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Comparative study on the induction of defense related enzymes in two different cultivars of chickpea (*Cicer arietinum* L) genotypes by salicylic acid, spermine and *Fusarium oxysporum* f. sp. *ciceri*

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Abstract

Induction of some defense related enzymes and phenolics in roots and shoots of two different genotypes of chickpea cultivars which were susceptible (L550) and resistant (ICCV10) to wilt disease treated with salicylic acid, spermine (Spm), SA+Spm and *Fusarium oxysporum* f. sp. *ciceri* was investigated. Higher levels of polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), β-1, 3-glucanase (PR-2) and phenolics were observed in roots and shoots of resistant cultivar than that of susceptible cultivar on treatment with elicitors and pathogen. However, no major changes were observed in susceptible cultivar after the treatments. β-1, 3-glucanase is constitutively present and is further induced in roots and shoots of resistant cultivar by *F. oxysporum* f. sp. *ciceri*. No induction of β-1, 3-glucanase was observed in susceptible cultivar. The structural changes during disease progression were observed by histochemical staining. However, the pathogen invasion was more in susceptible cultivar compared with resistant cultivar. Further, the invasion was restricted in roots of resistant cultivar treated with SA. These results suggest that induction of defense proteins and accumulation of phenolics might have contributed to restrict the invasion of *F.oxysporum* f. sp. *ciceri*, in resistant cultivar ICCV10.

Keywords: Polyphenoloxidase, phenylalanine ammonia-lyase, salicylic acid, spermine, chickpea, *Fusarium oxysporum* f. sp. *ciceri.*

Introduction

Induced resistance is defined as an enhancement of the plants defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation. Pre-treatment of plants with avirulent pathogens (biotic inducers) or chemical compounds (abiotic inducers) can enhance resistance to subsequent attack not only at the site of treatment, but also in tissues distant from the initial infection sites. Typically, this inducible resistance system known as systemic acquired resistance (SAR) is effective against diverse pathogens including viruses, bacteria and fungi (Ryals et al., 1996). Defense related genes encode a variety of proteins including enzymes controlling secondary metabolism, pathogenesis related proteins (PR) and regulatory proteins that control the expression of other defense related genes (Dixon et al., 1994). The defense gene products include polyphenol oxidase (PPO),

peroxidase (POD) that catalyzes the formation of lignin, and phenylalanine ammonia-lyase (PAL) that is involved in phytoalexins and phenolics synthesis. Other defense enzymes include pathogenesis-related proteins (PRs) such as β -1, 3-glucanases (PR-2 family) and chitinases (PR-3 family), which degrade the fungal cell wall and cause lysis of fungal cell. Chitin and glucan oligomers released during degradation of fungal cell wall act as an elicitor that elicit various defense mechanisms in plants (Frindlender et al., 1993). Induction of defense proteins makes the plant resistant to pathogen invasion (Van Loon, 1997), and has been correlated with defense against pathogen invasion in cucumber (Rasmussen, 1991), green gram (Ramanathan et al., 2000), tobacco (Beaudoin–Eagan and Thorpe, 1985) and tomato (Bashan et al., 1985). Polyphenol oxidases (PPOs, E C. 1.10.3.1) catalyze the oxygen-dependent oxidation of *o*-dihydroxyphenols to *o*-quinones, which are more toxic to pathogens than the former. Direct toxicity of quinones against pathogens has also been proposed (Mayer and Harel, 1979). In addition, many studies have shown that PPO is induced in response to mechanical wounding, fungal and bacterial infection, and by treatment with signaling molecules such as jasmonic acid /methyl jasmonate (MeJA), systemin and salicylic acid (Constabel et al., 2000); (Stewart et al., 2001). In addition, systemic induction of PPO expression in response to wounding and pathogens might provide an additional line of defense to protect plants against further attack by pathogen and insects (Thipyapong et al., 1995; Stout et al., 1999).

