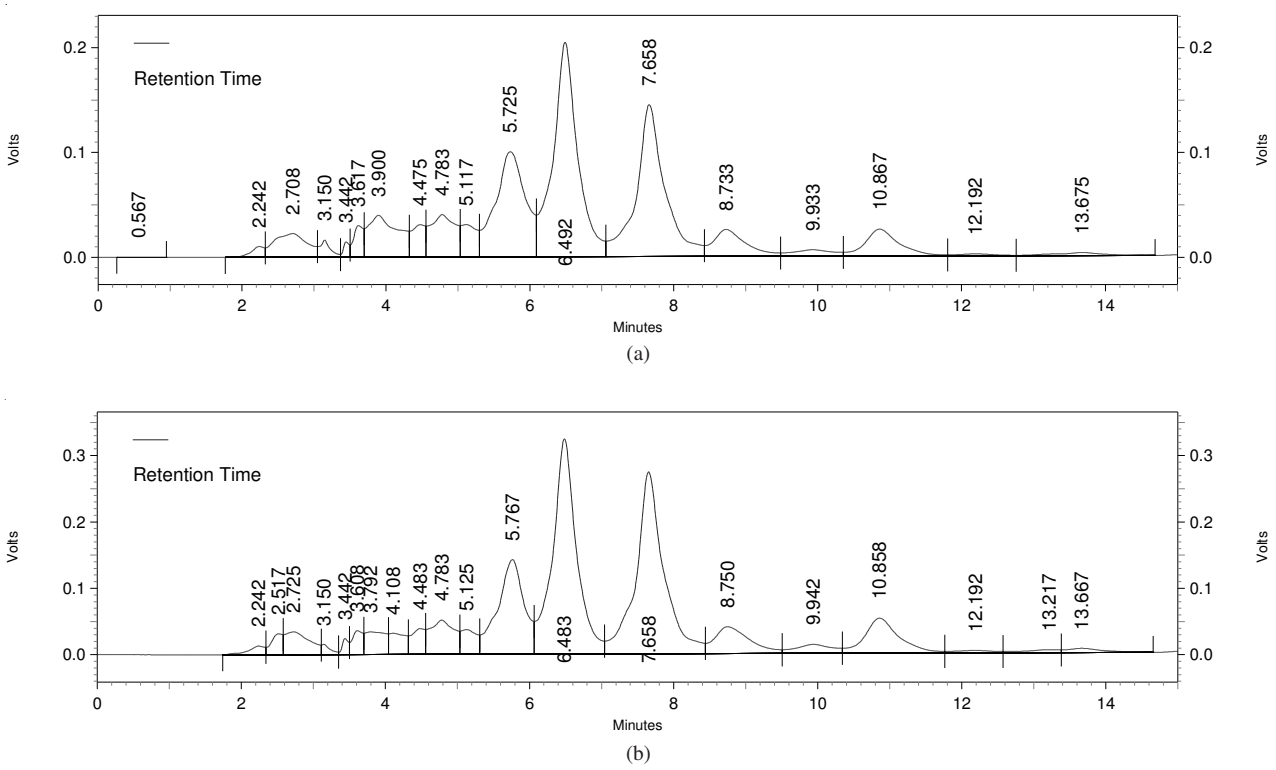


Fig. 4. (a) HPLC chromatogram of polyphenols obtained from 25 days old control plants of RSG-896 variety of chickpea after 120 h using *in vivo* system, (b) HPLC chromatogram of polyphenols obtained from 25 days old pathogen treated plants of RSG-896 variety of chickpea after 120 h using *in vivo* system

was increased by 53 %. Table-4 shows that similar results were obtained with rest of three varieties. The retention time of cinnamic acid, *p*-coumaric acid, *o*-coumaric acid, sinapic acid, ferulic acid, gallic acid, chlorogenic acid, kaempferol, myricetin and caffeic acid standards are shown in Table-5. A comparison of retention time of standards with Fig. 4 shows that 10 of the peaks in both the control and the inoculated plants separate with the authentic samples with a simultaneous increase in the integration area values of chickpea. Besides 10 phenolic acids, a few other peaks were also detected; however these could not be identified. This indicates the presence of other compounds, but in smaller concentration. Thus it is evident that on inoculation with the pathogen, not only the amount of total polyphenols increases, but a few new compounds appear to be synthesized. If the content of these identified phenols were considered individually, the healthy plants observed to contain significantly less phenols than the root-rot infected plants (Table-6). The results of TLC and HPLC confirm our results of presence of higher concentration of polyphenol in inoculated plants in comparison to control one.

Higher levels of total phenols following infection with pathogens have been reported by previous workers[11-13]. It is also found that phenols play an important role in disease resistance. Similarly, in chickpea the phenolic content showed a marked increase (*ca.* 36 %) post infection.

