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Pathogen Response of Chickpea varieties against Macrophomina phaseolina

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Abstract: **Host cell death occurs during many interactions between plants and the pathogens that infect them. Plants utilize defensive strategies that protect them against a multiplicity of attackers. This cell death can be associated with disease resistance or susceptibility, depending on the nature of the pathogen. The most well-known cell death response in plants is the hypersensitive response (HR) associated with a resistance response. Plants defend themselves against attack from insects and pathogens with various resistance strategies. For this polyphenol content, salicylic acid content were determined in control and after *Macrophomina phaseolina* treatment in chickpea var. 'PUSA- 391 and PUSA-1003'.The results indicated significant changes and a distinct role of polyphenols, salicylic acid in the defense response of chickpea after treatment. Maximal activity in elicited cells occurred after 48 h.**

Keywords: Chickpea, *Macrophomina phaseolina*, Charcoal root rot, Polyphenol, Salicylic acid

INTRODUCTION

The intimate interactions between cultivated crops and bacterial, viral and fungal pathogens often results in serious outbreak of disease. The plant defense response is a multi component system and is of broad spectrum (Lattanzio *et al*, 2006). Resistance in plants is manifestated by the inability of pathogen to grow or multiply and spread and often takes the form of hypersensitive reaction (HR) (Theis and Lerdau, 2003). HR is commonly regulated by direct or indirect interactions between a virulence proteins from pathogen and resistance proteins from plant and it can be the result of multiple signaling pathways (Elena *et al*, 2005). Exciting advances have been made in the identification of cellular protective components and cell death suppressors that might operate in HR. These local responses often triggers non-specific resistance throughout the plants known as systemic acquired resistance (SAR) providing durable protection against challenge infection by the broad range of pathogens (Delaney *et al*,1994). SAR can be distinguished from other disease resistance response by both the spectrum of the pathogen protection and the associated changes in the gene expression of a set of genes called SAR genes (Abramovitch and Martin, 2004). Chickpea (*Cicer arietinum.*) is the second most important cool season legume crop in the world. Chickpea is a self-pollinated crop. Cross-pollination is rare; only 0-1% is reported (Singh, 1987). Supplementation of cereals with high protein legume is potentially one of the best solutions to protein-calorie malnutrition particularly in the developing countries.

It not only serves as a good source of nutrition to the people but also improves the soil. However, this crop is susceptible to various biotic stresses and hence several strategies have been attempted to produce varieties resistant to these stresses and also to harvest high yield capacity. Fungi are of major significance as mutuality symbionts and parasites of plants, so their study is an important part of plant sciences and plant pathology (Choudhary *et al*, 2011). *Macrophomina phaseolina* (Tassi) good a soil inhabiting fungus is an important root pathogen and causes charcoal rot in over 500 plant species including chickpea (Patil, 2011). It remains to be a challenging task in terms of management, since it is soil-borne in nature. It is distributed worldwide and is prevalent in arid, sub-tropical and tropical climate, especially in the areas with low rainfall and high temperature. A number of phenolic compounds are involved in resistance reaction, though these are also present in normal plants but synthesis and accumulation occurs at a faster rate after infection in the resistance varieties than in susceptible ones. Polyphenols are present in a variety of plants utilized as important components of both human and animal diets (Duthie and crozier, 2000). Plants phenolics are the most important group of secondary

plant products that have been implicated in both constitutive and induced resistance (Cleberson *et al*, 2006).

They play an important role in reducing the susceptibility of a plant to pathogens. A distinct correlation between the degree of a plant resistance and phenolics present in plant tissues has been demonstrated (Onyilagha and Grotewold, 2004). Among the diverse roles of polyphenols, they protect the cell constituents against destructive oxidative damage thus limiting the risk of various degenerative diseases associated with oxidative stress (Villano *et al*, 2005). Salicylic acid (SA) levels drastically increases in the immediate vicinity of hypersensitive tissues of pathogen inoculated host plant and later on it can be recovered from the phloem indicating that it is translocated in plants (Mur *et al*, 1997). SA is involved in the production of pathogenesis related proteins and phytoalexins in response to pathogens (Murphy *et al*, 2000). Salicylic acid, several lines of evidence indicate that the salicylic acid may be involved in the development of SAR in tobacco and cucumber (Malamy *et al*, 1990).

