Int.J.Curr.Microbiol.App.Sci (2018) *7*(10): 2152-2160

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 10 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.710.247

Comparative Study on Biochemical Parameters among Ascochyta Blight Infected Moderately Resistant and Susceptible Chickpea Genotypes

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Keywords

Ascochyta, Defense, Genotype, Infection, Inoculation and resistance

Article Info

Accepted:
15 September 2018
Available Online:
10 October 2018

A B S T R A C T

The chickpea genotype ICCV 5530, which is moderately resistant and ICCV 4991, susceptible to *Ascochyta rabiei* were selected to identify the changes in biochemical activity in response to infection by the fungus *Ascochyta rabiei*. Twelve day old seedlings were inoculated with spore suspension of the pathogen at 5×10^4 spore/ml concentration using a Haemocytometer and incubated under Controlled Environmental Facility (CEF). Sampling was done at 2, 4 and 8 days after inoculation. Chlorophyll 'a', chlorophyll 'b' and total chlorophyll, total phenol, total soluble sugars, Peroxidase (PO), Polyphenol Oxidase (PPO) Phenylalanine Ammonia Lyase (PAL) activity was assayed by adopting the standard procedure described by various researchers. Higher total chlorophyll content was recorded in un-inoculated leaves of ICCV 4991 and ICCV 5530 genotypes compared to inoculated leaves. But higher total chlorophyll content was recorded in ICCV 5530 than susceptible at all the stages of sampling. The inoculated genotype ICCV 5530 had higher total phenolic content (11.57 mg/g fresh wt) than the ICCV 4991(7.32 mg/g fresh wt). The low concentration of total phenolics in leaves of ICCV 4991 genotype might be the reason for becoming highly susceptible to the disease. Lesser accumulation of the total soluble sugar was observed in ICCV 5530(7.95 mg/g fresh wt.) as compared to ICCV 4991(13.08 mg/h fresh wt). However, the expression level of defense related enzymes like PO, PPO and PAL were differed largely in moderately resistant and susceptible genotypes after infection with the pathogen. The increased activity of phenolic compounds, total sugars, CAT, PO, PPO and PAL were found in moderately resistant genotype compared to susceptible one. Therefore, these factors might be involved imparting disease resistance and could be considered for development of broad based resistance against ascochyta blight.

Introduction

Pulses are vital source of protein with high fibre content and provide ample quantity of vitamins and minerals to the human diet. Keeping in view large benefits of pulses for human health, the United Nations has proclaimed '2016' as the '*International Year of Pulses'*. Thus, due attention is required to enhance the production of pulses not only to meet the dietary requirement of protein but also to raise the awareness about pulses for

achieving nutritional food security and environmental sustainability.

Among the pulses, chickpea (*Cicer arietinum* L.) is a deep rooted, drought tolerant crop belonging to the family *Fabaceae* and contributed 48 per cent towards India's total pulses production. The production of chickpea is limited by various biotic and abiotic stresses throughout the world. Among the biotic stresses, Ascochyta blight is considered to be the most destructive diseases of the temperate environments of Indian subcontinent and world reducing the overall yield of the crop (Nene, 1982; Chongo *et al.,* 2000).

Present day cultivars often lose the resistance and become susceptible to diseases. Therefore, mechanism involved in the resistance against fungus during host-pathogen interaction in the resistant and susceptible genotypes are poorly understood. host pathogen Plants possess efficient defense mechanisms against various pathogens where static and dynamic secondary metabolites play important roles either as local or systemic resistance factors in protecting the plants against various pathogens (Redman *et al.,* 1999). The host-pathogen interaction induce signaling molecule in plants system, which lead to production of antimicrobial phenolic substances which are known to participate in a number of biochemical processes. Infection in certain diseases is characterized by increased synthesis of certain precursors of phenolic compounds and oxidation products of phenolics, such as quinines, which exhibit more toxicity to microorganisms.