Salicylic acid is a natural phenolic compound present in many plants, and an important component in the signal transduction pathway involved in local and systemic resistances to pathogens (Delaney et al., 1995). Exogenous application of SA was shown to be mimicking certain aspects of a pathogen infection, resulting in both SAR gene expression and induction of SAR (Vernooij et al., 1995). Murphy et al., (2000) have also shown that application of SA at non-toxic levels to susceptible plants could enhance resistance to necrotrophic fungal pathogen in tobacco. In addition, the application of SA, calcium chloride, and oxalic acid reduce the disease incidence in pear fruit caused by *Alternaria alternata* by inducing the activities of β-1, 3-glucanase, PAL, PPO and peroxidase (POD) (Tian et al., 2006). Polyamines (PAs), spermine, putrescine, and spermidine, are found in a wide range of organisms from bacteria to plants and animals. They are believed to promote plant growth and development by activating synthesis of nucleic acids (Walden et al., 1997). Role of PAs in plant-microbe interactions have also been proposed (Walters, 2003). In resistant wheat cultivars, fungal and bacterial pathogens elicit production of amide conjugate of phenolic acid and PAs, which may function as phytoalexins an antifungal compound (Samborski and Rohringer, 1970). Yamakawa et al., (1998) showed that Spm induce PR-protein and conferred resistance against tobacco mosaic virus (TMV) in host plants. These findings strongly suggest that Spm may additionally act as a signaling molecule to transduce defense responses, including hypersensitive (HR) cell death in plants and suggest a role for PAs in the resistance against bacterial or fungal attack in plants. Induction of pathogenesis–related proteins, which are capable of inhibiting pathogen development, would contribute to greater resistance (Fajardo et al., 1998).

In recent years, induced resistance following treatment with biotic and abiotic factors has been considered to be a great potential approach for the control of plant diseases. Therefore, induced resistance holds promise as a new technology for the control of plant diseases and has been proven to be effective in the laboratory and in a few field cases. Resistance inducing chemicals that are able to induce broad spectrum of disease resistance, offer an additional option for the farmer to complement genetic disease resistance.

Fusarium wilt of chickpea is a serious and wide spread disease in India, Iran, Pakistan, Nepal, Burma, Spain, Mexico, Peru, Syria and the USA (Nene et al., 1989). *F. oxysporum* is a soil borne, root pathogen colonizing the vessels and blocking them completely to effect wilting (Beckman and Roberts, 1995). It affects the susceptible cultivars within 25 days after sowing and affected seedlings exhibit drooping of leaves xylem and stem discoloration and root rotting followed by complete collapse. Various chemicals have been discovered that seem to act at various points, these defense activating networks mimic all or part of the biological activation of resistance.

In the present investigation an attempt was made to correlate and analyse the biochemical basis of disease resistance, by measuring levels of PPO, PAL, β-1, 3-glucanase and phenolics in root and shoot tissues of wilt resistant (ICCV10) and wilt susceptible (L-550) cultivars of chickpea, treated with salicylic acid (SA),

Table 1. Levels of total phenolics in roots and shoots of crude extracts of germinating seeds in presence of SA, Spm and SA+Spm at different time intervals.

	Susceptible cultivar L-550 phenols (µg /h/ gfw)				Resistant cultivar ICCV-10 phenols (µg /h/ gfw)			
Time (h)	Control	SA	Spm	SA+Spm	Control	SA	Spm	SA+Spm
48	62.64 ± 2.33	74.26±1.63	70.18±1.20	82.18±1.74*	80.22±1.73	120.17±4.63***	102.29±5.18**	164.17±2.91**
96	78.10±2.29	112.16±2.34**	102.76±2.29**	221.15±0.55*	106.28±4.64	142.30± 3.43**	128.16±3.45**	216.12± 1.70***
144	98.38±2.32	149.25±2.91**	143.69±2.87**	153.22±2.86**	129.84±3.43	218.18±2.32***	210.81±1.78***	283.42±4.08**
192	122.44±1.17	161.06±0.56**	152.30±3.47*	182.39±1.21**	162.46±2.42	243.17±2.27*	235.13±3.69**	311.19±0.52***
240	131.90±1.14	188.44±1.18**	172.16±2.88**	197.15±1.18**	174.40±2.61	286.40±2.84*	263.23±1.71*	351.33±7.98*
Shoots (240)	(40.84±0.60)	(58.22±1.73)***	(55.36±3.95)**	(64.28±3.23)**	(53.11±1.76)	(87.22±1.14)***	(83.25±3.49)**	(106.33±1.74)***

Values in parentheses indicate shoots and in the table represent the mean ± S.E from three independent experiments. Differences from control values were significant at. $^{*}_{P<}0.05$; $^{**}_{P<}0.01$ $^{***}_{P<}0.001$, according to ANOVA variance for regression. gfw-gram fresh weight

Table 2. Effect of *F. oxysporium* f sp *ciceri*, on phenolic contents in roots and shoots of susceptible and resistant cultivars at different time intervals

