Pak. J. Bot., 44: 417-422, Special Issue March 2012.

HPLC ANALYSIS FOR SECONDARY METABOLITES DETECTION IN *SCLEROTIUM ROLFSII* ISOLATED FROM CHICKPEA

PAKEEZA AMBER[1], ABIDA AKRAM[2], RAHMATULLAH QURESHI[2*] AND ZAHID AKRAM[3]

[1*]*Department of Biology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan*
[2]*Department of Botany, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan*
[3]*Department of Plant Breeding and Genetics, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan*
[*]*Corresponding author E-mail: rahmatullahq@yahoo.com*

Abstract

Chickpea (*Cicer arietinum* L.) is an economically important leguminous crop grown in Rabi season in barani areas of Pakistan. Among the biotic stresses responsible for its low production during last few decades, collar rot caused by fungus *Sclerotium rolfsii* Sacc., has caused serious economical losses to chickpea crop. The aim of the present research was to identify secondary metabolites among 12 isolates of *S. rolfsii* through HPLC analysis. The HPLC analysis of sclerotial filtrates revealed 4-17 peaks. Out of these, six were identified on the basis of their retention time (Rt) as gallic (Rt 2.86 min), oxalic (Rt 3.03 min), ferulic (Rt 3.30 min), indole-3 acetic (Rt 3.60 min), chlorogenic (Rt 4.16 min) and cinnamic (Rt 4.46 min) acids. Ferulic acid was identified in 9 isolates, IAA in 10 isolates, cinnamic acid in 11 isolates and chlorogenic acid in 5 isolates. Gallic and oxalic acids were found in all the isolates. Highest concentration in sclerotial filtrate of all isolates was gallic acid varying from 1.871-25.13µg/ml. Present study revealed that AM-04, AM-05, AM-09, AM-10 and AM-11 yielded severe collar rot infection in chickpea due to their ability to synthesize phenolic acids which act as a tool for their rigorous attack. AM-02 is less virulent strain which synthesized least number of phenolics and thus promotes mild infection against chickpea. So this information is helpful for plant breeders for the production of resistant varieties of chickpea against identified phenolic acids to inhibit or cease the infection caused by strains AM-04, AM-05, AM-09, AM-10 and AM-11 and thus reduce the economical losses to chickpea.

Introduction

Chickpea (*Cicer arietinum* L.), an important source of protein enriched human food and animal feed is a member of family Fabaceae (Suzuki & Konno, 1982) and is grown during Rabi season in Pakistan (Ilyas *et al.,* 2007). It is the 3rd most important pulse crop of the world and ranks first in the Indian subcontinent (Anon., 2004). In Pakistan, average yield per hectare of chickpea has been declined from 615 kg per hectare to 597 kg per hectare from the years 1995 to 2005 (Anon., 2009). Amongst diseases collar rot caused by *Sclerotium rolfsii* Sacc., is one of the several fungal diseases affecting this crop and is reported almost all over the world wherever chickpea is grown (Nene *et al*., 1984). *S. rolfsii.*, is a soil borne plant pathogenic fungus causing large economic losses (Kokub *et al*., 2007). Seedling mortality from 54.7 to 95.0% in chickpea due to infection of *S. rolfsii* has been reported by Mathur & Sinha (1970). In order to minimize infection, only practicable and cost-effective control is selection of disease resistant cultivars (Akram *et al*., 2008).

Fungi are famous for their ability to produce bioactive molecules called secondary metabolites. They act as enhancers of virulence and are not required for growth or development of the producing organism. Because of their bioactive properties, many fungal secondary metabolites have been used by humans in pharmaceutical industry (Shwab & Keller, 2008). Secondary metabolites serve as a chemical shield for fungus (Kempken & Rohlfs, 2010).

HPLC is a highly sensitive method for detection, identification and quantification of any chemical in a particular sample using ultraviolet and visible absorbance (Hanachi & Golkho, 2009). By comparing with the retention time of the standards, phenolic compounds can be identified (Sarma *et al.,* 2002).