Defensive enzymes are among the most influential and widely distributed products in the plants. Peroxidase, PPO and PAL were reported in plants treated with various biotic and abiotic inducer (Raghvendra *et al.,* 2007). The plants peroxidases have been implicated in a variety of defense-related processes, including the hypersensitive response, lignification, cross-linking of phenolics and glycoprotein, suberization and phytoalexin production. Catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules thus avoiding cellular disintegration (Bolwell and Wojtaszek, 1997). Polyphenol oxidase (PPO) is allowed to leave the chloroplast thylakoid membrane and come in contact with the accumulated phenolic compounds. In the presence of oxygen, PPO oxidizes the phenolic compounds that are in the form of odiphenol to o-quinone (Raj *et al.,* 2006). Higher polyphenol oxidase and peroxidase and lower catalase activity in chickpea's genotypes could be used as parameters for screening against *A. rabiei* (Kumar *et al.,* 2014). Kaur *et al.,* (2012) reported that higher peroxidase activity could be used as a marker for ascochyta blight resistance in chickpea genotypes Phenylalanine ammonialyase (PAL) is the key enzyme catalyzing the biosynthesis of phenolics and lignin from the aromatic amino acid phenylalanine (Cartea *et al.,* 2010). In resistance to chickpea ascochyta blight, biochemical defense enzymes such as polyamine, diamine oxidase and peroxidase and PAL suggested to play a significant role in regulating the accumulation of phenolics and phytoalexins in response to infection (Peltonen *et al.,* 1998).

The present study was focused on understanding the role of phenols and biochemical enzymes in defense against *Ascochyta rabiei* under compatible and incompatible reaction with chickpea genotypes.

Materials and Methods

The present investigation was carried out during 2015-16 under Controlled Environmental Facility (CEF) in the division

of Kanayo F Nwanze Crop Protection Division, Legume Pathalogy ICRISAT, Patancheru, Hyderabad.

Moderately resistant (ICCV 5530) and susceptible (ICCV 4991) to *Ascochyta rabiei* of chickpea genotypes were raised in a greenhouse under the conditions of 25±1°C temperature for 12 days. The fungus was mass multiplied from single conidial isolate by inoculating soaked and autoclaved kabuli seeds. The inoculated seeds were incubated at 20±1°C for 8 days with a 12-h photoperiod. Twelve days after incubation, spore suspension was prepared by soaking *A. rabiei* infected kabuli chickpea seeds in sterilized distilled water for 30 minutes and vortexed for 2-3 minutes to dislodge the spores from the seeds. The concentration of the double-layered muslin cloth filtered spore suspension was adjusted to 5×10^4 conidia/ml using a Haemocytometer. The trays with 12 day old seedlings were transferred to Controlled Environmental Facility (CEF) to acclimatize for 24 hours before inoculation.

After 24 hours; spore suspension of *A. rabiei* (5×10^4 conidia/ml) was sprayed on the moderately resistant and susceptible genotypes until runoff. The seedlings were allowed to dry partially for 30 minutes of post inoculation to avoid dislodging of spores. In the CEF air temperature (20±1°C), relative humidity (95-100% continuously for 96 hr and thereafter 6-8 h a day till the completion of experiment) and photoperiod (12h, ~1500 lux light intensity provided with fluorescent lights) was maintained throughout the experiment. An observation for the Per cent Disease Index (PDI) and sampling was done at 2, 4 and 8 days after inoculation. The plants were randomly collected and assessed for per cent disease severity using 1 to 9 rating scale as described by Reddy and Singh (1984) and further the grades were converted to PDI using the formula given by Wheeler (1969).

Sampling and enzymatic study

The chickpea leaves inoculated with *A.rabiei* were collected randomly at 2, 4 and 8 days after inoculation along with control plants to assess the biochemical changes and host pathogen interaction. The leaf samples collected were dipped in liquid nitrogen to stop plant metabolic activity, then placed in ice box containing ice cubes and brought to the laboratory.