	Susceptible cultivar L-550 phenols (µg /h/ gfw)				Resistant cultivar ICCV-10 phenols (µg /h/ gfw)			
Time (d)	Roots		Shoots		Roots		Shoots	
	Control	Pathogen treated	Control	Pathogen treated	Control	Pathogen treated	Control	Pathogen treated
1	129.15±2.28	138.73±1.13	34.52±0.12	41.35±0.61**	169.27±3.49	175.36±1.12	55.23±4.06	75.52±3.47**
2	137.32±1.14	154.77±2.89	39.16±1.14	47.25±2.90**	179.08±2.91	224.60±3.50**	62.13±1.74	88.66.0.60*
3	145.46±0.55	168.56±1.73	45.29±3.48	58.66±2.92**	188.81±1.11	243.20±1.74***	69.40±1.74	97.22±1.09*
4	152.19±1.15	188.23±2.86*	51.34±2.27	66.14±2.87*	196.18±4.09	291.27±1.14**	78.28±0.53	110.54±3.05*
5	157.84±1.70	209.13±4.05**	57.36±2.92	81.13±2.32**	214.22±1.16	345.60±2.84**	83.16±2.89	142.77±11***

Each value in the table represents the mean ± S.E from three independent experiments. Differences from control values were significant at $^{*}_{P<}0.05$; $^{**}_{P<}0.01$ $^{***}_{P<}0.001$, according to ANOVA variance for regression. gfw-gram fresh weight