Keeping in view the significance of chickpea and the threat of *S. rolfsii* to chickpea, the objective of the present study was to identify the secondary metabolites of the pathogen *S. rolfsii* through HPLC.

Materials and Methods

The present study was conducted in the Department of Botany, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi during the year 2010.

Sample collection

Isolation of pathogen: Experimental material comprised of 12 strains of *S. rolfsii* causing collar rot to chickpea plants. These strains were obtained from Crop Protection Laboratory, Crop Science Institute (CSI), National Agricultural Research Centre (NARC), Islamabad. The cultures of *S. rolfsii* were originally isolated from stem sections of diseased chickpea plants of infected crops in the experimental fields of NARC, with visible symptoms of collar rot of chickpea. The diseased samples were surface sterilized with Clorox. These samples were then inoculated to chickpea seed meal extract agar medium (dextrose 20grams, agar 20grams, chickpea seed meal extract 1litre, sterilized the media containing flasks in a electric autoclave at 15psi for 20 minutes and 121°C, cooled and poured in 9cm. Petri plates inside laminar flow and solidified). These inoculated plates were then incubated at 25±2°C under diffused light for about 5-7 days and observed daily for emergence of colonies.

Sub culturing and culture maintenance: These fungal strains were further sub-cultured by growing on freshly prepared chickpea seed meal extract agar medium. With the help of sterilized cutter, 10mm. diameter circular discs was punched out from margin of actively growing colony and placed onto the centre of media plate with mycelial

side facing downwards. Inoculation was done in replication of four and each plate was labeled for future identification. These plates were placed in incubator at 25±2°C under diffused light. After about 3-5 days, sclerotial formation started (Fig. 1A). Initially the colour of sclerotia was white which turned brown with the passage of time (Fig. 1B). After about 25 days petri plates were covered with mature sclerotia (Fig. 1C).

Fig. 1. Mycelial growth and developing sclerotia on chickpea seed meal extract agar medium depicting young white sclerotia which turned brown on maturation.

Preparation of pure cultures: Pure cultures were obtained by inoculating the media plates with 2-3 sclerotia of respective strains and incubated at 25±2°C for 7 days. Mycelia from these pure cultures were used for growing sclerotia for each strain.

Mass culture of sclerotia: Chickpea seed meal extract agar media was inoculated with 10 mm mycelial discs of respective strains of *S. rolfsii* taken from the margin of actively growing cultures using a sterilized cutter. It was incubated at 25±2°C under diffuse light for 5 days and then placed in environmental chamber at 28±2°C for 23 days. Inoculation was done in replication of four. After about 28 days, mature brown sclerotia were collected, dried for one day, labeled and stored in eppendorf tubes at 4°C for further analysis.

HPLC analysis

Standard preparation: Standards used in this study were oxalic, gallic, ferulic, chlorogenic, cinnamic and indole-3 acetic acids (IAA). Calculations for concentrations were made and solutions of each standard were prepared in distilled water with concentrations of 40µg/ml (1mg/25ml) in a 25ml volumetric flask and mixed well.

Ethyl acetate fractionation of sclerotia of *S. rolfsii*: The mature sclerotia produced on chickpea seed meal extract agar medium plates were collected separately. For the detection of secondary metabolites in samples, 1 gram of sclerotia from each isolate was taken separately thoroughly macerated with ethyl acetate and crushed in a sterilized pestle and mortar. The finely crushed material was collected in screw capped bottles with an additional 5 ml of ethyl acetate and kept overnight at 4°C.

The crude solution of sclerotia was allowed to settle down and clear supernatant was recovered carefully in another tube. Re-extraction was done twice and pooled fractions of sclerotia were collected in screw capped bottles. It was then filtered through Whatman No. 1 filter paper, and the filtrate was evaporated under vacuum in rotary vacuum evaporator (Buchi Rotavor Re Type). Dried samples were suspended in 1ml of HPLC grade methanol by vortexing and were stored at 4°C for HPLC analysis.