Chlorophyll 'a', chlorophyll 'b' and total chlorophyll were estimated following the method described by Arnon (1949). The total phenol in plant samples was estimated following Folin- Ciocalteau reagent (FCR) method (Bray and Thorpe, 1954). In Catalase (CAT) estimation, 500 mg of fresh frozen leaf sample were homogenized with 3 ml of 25 mM sodium phosphate buffer (pH 7.8). Homogenate was transferred into an Eppendorf tube and centrifuged at 20,000 rpm for 30 min at 4˚C, and then, the supernatant was used for enzyme assay. The reaction mixture (2,000 µL) consisted of 1,400 µL 25 mM Na-phosphate buffer, 500 µL, 10 mM H_2O_2 and 100 µL crude enzyme extract. CAT activity was assayed by measuring H_2O_2 consumption at 240 nm for 1 min in spectrophotometer. Enzyme activity was expressed as CAT units' min-1 mg-1 protein.

Total soluble sugars were estimated according to the method described by Yoshida *et al.*, (1971) using anthrone reagent. The peroxidase activity was assayed by measuring the oxidation of guaiacol in the presence of hydrogen peroxide into water at 470nm as described by Hammer Schmidt *et al.,* (1982). Activity was expressed as the increase in absorbance at 470nm in min-1mg-1 of protein. PPO activity was determined as per the procedure given by Mayer *et al.,* (1965). Five hundred milligram of leaves was used for polyphenol oxidase estimation: the reaction

mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extracts. To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm/min/g fresh weight of leaf tissue. Assay and determination of PAL was carried out by adopting the procedure given by Sadasivam and Manickam (1996). The absorbance was read at 290 nm in UV Spectrophotometer. The quantity of cinnamic acid formed was calculated by using a standard curve of cinnamic acid. The results of each experiment are average of two replications.

Results and Discussion

The inoculation of *Ascochyta rabiei* fungus onto chickpea genotypes, ICCV 4991(S) and ICCV 5530(MR) results in extreme changes in biochemical factors and disease severity were observed. The *Ascochyta rabiei* inoculated genotypes (ICCV 4991 and ICCV 5530) showed a wide range of symptoms depending on their level of resistance and genetic makeup. Susceptible genotype ICCV 4991 start developed disease symptoms at 4 days after inoculation as water-soaked lesions on the upper leaves and stem. At 10 days after inoculation, 100 per cent disease severity was observed on susceptible genotype ICCV 4991 but moderately resistant genotype ICCV 5530 did not show much increase in disease severity.

The study on effect of chlorophyll content (mg/g fresh wt) in resistant and susceptible genotypes after inoculation with *Ascochyta rabiei* revealed that at different DAI, the chl-'a' content was differed. In susceptible genotypes, there was decrease of 23.33, 10.98 and 55.68 per cent in chl-'a' content was observed in diseased leaf over healthy ones (Table 1). Overall, 6.20 per cent more of chl-'a' was recorded in resistant genotypes than susceptible ones. Over all, there was 37.24 per cent more of chl-b was recorded in resistant genotypes than susceptible ones. The study revealed that at different days after inoculation with the pathogen, the total chlorophyll content differed significantly. Among the genotypes, resistant genotypes recorded higher total chlorophyll content than susceptible genotype. Whereas, in diseased leaf showed 6.82 per cent more of total chlorophyll in moderately resistant as compared to susceptible genotype. Overall, there was 18.86 per cent change in total chlorophyll content in resistant genotype as compared to susceptible one.

Total soluble sugars (mg/g fresh wt.) of leaves of resistant and susceptible genotypes were estimated. Among the genotypes resistant genotype recorded 7.81, 7.99 and 8.06 mg/g fresh wt of total soluble sugars and susceptible genotype recorded 13.09, 13.14 and 13.01 mg/g fresh wt of total soluble sugars when inoculated with *A. rabiei* at 2, 4, and 8 DAI. The total soluble sugar content of resistant genotype ICCV 5530 was less than susceptible genotype ICCV 4991 (Table 2).