MATERIALS AND METHODS

Plant material as per PUSA-391 and PUSA-1003 cultivar of chickpea (*Cicer arientium*) were procured from IARI, PUSA New Delhi. The surface sterilized seeds were grown in sterilized garden soil in plastic pots in the botanical garden, Department of Bioscience and Biotechnology Banasthali University. The seeds germinated within 2-3 days after sowing and 15 days old plants were taken for different experimental assay.

Pathogen

Macrophomina phaseolina was obtained from IMTECH Chandigarh (MTCC 2165).

Mode of plant infection

Cultivars of chickpea which were of contrasting type were used in this study. Seeds were surface sterilized with 1% $HgCl_2$ for 2 minutes and then washed several times with distilled water. For sowing seeds were placed in pots of 15cm diameter containing 1kg sterilized soil and vermicompost (3:1) in growth chamber under 16h/18h day/night photoperiod of 220±20 $\mu E/m^2/s$ at changing day/night temperature of 24 ºC/20 ºC .15 days old seedling were used for inoculation with 10 ml of mycelia suspension of *M. phaseolina* using thin layer chromatography sprayer. The mock infected plants were sprayed with sterile water after rubbing the leaves with sand paper. The infected and the mock infected plants were then placed in the growth chamber. After treatment, the experiments were performed in both control and infected plants at interval of 0 hr, 24 hrs, 48 hrs, 72 hrs and 96 hrs up to 168 hrs.

Assay of polyphenols:

0.5 ml of the polyphenol extract was mixed with 8.5 ml of distilled water and 0.5 ml of folin-ciocalteu reagent. After 3 min of incubation, 1 ml of 25% sodium carbonate was added. This was mixed well and the reaction mixture was incubated at room temperature for one hour. The absorbance was read against blank at 725nm using UV-spectrophotometer (spectronic-119). The polyphenol content was calculated as micro gram fresh weight of plant tissue by using a standard curve of catechol.

Assay for salicylic acid:

1ml of the extract was taken to which 5 ml of color reagent was added. This color reagent was prepared by dissolving 4gm of $HgCl_2$ in 80 ml of warmed distilled water, then 12 ml of HCl was added and 4gm of ferric nitrate. The volume was then made up to 100 ml. 1ml of supernatant and 5ml of color reagent were mixed and then centrifuged at 2000 rpm for 2 minutes. Methanol was used as a blank. The supernatant was read against the blank at 540 nm. The amount of salicylic acid in the samples was calculated using standard value obtained from standard curve of salicylic acid.

RESULTS:

The surface sterilized seeds of both varieties of chickpea were taken and sown in sterilized soil pots and vermicompost (3:1). After germination 15 days old plants were taken for further studies. The samples were analyzed with a time interval of 24 hours after inoculation with pathogen up to 168 hours.

Changes in polyphenol content: The polyphenol content was determined in *Cicer arientium* var. 'PUSA 391 and PUSA 1003' at different time intervals using gallic acid as standard. The phenolic content of methanolic extract of leaves of both plants was calculated as gallic acid equivalents. From the data given in Table 1, it is found that the polyphenol content was higher in the *M. phaseolina* treated plants as compared to the control. The polyphenol content was determined in both varieties of chickpea at different time of fungal inoculation using *in vivo* system. A fold increase was recorded at 48 hrs after pathogen infection in one month old plants (figure 1). The content first increases, reaches a maximum and then decreases. A positive correlation was found between polyphenol activity and resistance response of the variety. Correlation is significant at the 0.01 level. This is an agreement with results reported by Kuc (1982). Changes in phenolic acid content in tomato fruits in response to pathogen attack was determined by Ruelas *et al*, (2006). A number of phenolic acids were synthesized or significant increase in the concentration of phenolic acids was observed after inoculation with inoculums of *M. phaseolina* (Bray, 1954). The induction of phenolic compounds as part of the defense system against pathogen using elicitors has also been demonstrated by Pearce *et al*, (1998).