HPLC system: Qualitative and quantitative HPLC analysis of the sclerotial sample was performed according to the method of Sarma *et al*., (2002). The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with 2 Shimadzu LC-20 ATVP reciprocating pumps, a variable Shimadzu SPD-20 AVP UV VIS detector and a Rheodyne Model 7725 injector with a loop size of 20µl. The peak area was calculated with a Winchrom integrator. Software package used for analyzing results was LC Solutions. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250 x 4.6mm i.d., particle size 5µm, Luna 5µ C-18; phenomenex, USA) at 25°C. Running conditions included: injection volume, 20µl; mobile phase, methanol: 0.4% acetic acid (80: 20 v/v); flow rate, 1ml/min; and detection at 290mm. Samples were filtered through an ultra membrane filter (pore size 0.45µm; Merck, Germany) prior to injection in the sample loop. Oxalic, gallic, ferulic, cinnamic, chlorogenic and indole-3 acetic acids were used as external standards.

Data analysis: Phenolic acids present in each sample were identified by comparing retention time (Rt) of the samples with the retention time of individual standards and further confirmed by co-injection. The amount of each phenolic acid was expressed as µg/ml.

Results

HPLC analysis of sclerotial filtrate of *S. rolfsii* revealed 4-17 peaks. Out of these, six were identified on the basis of their retention time (Rt) as well as co-injection. All these peaks consistently appeared in the sclerotial filtrate of most of the isolates. The peaks identified were of gallic acid (Rt 2.86 min), oxalic acid (Rt 3.03 min), ferulic acid (Rt 3.30 min), indole-3 acetic acid (Rt 3.60 min), chlorogenic acid (Rt 4.16 min) and cinnamic acids (Rt 4.46 min).

Out of 12 isolates, ferulic acid was identified in 9 isolates, IAA in 10 isolates, chlorogenic acid in 6 isolates and cinnamic acid in 11 isolates. Gallic acid and oxalic acid were detected in all the isolates. The highest concentration in sclerotial filtrate of all the isolates was of gallic acid and its amount varied from 1.871 to 25.13µg/ml (Table 1). Chlorogenic acid was found in least number of isolates with concentration ranging from 1.678 to 3.45µg/ml.

Table 1. Concentrations of phenolic acid (secondary metabolites) in sclerotial filtrates of *S. rolfsii* strains isolates from chickpea.

	Concentration (µg/ml)					
Isolates	**Gallic acid**	**Oxalic acid**	**Ferulic acid**	**IAA**	**Cinnamic acid**	**Chlorogenic acid**
AM 1	25.138	1.11	2.161	3.542	1.496	-
AM 2	5.112	2.231	-	-	-	-
AM 3	16.224	0.506	1.399	7.586	1.113	3.45
AM 4	13.702	1.213	1.116	9.562	1.891	1.678
AM 5	3.194	2.341	0.207	1.584	1.123	2.348
AM 6	2.038	4.65	-	1.339	0.306	-
AM 7	1.871	2.546	-	0.596	3.313	-
AM 8	3.345	1.123	0.206	1.602	0.525	-
AM 9	4.583	1.056	0.462	1.112	0.238	3.11
AM 10	10.732	1.358	1.148	3.395	1.169	2.346
AM 11	8.940	1.476	1.094	8.884	1.086	2.9
AM 12	3.240	1.245	3.015	-	1.187	-

The HPLC profile of AM-01 (Fig. 2A) revealed 13 peaks. Out of which 5 peaks were identified on the basis of their retention time as gallic acid (Rt 2.611), oxalic acid (Rt 3.09), ferulic acid (Rt 3.491), IAA (Rt 3.835) and cinnamic acid (Rt 4.525). The highest concentration (25.138µg/ml) of gallic acid was detected in this isolate. It was followed by oxalic acid (1.11 µg/ml) and ferulic acid (2.161 µg/ml). IAA had concentration 3.542µg/ml. Cinnamic acid had concentration 1.496µg/ml. Chlorogenic acid was absent in the sclerotial filtrate of AM-01.