The plant-pathogenic fungal, bacterial and viral interactions, which showed that certain common phenols and phenolic substances are toxic to pathogens, which have long been considered as important defense related compounds whose levels are naturally high in the resistant varieties of many crops (Gogoi *et al.*, 2001) and accumulates in plants after infection, especially in resistant varieties. Sugars could limit the spread of the pathogen by isolating the infected cells, and protect the tissues against water loss (Herbers *et al.*, 1996). Soluble sugars directly and indirectly play a significant role in resistance processes. A high concentration of soluble sugars may directly limit the pathogen colonization of the cells as a result of increased osmotic potential (Farrer, 1989).

Phenolic compounds may involve in enhancing the mechanical strength of host cell walls by the synthesis of lignin and suberin compounds that are involved in the construction of physical barriers that can obstruct the spread of pathogens (Ngadze *et al.,* 2012). The total phenol content at different days after inoculation is depicted in Table 2. In the present study, the amount of total phenols differed significantly among the two genotypes. The resistant genotype ICCV 5530 recorded highest amount of phenol (mg/g fresh wt) both in healthy (8.295, 9.525, 12.33) and inoculated one (10.21, 11.05, 13.45) at 2, 4 and 8 days after inoculation. And also, the amount of total phenols was found decreased in inoculated susceptible genotype ICCV 4991 at all the stages of inoculation, while increased in inoculated resistant genotype ICCV 5530 as compared to their non-inoculated plants. This higher amount of phenols in these plants has been correlated with enhanced resistance to *A.rabiei* as the accumulation of total phenols is usually found to be higher in resistant genotypes compared to susceptible ones (Velazhahan and Vidhyasekaran, 1994).

Catalase activity (change in absorbance min-1 mg-1 protein) increased in resistant genotype (ICCV 5530) after inoculation with *A. rabiei* at all the stages (2, 4 and 8 DAI) of sampling (11.39, 13.90, and 15.93). In susceptible genotype (ICCV 4991) there was slight increase at 2 DAI (9.48) and then declined (8.65, 6.44) to the level below the enzyme activity present in uninoculated control plants (9.00, 8.75) at 4 and 8DAI (Table 3). These results suggests that catalase might also be involved in defense mechanism of chickpea against *A. rabiei* by detoxifying the toxic oxygen derivatives, that are considered as common features of stress conditions (Foyer *et al.,* 1994).When plants are exposed to different stresses either biotic or abiotic, results in shifting of their metabolism towards oxidative direction and plant mobilize the anti-oxidative defence mechanisms by several stress enzymes like peroxidase and catalase, in order to eliminate the effect of free radicals (Gill and Tuteja, 2010).

Increased PO activity (change in absorbance at 470 min-1mg-1 of protein) was recorded in the resistant genotype ICCV 5530 (1.10) compared to susceptible genotype ICCV 4991 (0.72) (Table 3). Initially there was a slight increase in the PO activity (0.40) in the leaves of susceptible genotype but after 4 and 8DAI it was declined (0.25, 0.11). In resistant variety, however activity continued to increase till 8DAI with *A. rabiei*. The activity of PPO in healthy leaves of resistant genotype ICCV 5530 was higher (0.87, 0.64, 0.05) than the corresponding leaves of susceptible genotype ICCV 4991 (0.34, 0.23, 0.01). Two days after inoculation, increased PPO activity was observed in susceptible genotype inoculated with *A. rabiei* (0.61), and then declined at later stages of sampling (0.04, 0.11) (Table 3).