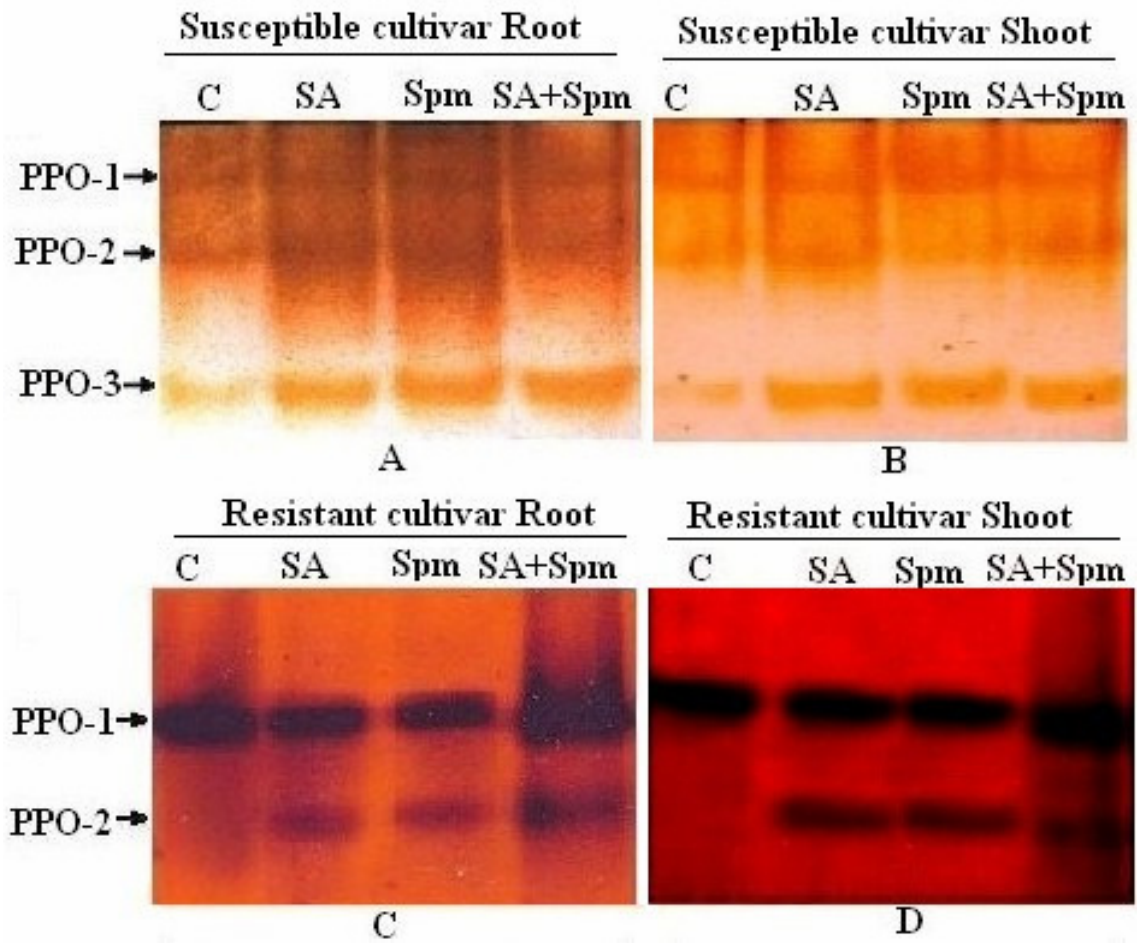

Fig 1. Native gel stained for PPO activity: (A) roots and (B) shoots of susceptible cultivar L-550. (C) roots and (D) shoot of resistant cultivar ICCV-10. Equal amount of protein (300µg) loaded on gels. Lane C: control, lane SA: salicylic acid, lane Spm: spermine, lane SA+Spm: salicylic acid and spermine treated samples.

spermine (Spm), in combination of SA+Spm and *F. oxysporum* f. sp. *ciceri.*

Materials and methods

Treatment

Seeds of chickpea (*Cicer arietinum* L.) susceptible (L-550, GBC-6, GCP-101, GCP-107), resistant (ICCV-10, GBS-963, GBC-2, A-1) and moderately resistant (GBS-11) cultivars to wilt disease respectively were procured from the agriculture Research Station Gulbarga, India. The seeds were surface sterilized with 0.01% aqueous $HgCl_2$ for 1-2 min and then thoroughly washed with double distilled water. There were four replications with 60 seeds for each treatment. The first set was supplied with distilled water to serve as a control, while set two was supplied with 0.8 mM Salicylic acid (SA), set three with 0.01 mM Spermine (Spm), and set four with 0.8 mM SA + 0.01 mM Spermine (SA+Spm). All the Petri-plates lined with double layer of filter paper were kept wet by supplying respective solutions and allowed to germinate at 26 ^{0}C up to 10 days. Roots and shoots were separated at different time intervals (48, 96, 144, 192 and 240 h). Enzymes extracted and their activities were determined.

Inoculation with F. oxysporum** f. sp. **ciceri (race1)

Culture of *Fusarium oxysporum* f. sp. *ciceri* race 1 causing wilt disease in chickpea was obtained from Agriculture Research Station Gulbarga, India. The culture was maintained on sterilized sandy loam soil mixed with maize powder at 19:1 w/w**.** The pathogen inoculum was prepared by culturing the fungus on potato dextrose agar (PDA) medium for 7 days in petri-plates. The micro conidial suspension was prepared by pouring 20 mL of sterile distilled water in each petri-plate. The concentration of micro conidia was adjusted to 1000 conidia mL^{-1}. Then, the ten days old seedlings of resistant (root 12 cm, shoot 10 cm in length) and susceptible cultivars (root 10 cm, shoot 8 cm in length) were sprayed with a spore suspension prepared as indicated above. The roots and shoots of both the cultivars were collected at different time intervals (1-5 days), to determine activities of PPO, PAL and phenolics.

Estimation of Phenol

Root and shoot samples (1 g) were homogenized in 10 mL of 80% methanol and agitated for 15 min at 70 ^{0}C. One mL of the methanolic extract was added to 5 mL of distilled water and 250 µL of Folin Ciocalteau reagent (1 N) and the solution was kept at 25 ^{0}C. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg catechol mg^{-1} protein (Zieslin and Ben-Zaken, 1993).

Enzyme extraction

Weighed samples (g) (roots or shoots) were ground in a pre-chilled mortar with pestle in 5 mL of ice cold 0.1 M Tris-HCl buffer, (pH 6.0), containing 5×10^{-3} M 2-mercapto ethanol. The extract was centrifuged at 10,000 g for 25 min at 4 ^{0}C. The supernatant thus obtained was used as an enzyme source for the determination of PPO and PAL. The protein concentration of supernatant was determined according to Lowry et al., (1951) method using bovine serum albumin as a standard.

Assay of PPO

PPO activity was determined according to Mayer et al., (1965). The reaction mixture consisted of 200 µL enzym*e* extract and 1.5 mL of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm $min^{-1}mg^{-1}$ protein.