The HPLC analysis of AM-02 (Fig. 2B) revealed 4 peaks. Out of which 2 were identified on the basis of their retention time as gallic acid (Rt 2.645) and oxalic acid (Rt 3.135). The concentration of gallic acid was 5.112µg/ml. Oxalic acid in this isolate had concentration 2.231µg/ml. Ferulic acid, IAA chlorogenic acid and cinnamic acid were absent in the sclerotial filtrate of AM-02.

The sclerotial filtrate of AM-03 (Fig. 2C) revealed 12 peaks. Out of which 5 peaks were identified on the basis of their retention time as gallic acid (Rt 2.664), oxalic acid (Rt 3.132), ferulic acid (Rt 3.376), IAA (Rt 3.571) and cinnamic acid (Rt 4.412). Gallic acid had concentration 16.226µg/ml. Oxalic acid had concentration 0.506µg/ml. The concentration of ferulic acid was 1.399µg/ml. IAA had concentration 7.586µg/ml. Cinnamic acid in this isolate had concentration 1.113 µg/ml. Whereas chlorogenic acid was absent in the sclerotial filtrate of AM-03.

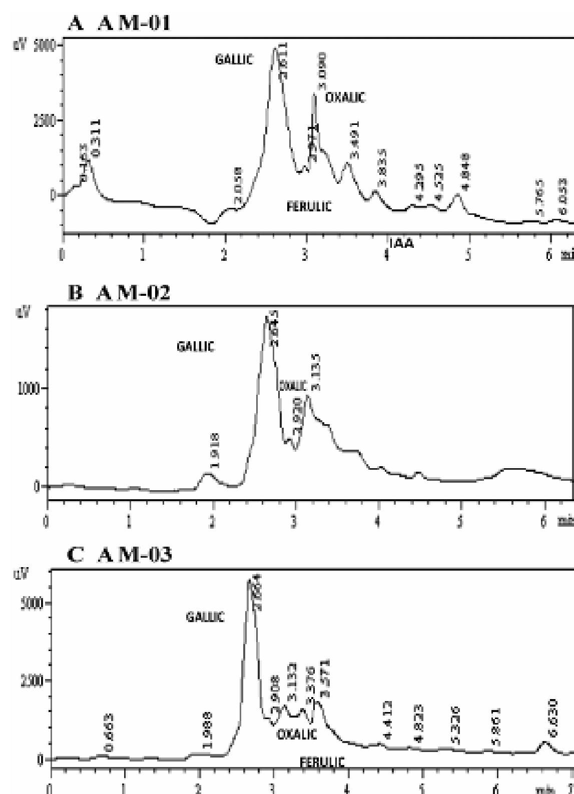

Fig. 2. Peaks of secondary metabolites obtained by HPLC analysis of ethyl acetate fractions of sclerotial filtrate (A) AM-01, (B) AM-02 and (C) AM-03.

The HPLC profile of AM-04 (Fig. 3D) revealed 15 peaks. Out of which 6 peaks were identified on the basis of their retention time as gallic acid (Rt 2.668), oxalic acid (Rt 3.133), ferulic acid (Rt 3.384), IAA (Rt 3.580), chlorogenic acid (Rt 4.310) and cinnamic acid (Rt 4.795). In this strain gallic acid had concentration 13.702µg/ml. Oxalic acid had concentration 1.213µg/ml The concentration of ferulic acid was 1.116µg/ml. IAA was detected in AM-04 having concentration 9.562µg/ml. Chlorogenic acid with concentration 1.678µg/ml and cinnamic acid in this isolate had concentration 1.891µg/ml.

AM-05 (Fig. 3E) revealed 10 peaks through HPLC analysis. Out of which 6 peaks were identified on the basis of their retention time as gallic acid (Rt 2.686), oxalic acid (Rt 3.136), ferulic acid (Rt 3.387), IAA (Rt 3.674), chlorogenic acid (Rt 4.395) and cinnamic acid (Rt 4.608).