Table.1.Quantitative changes in Polyphenol content in control and pathogen (*M. phaseolina*) treated plants of *Cicer arientium* var. 'PUSA -391 and PUSA-1003.

Time (hr)	PUSA – 391			PUSA – 1003		
	C	I	Decrease (%)	C	I	Decrease (%)
0	4.35±0.03	7.54±0.04	42.3	4.47±0.03	6.18±0.04	27.6
24	4.84±0.06	8.30±0.06	41.6	6.07±0.04	6.78±0.06	10.4
48	6.23±0.06	9.02±0.04	30.9	6.75±0.09	8.93±0.06	24.4
72	5.83±0.05	10.1±0.04	42.2	4.66±0.07	8.13±0.03	42.6
96	5.38±0.08	8.17±0.04	34.1	3.68±0.07	6.17±0.13	40.3
120	3.98±0.06	7.23±0.04	44.9	2.62±0.05	3.14±0.06	16.5
144	3.59±0.05	6.58±0.04	45.4	2.33±0.05	2.73±0.06	14.6
168	2.88±0.05	4.99±0.04	42.2	2.18±0.05	2.39±0.05	8.7

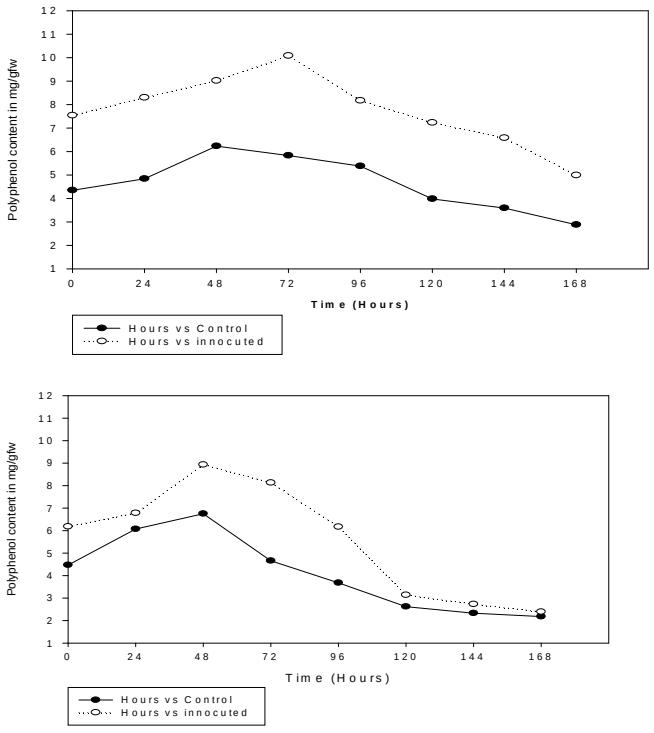

Fig 1: Polyphenol activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Cicer arientium* plants *viz;* PUSA 391 and PUSA 1003.

Changes in Salicylic acid (SA) content:

Salicylic acid content was found to be increased in both varieties after infection with *M. phaseolina in vivo* conditions. The data of *in vivo* investigation revealed that the SA content in the inoculated plant increased first and then followed by a subsequent decrease in its amount both varieties in 15 days old plants. Table 2 shows detailed comparison of control plants of both varieties with infected one. A fold increase was recorded at 48hrs after pathogen infection in one month old plants (fig.2). A positive correlation was found between salicylic activity and resistance response of the variety. Pearson Correlation is significant at the 0.01 level**.** This shows that concentration of salicylic acid rises after pathogen attack in *in vivo* conditions. SA plays a central role in plant disease resistance response including activation of SAR, an inducible defense response that confers a long lasting enhanced resistance against a broad spectrum of pathogens. Elevated levels of SA have been found to be required for SAR induction (Alvarez, 2000).