Estimation of PAL activity

PAL activity was determined as described by Burrell and Rees (1974). The reaction mixture contained 0.03 M L-phenylalanine and 0.2 mL enzyme extract in a total 2.5 mL of sodium borate buffer (pH 8.8). The mixture was placed in a water bath at 37 ^{0}C for 1 h and add 0.5 mL of 1 M (trichloro acetic acid) TCA. The amount of trans-cinnamic acid formed from L- phenylalanine was measured spectrophotometerically at 290 nm. Enzyme activity was expressed as µg of trans-cinnamic acid h^{-1} mg^{-1} protein.

Immunoblot

Treated and untreated roots and shoots of chickpea seedlings were ground in a chilled mortar with pestle in 5 mL of potassium phosphate buffer (0.1 M), pH 6.5 containing 0.5 mM phenyl methyl sulphonyl fluoride. Proteins (100 µg) in aliquot of extracts were separated on 12% SDS-PAGE (Laemmli, 1970) and electro blotted on to nitrocellulose membrane at 58 V for 14 h at 4 ^{0}C using submerged Genei blot mini electro-transfer system, Bangalore, India. According to the manufacturer instructions, western blotting was carried out by blocking the membrane with blocking buffer for 2 h in Tris-buffer saline containing Tween 20 (TBS-T) (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20 (v/v). After blocking, the membrane was incubated with tobacco glucanase antiserum (kindly provided by Prof. Chinta Sudhakar, Department of Biotechnology S.K University Anantapur, India) at a 500 fold dilution in TBS-T buffer for 1 h. Antigenic bands were visualized using a 1000 fold dilution of horse radish peroxidase conjugated anti rabbit secondary antibody, for 1 h before being developed colour as per the instruction manual of Bangalore Genei, India.

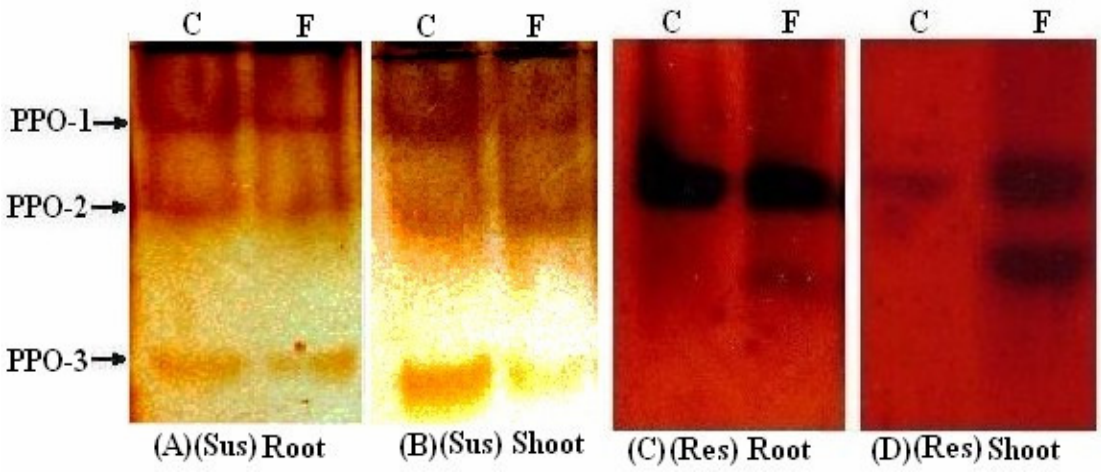

Fig 2. Native gel stained for PPO activity (A) roots, and (B) shoots of susceptible cultivar L-550 and (C) roots, (D) shoots of resistant cultivar ICCV-10, after pathogen treatment. Equal amount of protein (300µg) loaded on gels. Lane C: control, lane F: pathogen treated roots and shoots.

Native-PAGE analysis

The isozyme profile of PPO was examined by native polyacrylamide gel electrophoresis (Laemmli, 1970) without SDS. Root and shoot samples were collected after 10 days of treatment with SA, Spm, SA+Spm and 5 days after pathogen inoculation respectively. The protein extract was prepared by homogenizing 1 g of root or shoot samples in 2 mL of 0.1 M sodium phosphate buffer pH 7.0 and centrifuged at 16,000 *g* for 20 min at 4 ^{0}C. Samples (100 µg protein) were loaded onto 8% polyacrylamide gel. After electrophoresis, PPO isoforms were visualized by equilibrating the gel for 30 min in 0.1% p-phenylene diamine followed by addition of 10 mM catechol in the same buffer (Jayaraman et al., 1987).