The concentration of gallic acid was 3.194µg/ml. The concentrations of oxalic acid was 2.341µg/ml and ferulic acid was 0.207µg/ml. IAA had concentration 1.584µg/ml. Chlorogenic acid was 2.348µg/ml. Cinnamic acid in this isolate had concentration 1.123µg/ml.

AM-06 (Fig. 3F) yielded 7 peaks through HPLC. Out of which 4 peaks were identified on the basis of their retention time as gallic acid (Rt 2.683), oxalic acid (Rt 3.128), IAA (Rt 3.689) and cinnamic acid (Rt 4.772). In this strain gallic acid, IAA and cinnamic acid had concentrations 2.038µg/ml, 1.339µg/ml and 0.306µg/ml respectively. Oxalic acid had concentration 4.65µg/ml. Ferulic acid and chlorogenic acid were absent in this strain.

The HPLC fingerprints of AM-07 (Fig. 4G) revealed 7 peaks. Out of which 4 peaks were identified as gallic acid (Rt 2.707), oxalic acid (Rt 3.138), IAA (Rt 3.765) and cinnamic acid (Rt 4.751). Gallic acid had concenteration 1.871µg/ml. Oxalic acid, IAA and cinnamic acid had concentration 2.546µg/ml, 0.596µg/ml and 3.313µg/ml respectively. Ferulic acid and chlorogenic acid were absent in this strain.

The HPLC analysis of AM-08 (Fig. 4H) revealed 10 peaks. Out of which 5 peaks were identified as gallic acid (Rt 2.689), oxalic acid (Rt 3.132), ferulic acid (Rt 3.385), IAA (Rt 3.660) and cinnamic acid (Rt 4.84). Oxalic acid had concentration 1.123µg/ml. Gallic acid was 3.345µg/ml. The concentration of ferulic acid was 0.206µg/ml. IAA was detected in AM-08 at concentration 1.602µg/ml. Cinnamic acid was found at concentration of 0.525µg/ml. Chlorogenic acid was absent in this isolate.

The HPLC profile of AM-09 (Fig. 4I) yielded 15 peaks. Out of which 6 peaks were identified as gallic acid (Rt 2.66), oxalic acid (Rt 3.142), ferulic acid (Rt 3.385), IAA (Rt 3.744), chlorogenic acid (Rt 4.225) and cinnamic acid (Rt 4.645). Phenolic compound which was present in highest concentration was gallic acid having 4.583µg/ml and lowest concentration was of cinnamic acid having 0.238µg/ml. Oxalic acid had concentration 1.056µg/ml. The concentration of ferulic acid was 0.462µg/ml. IAA was detected at concentration 1.112µg/ml. Chlorogenic acid had concentration of 3.11µg/ml.

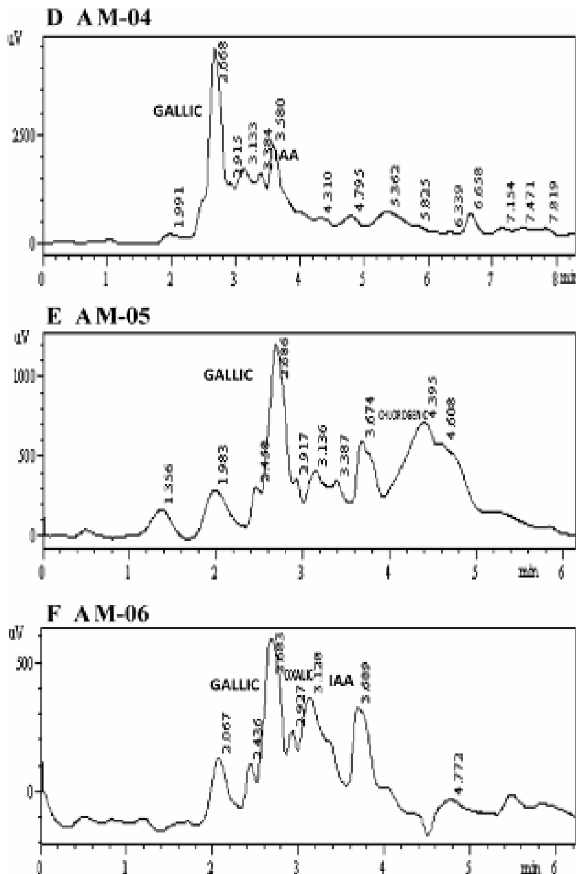

Fig. 3. Peaks of secondary metabolites obtained by HPLC analysis of ethyl acetate fractions of sclerotial filtrate (D) AM-04, (E) AM-05 and (F) AM-06.