The polyphenols content which was further confirmed by TLC and HPLC. The greater $R_f$ value of infected samples than that of control was observed. Further, the analysis of HPLC chromatograms showed 10 major peaks which indicate the presence of ten polyphenols which were confirmed by comparing with retention time of known standards. The area of peaks was found to be high in case of infected when compared with control. The increased area shows the higher production of cinnamic acid in infected plants after pathogen attack. Its content was raised in infected plants from 3068.9-3812.9 mg gfw$^{-1}$ in RSG-896. Small variations were found owing to the different varieties used in the analysis. The content

TABLE-4
A COMPARATIVE DATA OF HPLC OF POLYPHENOLS EXTRACTED FROM CONTROL AND INFECTED 25 DAYS OLD PLANTS OF CHICKPEA *IN VIVO* CONDITION AT 120 h

| Varieties | Treatment | Peak No. | Area | Height |
|---|---|---|---|---|
| RSG-931 | Control | 18 | 15505628 | 594031 |
| | Infected | 18 | 40114432 | 1740466 |
| RSG-945 | Control | 18 | 11662245 | 764291 |
| | Infected | 19 | 23471396 | 883938 |
| RSG-896 | Control | 18 | 18244388 | 749185 |
| | Infected | 20 | 28000033 | 1207846 |
| CSJD-884 | Control | 20 | 19416604 | 779303 |
| | Infected | 20 | 26480695 | 1104550 |

TABLE-5
HPLC OF STANDARDS AND THEIR RETENTION TIMES

| S. No. | Standard compound | Retention time |
|---|---|---|
| 1 | Caffeic acid | 4.33 |
| 2 | Chlorogenic acid | 5.192 |
| 3 | Cinnamic acid | 5.725 |
| 4 | Ferulic acid | 6.258 |
| 5 | Kaempferol | 7.508 |
| 6 | Myricetin | 9.183 |
| 7 | *o*-Coumaric acid | 6.700 |
| 8 | *p*-Coumaric acid | 6.933 |
| 9 | Sinapic acid | 5.742 |
| 10 | Gallic acid | 3.692 |

TABLE-6
CONTENT OF PHENOLIC COMPOUNDS IN mg $gfw^{-1}$ IN HEALTHY AND DISEASED LEAVES OF INFECTED 25 DAYS OLD PLANTS OF CHICKPEA *IN VIVO* CONDITION AT 120 h

| Phenolics | RSG-931 | | RSG-945 | | RSG-896 | | CSJD-884 | |
|---|---|---|---|---|---|---|---|---|
| | Control | Infected | Control | Infected | Control | Infected | Control | Infected |
| Caffeic acid | 0.016 | 0.045 | 0.023 | 0.029 | 0.035 | 44.1 | 22.8 | 32.1 |
| Chlorogenic acid | 0.021 | 0.057 | 0.025 | 0.030 | 0.035 | 42.6 | 25.5 | 32.5 |
| Cinnamic acid | 2.463 | 6.906 | 2.103 | 3.985 | 3.068 | 3812.9 | 3206.2 | 4111.3 |
| Ferulic acid | 0.428 | 1.333 | 0.346 | 0.674 | 0.439 | 674.6 | 519.3 | 729.7 |
| Kaemferol | 0.488 | 1.315 | 0.334 | 0.755 | 0.476 | 883.3 | 574.9 | 845.6 |
| Myrecetin | 0.031 | 0.057 | 0.040 | 0.020 | 28.1 | 54.9 | 40.2 | 44.5 |
| *o*-Coumaric acid | 0.305 | 0.323 | 0.246 | 0.480 | 313.6 | 481.2 | 370.4 | 520.5 |
| *p*-Coumaric acid | 0.740 | 2.298 | 0.598 | 1.164 | 759.7 | 1165.7 | 897.4 | 1260.9 |
| Sinapic acid | 0.256 | 0.719 | 0.219 | 0.415 | 319.6 | 397.1 | 333.9 | 428.2 |
| Gallic acid | 0.567 | 0.569 | 0.579 | 0.219 | 865.3 | 1084.1 | 510.1 | 722.5 |

of the remaining hydroxycinnamic acids analyzed was lower than that of cinnamic acid. From Table-6 it can be observed that in chickpea, caffeic acid, myrcetin and chlorogenic acid were present in lower amounts, but their concentration also increases post-infectionally. The ferulic acid, kaemferol, *o*-coumaric acid, *p*-coumaric acid, sinapic acid and gallic acid of the phenolics showed a significant increase in their content after *M. phaseolina* infection.

Püssa *et al*.[14] and Bruno and Sparapano[15] reported that phenolic content in grapevine was cultivar-specific and that may be directly linked to host resistance to pathogenic attack. This confirms our results, in which the RSG-896 variety was found to be more active to fight against the infection as compared to rest of the three varieties.

From the results presented here, it can be concluded that the infection of chickpea plants with *M. phaseolina* induced an upregulation of plant defense mechanisms. This resulted in increased accumulation of phenolic compounds, which are known to inhibit fungal growth. The accumulation of phenolics would be a fundamental step in controlling the spread of the infection within the host. Perhaps, phenolics are probably not the only compounds that contribute to resistance of chickpea to root rot but, our results shows that their synthesis is increased due to the infection. The results achieved represent an important contribution to understanding plant-pathogen interaction and may be useful for further investigation. For further studies, the time scale in which other specific phenolics as well as other compounds are synthesized could be of great importance as a mechanism of quick response to *Macrophomina phaseolina* infection, which can lead to a higher degree of resistance.

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