Histochemical staining and measurement of fungal colonization

In order to monitor the influence of SA on development of *F. oxysporum* in growing roots at 15 days after treatment were processed for histochemical staining. The surface sterilized, presoaked seeds of L-550 and ICCV10 were grown in pots containing soil collected from the wilt sick plot at the Agriculture Research Station Gulbarga, India. Wilt sick soil is impregnated with spores of *F. oxysporum,* which bring about wilting in the susceptible cultivar L-550, with in 25 days. Sickness in each pot was confirmed by sowing a few seeds of L-1550 as a check (wilting within 25 days), in the center of the pot along with the test material. The pot experiment was laid out in a randomized block design having four different treatments [control (C), salicylic acid (SA), pathogen (P), and SA followed by pathogen inoculation (SAP), in both susceptible and resistant chickpea cultivars having five replicates. A freshly prepared spore suspension of *F. oxysporum,* was added to autoclaved pots containing 1:1 sand. After two days the sand in the pot was thoroughly mixed with a sterile glass rod to ensure uniform spread of the fungus. In all the cases seeds were presoaked in SA or water for 15 h and used for experiments. Pots with autoclaved soil containing no pathogen served as a control. For SA treatment, seeds presoaked in SA (15 h) were sown in pots containing autoclaved soil without pathogen, whereas for pathogen inoculation, the presoaked seeds were sown in pots containing autoclaved soil with pathogen. For SA followed by pathogen inoculation, the SA presoaked seeds were sown in pots containing autoclaved soil with pathogen and allowed to germinate for 15 days. After 8 days of

Table 3. Effect of SA, Spm and SA+Spm on polyphenol oxidase of roots and shoots of susceptible and resistant cultivars at different time intervals

	Susceptible cultivar L-550 phenols (µg /h/ gfw)				Resistant cultivar ICCV-10 phenols (µg /h/ gfw)			
Time (h)	Control	SA	Spm	SA+Spm	Control	SA	Spm	SA+Spm
48	6.30±3.3	8.30±3.33	7.10±1.12	10.21±0.58	11.21±0.57	19.16±5.50	16.20±0.64	25.35±0.60**
96	7.39±0.51	10.60±0.17	8.50±0.17	11.29±5.16	15.29±5.77	24.36±5.50	22.44±1.33***	36.15±0.54***
144	11.17±0.61	15.43±1.73	13.13±0.51*	19.24±8.08	18.42±6.35	31.27±3.33***	27.20±0.62***	45.18±0.58***
192	16.28±0.61	21.46±1.15**	19.22±2.27	24.65±0.51***	25.27±0.62	42.29±0.54*	38.25±0.53*	59.23±0.51*
240	19.87±3.90	29.54±2.27*	27.14±0.55*	31.40±0.23*	37.24±0.62	72.17±0.53**	66.17±1.14**	98.20±1.17**
Shoots (240)	(15.30±0.49)	(24.40±0.69)*	(21.43±1.73)*	(27.36±0.54)***	(31.28±0.54)	(68.35±1.74)**	(58.20±0.54)**	(83.20±2.88)**

Activity is expressed as Unit min^{-1} mg^{-1} protein. Values in parentheses indicate shoots and in the table represent the mean ± S.E from three independent experiments. Differences from control values were significant at $^{*}_{P<}0.05$; $^{**}_{P<}0.01$ $^{***}_{P<}0.001$, according to ANOVA variance for regression.

sowing, plants were sprayed with 0.8 mM SA for 7 days alternatively. Method suggested by Myers and Fry (1978) was followed to study the infection process. The treated roots tissues were cut in small sections (10 μm) and immediately immersed in trichloroacetic acid: phenol (2:1) for 30 min, and then transferred to the lacto phenol solution (phenol 20g + lactic acid 40 mL) and stained with cotton blue. The stained sections were mounted in Depex Polystyrene Xylene and observed under the light microscope (400X, Polyvar).

Lignin staining

Tissue for lignin staining was fixed in 95% (v/v) ethanol and mounted on a slide in a solution of saturated aqueous phloroglucinol in 20% HCL and observed with light microscope, with positive lignin staining indicated by red-violet colour (Jensen, 1962).

Effect of SA, Spm and SA+Spm on chickpea seedlings treated F. oxysporum f. sp. ciceri (race 1)

The surface sterilized seeds of L-550 and ICCV10 were presoaked in SA, Spm, SA+Spm and water (control) and sown in pots inoculated with a conidial suspension of *F. oxysporum* f. sp. *ciceri* race 1 for 25 days. After 8 days of sowing plants were sprayed (200 μL/ seedling) with respective solutions of 0.8 mM SA, .01mM Spm and the mixture of SA+Spm containing (0.8mm SA+ 0.01mM Spm) for once in a week.