Table.2. Quantitative changes in Salicylic content in control and pathogen (*M. phaseolina*) treated plants of *Cicer arientium* var. 'PUSA -391 and PUSA-1003.

Time (hr)	PUSA – 391			PUSA – 1003		
	C	I	Decrease (%)	C	I	Decrease (%)
0	15.0±0.50	16.4±0.6	8.5	8.93±0.4	10.0±0.4	10.8
24	17.1±0.50	18.9±0.2	9.5	10.3±0.3	12.9±0.5	20.0
48	19.0±0.97	20.6±0.2	7.7	11.9±0.4	16.4±0.5	27.7
72	19.5±0.18	22.5±0.4	13.3	12.7±0.4	15.6±0.5	18.2
96	18.9±0.91	23.6±0.2	19.9	11.7±0.4	13.1±0.1	10.9
120	18.5±0.25	19.7±0.1	6.0	10.3±0.3	12.5±0.3	17.2
144	16.2±0.48	17.9±0.2	9.4	8.33±0.3	10.5±0.4	20.9
168	15.1±0.50	16.5±0.4	8.4	6.40±0.3	8.05±0.2	20.5

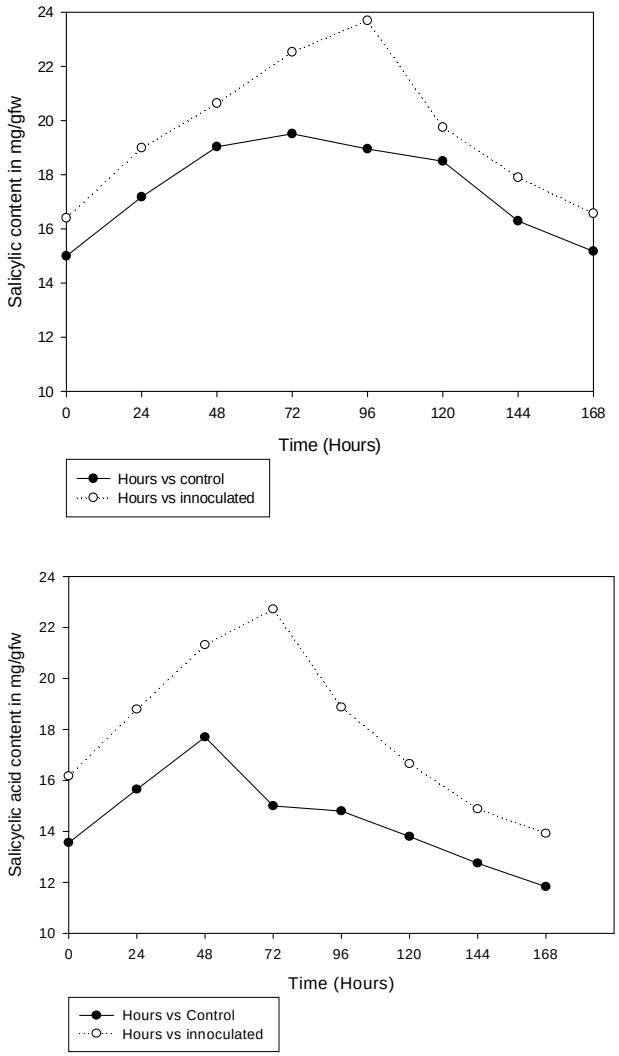

Fig 4: Salicylic activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Cicer arientium* plants *viz*; PUSA- 391 and PUSA- 1003.