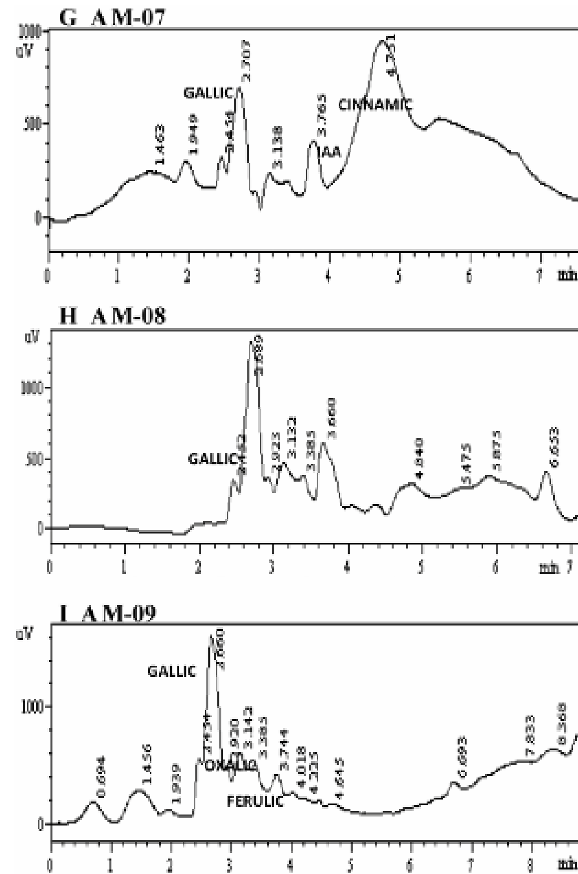

Fig. 4. Peaks of secondary metabolites obtained by HPLC analysis of ethyl acetate fractions of sclerotial filtrate (G) AM-07, (H) AM-08 and (I) AM-09.

AM-10 (Fig. 5J) revealed 16 peaks. Out of which 6 peaks were identified on the basis of their retention time as gallic acid (Rt 2.673), oxalic acid (Rt 3.162), ferulic acid (Rt 3.398), IAA (Rt 3.657), chlorogenic acid (Rt 4.217) and cinnamic acid (Rt 4.497). In this strain highest amount of phenolic acid gallic acid was found which was 10.732 µg/ml and lowest concentration of ferulic acid 1.148µg/ml. The concentration of oxalic acid and IAA

were 1.148µg/ml and 3.395µg/ml respectively. Cinnamic acid and chlorogenic acid had concentrations 1.169µg/ml and 2.346 respectively.

The HPLC analysis of AM-11 (Fig. 5K) revealed 17 peaks. Out of which 6 peaks were identified as gallic acid (Rt 2.683), oxalic acid (Rt 3.140), ferulic acid (Rt 3.372), IAA (Rt 3.506), chlorogenic acid (Rt 4.308) and cinnamic acid (Rt 4.430). Gallic acid had concentration 8.940µg/ml Cinnamic acid was 1.086µg/ml. Oxalic acid had concentration 1.476µg/ml. The concentration of ferulic acid was 1.094µg/ml in this strain. IAA and cinnamic acid had concentrations 8.884µg/ml and 1.086µg/ml respectively. Chlorogenic acid had concentration 2.9µg/ml.