Statistical analysis

The results are the means of three independent experiments. The data were statistically evaluated using statistic programme, SPSS version 7.5. To test the difference of the treatments, the data were analyzed by ANOVA variance for linear regression. Differences at $p<0.05$, $p<0.01$ and $p<0.001$ were considered as significant, moderate significant and highly significant.

Results

Phenolics

Studies on induction of defense mechanism revealed that high accumulation of phenolics was observed in SA, Spm and pathogen treated roots and shoots of both the cultivars in a dose and time dependant manner. The levels of phenolics increased by 1.4, 1.3 and 1.5 fold in roots and 1.4, 1.3 and 1.7 fold in shoots of susceptible cultivar L550 at 240 h in response to the said chemical treatment. In resistant cultivar ICCV10, levels of phenolics were 1.6, 1.5 and 2.0 fold in root and 1.6, 1.5 and 2.0 fold in shoots compared to their respective water treated controls (Table 1). Plants inoculated with the pathogen also record increased accumulation of phenolics, by 1.3 fold in roots and 1.4 fold in shoots of susceptible cultivar L550. Whereas, 1.6 and 1.7 fold respectively, in roots and shoots of the resistant cultivar ICCV10 after 5 days of treatment (Table 2). Overall, with respect to their controls the accumulation of phenolics was less in susceptible cultivar than that of resistant cultivar in all the treatments.

Polyphenol oxidase activity

The PPO activities in susceptible cultivar L-550 were increased by 1.5, 1.4 and 1.6 fold in roots and 1.6, 1.4 and 1.8 fold in shoots after SA, Spm and SA+Spm treatment respectively with respect to their water treated samples. Whereas, in the resistant cultivar ICCV10, PPO activity increased by 1.9, 1.7 and 2.6 fold in roots and 2.1, 1.8 and 2.6 fold in shoots with respect to their water treated samples at 240 h (Table 3). Up on pathogen infection the activities of PPO increased by 1.4 and 1.5 fold in roots and shoots of susceptible cultivar L550, whereas, 1.7 and 1.9 fold in both the roots and shoots of resistant cultivar respectively compared to their corresponding controls after 5 days of treatment (Table 4).

Phenylalanine ammonia-lyase activity

The PAL activities were increased by 1.5, 1.4 and 1.9 fold in roots and about 1.7, 1.5 and 1.9 fold in shoots of susceptible cultivar and 2.0, 1.9 and 2.4 fold in roots and 2.3, 2.0 and 3.3 fold in shoots of resistant cultivar respectively, treated with SA, Spm and SA+Spm compared with their untreated controls at 240 h (Table 5). The PAL activity was increased by 1.3 fold in roots and 1.5 fold in shoots of susceptible cultivar L550 and 1.5 fold in roots and 1.8 fold in shoots of resistant cultivar ICCV10 compared with their respective controls at 5^{th} of pathogen inoculation (Table 6).