DISCUSSION

Experiments were done to investigate the changes after inoculation with fungal pathogen *M. phaseolina*. The result indicate an increases in polyphenol, salicylic acid content in the infected plants compared to control plants *in vivo* system. The response of variety PUSA 391 was found to be more pronounced in comparison to other variety. This variety fights more actively by responding fast and producing higher concentration of polyphenols, Salicylic acid to combat pathogen infection. The high level of production of polyphenols, and salicylic acid content in PUSA-391 after pathogen attack attributes to varietals difference. Both were found to be responding to infection by *M. phaseolina* but the response of PUSA-1003 was more pronounced. This variety was found to be more efficient in fighting against the pathogen attack. So, working on this variety can help in developing the resistant variety, which can not only survive but grow efficiently against pathogen attack (Smart *et al*, 2003), increase in the phenolic contents of plant, which plays an important role in the resistance of cultivar (Mahmood and Saxena, 1986). The present study would be helpful in understanding the defense response of chickpea which is an economically important crop.

Phenolic compounds may accumulate in plants as low molecular weight compounds called phytoalexins as a result of microbial attack (Mattila and Hellstrom, 2007). It is supposed that these phenolic compounds might already be present at low concentration in plant, but these rapidly accumulate upon attack as induced compounds. This is an agreement with results reported by Kuc (1982). Changes in phenolic acid content in tomato fruits in response to pathogen attack was determined by Ruelas *et al*, (2006). A number of phenolic and salicylic acids were synthesized or significant increase in the concentration of phenolic acids was observed after inoculation with inoculums of *M.phaseolina*. The induction of phenolic compounds as part of the defense system against pathogen using elicitors has also been demonstrated by Pearce *et al*, (1998). The result of the present study revealed that plants have evolved multiple defense mechanisms against microbial pathogens and various types of environmental stress. Besides anti-microbial secondary metabolite, some of which are performed and some of which are induced by infection.

The polyphenol compounds were detected in ethanol and water extracts of leaves and stem and it was suggested by Huang *et al*, (2005) that they might contribute directly to antioxidant action. The compounds such as flavonoid, which contain hydroxyls, are responsible for the radical scavenging effect in the plants (Das and Pereira, 1990). According to studies, the high contents of these phytochemicals can explain its high radical scavenging activity and the role of polyphenols in activation of the defense response (Williams *et al*, 2002). The increase in protein content may be because of increase in PR protein after infection with pathogen (Bailey and Mansfield, 1992). Induction of PR proteins following elicitor treatment, wounding or tissue infection is accompanied by a massive alteration in the pattern of gene expression and activation of other defense responses such as phytoalexin accumulation, lignin deposition and synthesis of cell wall hydroxyproline rich glycoproteins (Lawton, 1996).

These results can be compared to those reported by Van Loon and Van Strien (1999) who reported that PR protein preparation contain varying amounts of proteins extracted from different plants. The metabolism of the phenolic compounds also involves oxidative enzymes, such as peroxidase and PPO, the latter catalyzes the oxidation of phenolics to quinones (Thypyapong *et al*, 1995). A large number of studies have demonstrated that these enzymes increase in response to biotic and abiotic stress (Sanchez *et al*, 2000). One of the principal events in the early phase of plant-pathogen interactions is the rapid and transient production of

active oxygen species by the plant. This response has been reported in numerous plant-pathogen systems involving fungi (Vera-Estrella *et al*, 1992). Ample evidence indicates that active oxygen species perform multiple important functions in early defense responses of the plant the increase in salicylic acid perhaps being the most important (Wu and Shortt, 1997). Much evidence suggests that the increases in salicylic acid levels are essential to induce systemic acquired resistance (Tenhaken and Ru, 1997). On the other hand Nemat Alla and Younis, 1995 related the changes and more specifically the decline in secondary metabolic processes with the growth reduction of the plants treated with certain herbicides and fungicides. Similar kinetics have been found in cells and tissues of many resistant plant-pathogen interactions ranging from potato leaves and *Phytophthora infestans* to Arabidopsis suspension cells and elicitors (Davis and Ausubel, 1989).

While the increased fungal growth upon inhibition of lignifications clearly demonstrates a direct consequence relationship between lignification and resistance (Mithofer *et al*, 2002) the proposed causal relationship between cell death and resistance (Health, 1976). Screening of germplasms to get resistant or tolerant cultivars may be important because it can be used as a source of resistance in plant breeding programs.

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