The HPLC analysis of AM-12 (Fig. 5L) revealed 9 peaks. Out of which 4 peaks were identified as gallic acid (Rt 2.715), oxalic acid (Rt 3.158), ferulic acid (Rt 3.434) and cinnamic acid (Rt 4.633). Gallic acid and oxalic acid had concentrations 3.240µg/ml and 1.245µg/ml respectively. Cinnamic acid had concentration 1.187µg/ml. The concentration of ferulic acid was 3.015µg/ml. IAA and chlorogenic acid were absent in AM-12.

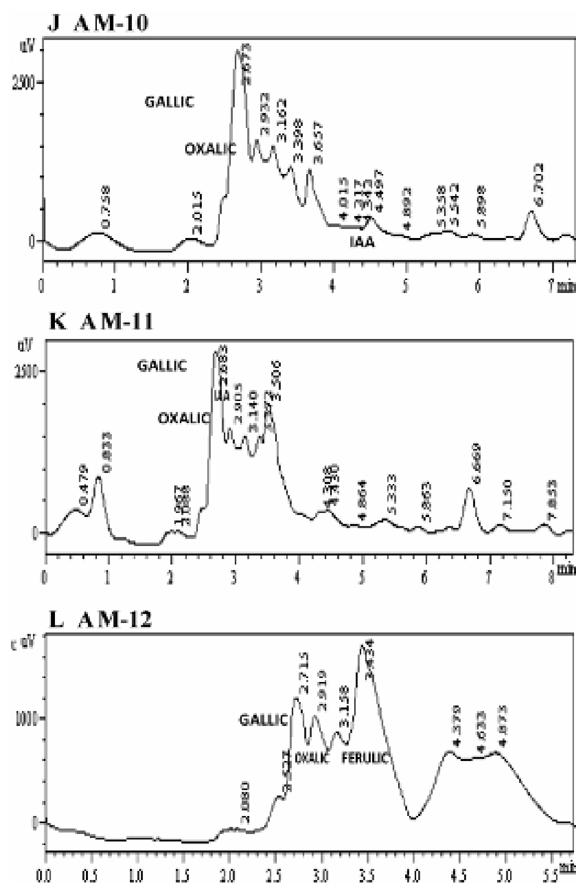

Fig. 5. Peaks of secondary metabolites obtained by HPLC analysis of ethyl acetate fractions of sclerotial filtrate (J) AM-10, (K) AM-11 and (L) AM-12.

Discussion

HPLC analysis of 12 strains of *S. rolfsii* revealed that the lowest numbers of 2 peaks were present in AM-02 as oxalic and gallic acid. Highest numbers of six peaks were identified in AM-04, AM-05, AM-09, AM-10 and AM-11 as oxalic, gallic, ferulic, cinnamic, chlorogenic and indole-3 acetic acid. Gallic, ferulic, chlorogenic and cinnamic acids are phenolic acid that synthesizes following the shikimic acid pathway (Salisbury & Ross, 1986). Ethyl acetate fractions of sclerotial filtrates of 12 strains of *S. rolfsii* showed the qualitative and quantitative variation in their phenolic acid composition revealed by HPLC. No definite pattern of the occurrence of phenolic acids (secondary metabolites) was observed in sclerotial filtrates, though gallic acid and oxalic acid were present in varying amounts in sclerotial filtrates of all strains. However this study reports higher concentration of gallic acid amongst all phenolic acids.

The results showed that sclerotial filtrates of all strains possessed high amount of gallic acid. There are no previous reports on the gallic acid production by fungi *S. rolfsii* and the present work is supposed to be pioneer study which reported the presence of such high levels of gallic acid in *S. rolfsii*. So its role and functions in the fungal survival and pathogenicity is unknown, however it may synergize with rest of the phenolic acids to promote infection and resist control measures.

Phenolic compounds in sclerotia of *S. rolfsii* were reported by Punja & Damiani (1996), but identification of such high levels of some of the phenolics is reported for the first time in the present investigation.

The sclerotia of *S. rolfsii* contained some phenolic acids needed for their growth and resistance against adverse environmental conditions. The role of phenolics as secondary metabolites in living organisms is to induce resistance among the biota for their existence (Harborne, 1988).