The above results are supported by Nawar and Kuti (2003), according to them an increase in peroxidase activity is considered as preliminary indicator for resistance to chocolate spot disease of broad beans. These compounds act as a barrier against pathogen invasion. Accumulation of protein and peroxidase are the part of defense mechanisms against plant pathogens (Sarwar *et al.,* 2011). Hassan *et al.,* (2007) also revealed that increased peroxidase activity in faba bean in response to inducer molecule showed resistance against chocolate spot disease. Plants have complex systems to adapt to biotic and abiotic stresses. Among the response to stresses, increased production of Reactive Oxygen Species (ROS) is one of them. ROS are formed due to incomplete reduction of oxygen. ROS include a variety of short and long-lived molecules such as superoxide, hydroxyl radicals and hydrogen peroxide (Apel and Hirt, 2004).

Table.1 Content of chlorophyll a, chlorophyll b and total chlorophyll (mg/ g fresh wt.) in chickpea genotypes at different days after inoculation (DAI) with *A. rabiei*

Parameter assayed	Days after inoculation (DAI)	Genotype ICCV 4991		Per cent change	Genotype ICCV 5530		Per cent change
		Healthy (mg/g)	Inoculated (mg/g)		Healthy (mg/g)	Inoculated (mg/g)	
Chl a	2	29.03	23.53	23.33	27.91	26.66	4.66
	4	26.92	24.25	10.98	29.30	23.46	24.88
	8	30.96	19.88	55.68	29.47	20.70	42.31
	Mean	**28.97**	**22.56**	**28.41**	**28.89**	**23.61**	**22.36**
Chl b	2	20.41	17.51	16.54	22.77	22.52	1.09
	4	21.22	14.10	50.52	21.15	20.47	3.31
	8	20.93	12.75	64.17	22.45	20.95	7.15
	Mean	**20.85**	**14.79**	**41.03**	**22.12**	**21.31**	**3.79**
Total chl	2	47.53	39.39	20.67	47.04	45.18	4.12
	4	45.84	35.90	27.68	49.61	42.35	17.13
	8	50.21	32.66	53.71	50.06	40.94	22.26
	Mean	**47.86**	**35.98**	**32.99**	**48.90**	**42.82**	**14.19**

Table.2 Concentration of Total soluble sugars and Total phenols in chickpea genotypes at different days after inoculation (DAI) with *A. rabiei*

Total soluble sugars (mg/g fresh wt)	2	20.12	13.09	53.71	11.22	7.81	43.66
	4	19.82	13.14	50.84	12.71	7.99	59.07
	8	20.96	13.01	61.11	12.08	8.06	49.88
	Mean	**20.30**	**13.08**	55.20	**12.00**	**7.95**	50.92
Total phenols(mg/g fresh wt)	2	8.23	7.32	-11.11	8.295	10.21	23.14
	4	9.42	5.80	-38.37	9.525	11.05	16.06
	8	11.28	8.83	-21.67	12.33	13.45	9.12
	Mean	**9.64**	**7.32**	-23.72	**10.05**	**11.57**	16.11

Table.3 Concentration of defensive enzymes viz., PO, PPO, PAL and catalase activity in resistant and susceptible chickpea genotypes at different days after inoculation (DAI) with *Ascochyta rabiei*

Enzymes assayed	DAI	Genotype ICCV 4991		Genotype ICCV 5530	
		Healthy (mg/g)	Inoculated (mg/g)	Healthy (mg/g)	Inoculated (mg/g)
Peroxidase (PO) change in absorbance/min/mg of protein	2	0.35	0.40	0.53	1.00
	4	0.70	0.25	0.71	0.85
	8	0.19	0.11	1.35	1.45
	Mean	**0.41**	**0.72**	**0.86**	**1.10**
Polyphenol oxidase (PPO) (Change in absorbance/min/mg fresh weight	2	0.34	0.61	0.87	0.93
	4	0.23	0.04	0.64	0.71
	8	0.01	0.11	0.05	0.33
	Mean	**0.19**	**0.25**	**0.52**	**0.66**
Phenylalanine ammonia lyase (PAL) nmol transcinnamic acid/min/mg protein	2	1.71	2.04	2.17	2.55
	4	2.12	1.72	1.88	2.11
	8	1.79	1.77	1.79	2.63
	Mean	**1.87**	**1.84**	**1.951**	**2.436**
Catalase(CAT) (Change in absorbance/min/mg of protein)	2	7.38	9.48	10.01	11.39
	4	9.00	8.65	10.84	13.90
	8	8.75	6.44	14.42	15.93
	Mean	**8.38**	**8.19**	**11.76**	**13.74**

The level of ROS is maintained to optimum level by ROS-scavenging enzymes such as PPO, PO and CAT. Peroxidase also produces free radicals and hydrogen peroxide which are toxic to many microorganisms (Pena and Kuc, 1992). In chemically induced plants, Peroxidase activity was significantly increased as compared to un-inoculated plants (Chaudhary *et al.,* 2001). Early and enhanced expression levels of many defensive enzymes are the main factors of plant resistance to fungus. The PO and PPO activity was observed higher in resistant seedlings than in susceptible seedlings indicates that PO and PPO might have played a considerable role in enhancing host resistance.

In the phenylpropanoid metabolism, PAL is the primary enzyme plays a major role in the synthesis of a number of defense-related secondary metabolites such as phenols and lignin (Hemm *et al.,* 2004). The activity of PAL affects biosynthetic pathways of phenolic compounds (Jayaraj *et al.,* 2010). The activity of PAL increased at 2, 4 and 8 days after inoculation with *Ascochyta rabiei* in resistant genotype ICCV 5530 (2.55, 2.11 and 2.63). Increase was more pronounced in resistant genotype ICCV 5530 than in susceptible ones (Table 3). PAL activity was higher in inoculated plants of resistant genotype (2.55, 2.11 and 2.63) than in the susceptible genotype ICCV 4991 (2.04, 1.72, and 1.77). There was an increase in the activity of PAL in susceptible genotype at 2 DAI (2.04) with *A. rabiei* pathogen then declined (1.72, 1.77) at 4 and 8 DAI.

Our findings indicated that phenolic compounds, total sugars, CAT, PO, PPO and PAL played an active role in disease resistance against ascochyta blight of chickpea. The pre-infectional presence of high level of phenolics and post-infectional increase in defense enzymes in incompatible reaction may have further role in disease resistance; thus, additional studies are needed to characterize the phenolic compounds and antioxidative enzymes involved in host pathogen interaction. The higher quantities of defense related proteins such as PAL, PPO and PO in insect infested tissues governs stronger resistance. These enzymes are also actively involved in biosynthesis of defensive enzymes e.g. PAL is involved in the formation of phenolics through phenylpropanoid pathway (Shafique *et al.,* 2014).

Increased biochemical activity during plant fungus interaction is an important feature of resistance to pathogen. Constitutively induced defensive enzymes and level of phenolic compounds expressed in the genotypes are actively involved in imparting resistance to *A.rabiei* and also be considered as biochemical markers for development of broad based and durable resistance against ascochyta blight disease.

Acknowledgment

Authors are grateful to ICAR for providing fund for professional attachment training at ICRISAT. I sincerely acknowledge Dr G. P. Dixit, Coordinator (AICRP on chickpea), IIPR, Kanpur for their moral support and encouragement towards my research goals.

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How to cite this article:

Manjunatha, L., Mamta Sharma, P.R. Saabale, B.M. Ravikumara and Singh, N.P. 2018. Comparative Study on Biochemical Parameters among Ascochyta Blight Infected Moderately Resistant and Susceptible Chickpea Genotypes. *Int.J.Curr.Microbiol.App.Sci.* 7(10): 2152-2160. doi: https://doi.org/10.20546/ijcmas.2018.710.247