Phenolic acids are believed to contribute resistance in fungus against certain pathogens and biocontrol agents. The presence of phenolic acids in *S. rolfsii* might be attributed to its self-defense against other microbes during its survival in soil. As sclerotia is a resistant structure produced by *S. rolfsii* to overcome unfavorable conditions which require high amounts of phenolic acids for their survival (Sarma *et al*., 2002).

Phenolic acids are believed to act as chemical shield for fungus during its attack on host and under unfavorable environmental conditions. So, the detection of highest number of phenolic acids in AM-04, AM-05, AM-09, AM-10 and AM-11 indicated that these were the most pathogenic fungal strains against chickpea that confer high losses in chickpea crops. These strains degenerate defensive mechanism and synthesize excessive phenolic acids if controlling measures are applied. AM-02 is less virulent strain which had least ability to synthesize phenolic acid molecules and thus promoted mild infection against chickpea and can be easily restricted by controlling measures because of its least ability to overcome unfavorable environmental conditions.

References

Akram, A., S.M. Iqbal, C.A. Rauf and R. Aleem. 2008. Detection of resistant sources for collar rot disease in chickpea germplasm. *Pak. J. Bot*., 40(5): 2211-2215.

Anonymous. 2009. *Agriculture statistics of Pakistan 2008-09*. Finance Division, Economic Advisor's Wing, Islamabad, Pakistan.
Anonymous. 2004. Food and Agriculture Organization. http://faostat.fao.org.
Hanachi, P. and S.H. Golkho. 2009. Using HPLC to determination the composition and antioxidant activity of *Berberis vulgaris*. *Euro. Journals*, 29(1): 47-54.
Harborne, J.B. 1988. *Introduction to ecological biochemistry*. (3rd Ed) Academic Press, London.
Ilyas, M.B., M.A. Chaudhry, N. Javed, M.U. Ghazanfar and M.A. Khan. 2007. Sources of resistance in chickpea germplasm against *Ascochyta* blight. *Pak. J. Bot*., 39(5): 1843-1847.
Kempken, F. and M. Rohlfs. 2010. Fungal secondary metabolite biosynthesis a chemical defence strategy against antagonistic animals. *Fungal Ecology,* 3(3): 107-114.
Kokub, D., F. Azam, A. Hassan, M. Ansar, M.J. Asad and A. Khanum. 2007. Comparative growth, morphological and molecular characterization of indigenous *S. rolfsii* strains isolated from different locations of Pakistan. *Pak. J. Bot*., 39(5): 1849-1866.
Mathur, S.B. and S. Sinha. 1970. Role of manuring in control of root rot of guar (*Cyamopsis psoraloides* DC.) and wilt of gram (*Cicer arietinum* L.) caused by *S. rolfsii* Sacc. *Mycopath*., 40: 155-159.
Nene, Y.L., V.K. Sheila and S.B. Sharma. 1984. A world list of chickpea (*C. arietinum* L.) and pigeonpea (*Cajnus cajan* (L.) Millsp.) pathogens. *ICRISAT Pulses Pathology Progress Report*, 32, pp.19.
Punja, Z.K. and A. Damiani. 1996. Comparative growth, morphology and physiology of three *Sclerotium* species. *Mycologia*, 88: 694-706.
Salisbury, F.B. and C.W. Ross. 1986. Lipids and other natural products. *In: Plant physiology*. CBS Publishers and Distributors, Shahdara, Delhi, pp. 268-289.
Sarma, B.K., U.P. Singh and K.P. Singh. 2002. Variability in Indian isolates of *S. rolfsii*. *Mycologia,* 94(6): 1051-1058.
Shwab, E.K. and N.P. Keller. 2008. Regulation of secondary metabolite production in filamentous ascomycetes. *Mycol. Res*., 112: 225-230.
Suzuki, F. and S. Konno. 1982. *Regional report on Grain Legumes Production in Asia*. Tokyo Japan: Asian Productivity Org., pp. 93.

(Received for publication 12 February 2011)