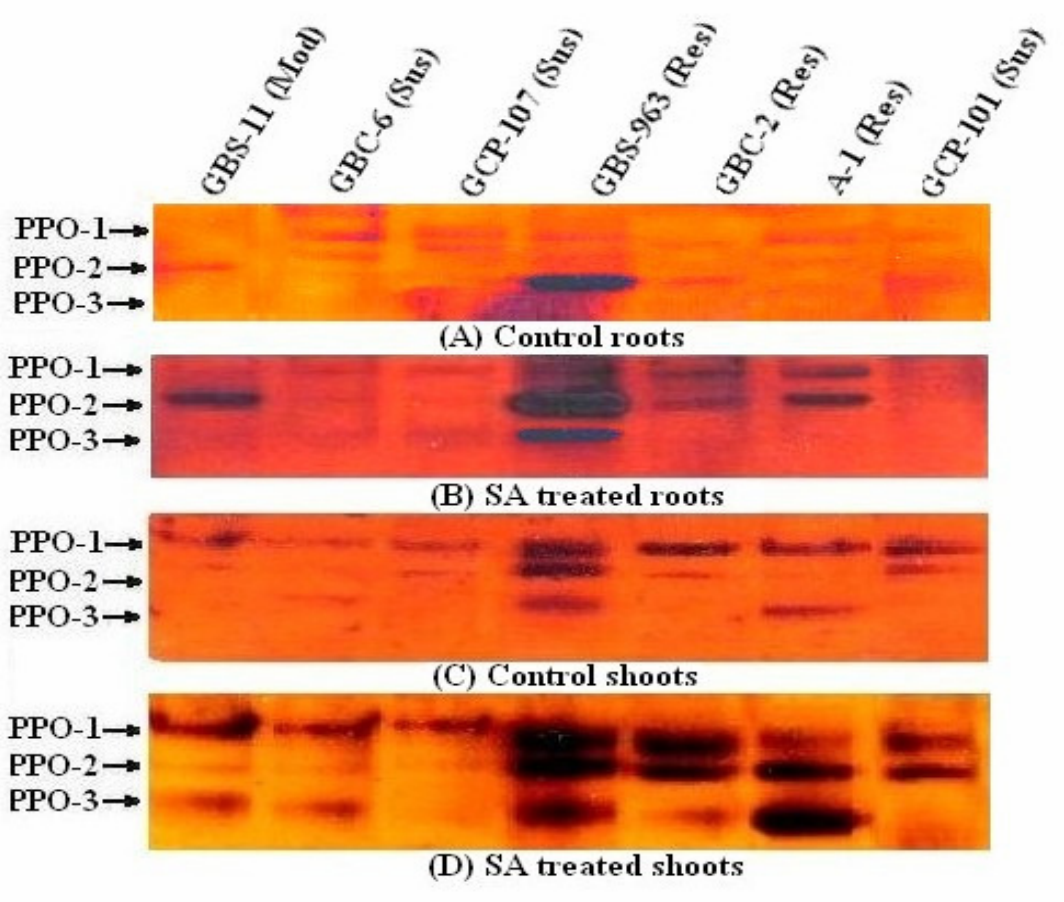

Fig 3. Native gel stained for PPO activity in roots and shoots of different chickpea genotypes. Equal amount of protein (300µg) loaded on the gel. Lane GBS-963, Lane GBC-2 and Lane A-1 are resistant cultivars. Lane GCP-101, Lane GCP107 and Lane GBC-6 are susceptible cultivars. Lane GBS-11 a moderately resistant cultivar. (**A**) Control roots treated with water. (**B**) roots treated with salicylic acid. (**C**) shoots treated with water. (**D**) shoots treated with salicylic acid.

Isozymes

In plant cells PPO exhibit as multiple isoforms and the spectrophotometer analysis indicate only the combined activity of different isoforms. Therefore, we analyzed the isoform composition of PPO by native PAGE. Native gel stained for PPO activity revealed three isoforms PPO-1, PPO-2 and PPO-3 in control as well as in treated roots and shoots of susceptible cultivar. However, the activity of PPO-3 isoform was induced slightly in treated compared to untreated roots and shoots (Fig. 1A and B). Similar pattern of PPO isoform composition was observed in roots and shoots of susceptible cultivar treated with pathogen (Fig. 2A and B). Whereas, an induction of a newly synthesized PPO-2 isoform was observed in roots and shoots of resistant cultivar treated with SA, Spm and SA+Spm as well as with pathogen, (Fig. 1C and D) and (Fig. 2C and D).Different genotypes of chickpea, which were susceptible, moderately resistant and resistant to wilt, treated with SA and screened for the induction of PPO isoforms. In resistant genotype, GBS-963 three isoforms PPO-1, PPO-2 and PPO- 3, in GBC-2 and

A-1 two isoforms PPO-1 and PPO-2 (Fig. 3B) were induced by SA in roots, compared with their controls (Fig. 3A). In the moderately resistant cultivar GBS-11, PPO-2, was induced in roots treated with SA. No major changes were observed in roots and shoots of susceptible genotypes, GCP-101, GCP-107 and GBC-6 treated with SA either in roots or shoots compared with their respective controls (Fig. 3A and B). Similarly induction of new PPOs was observed in shoots of resistant cultivar compared to moderate and susceptible cultivars (Fig. 3C and D).

Immunoblot

Western blot showed constitutive expression of β-1, 3-glucanase in roots and shoots of resistant cultivar ICCV10 treated with pathogen. Expression of β-1, 3-glucanase was at a low level in control and induced up on pathogen inoculation (Fig. 4). In contrast, no β-1, 3-glucanase expression was detected in either roots or shoots of susceptible cultivar before and after treatment with the pathogen.

Influence of SA on the structural changes in roots of chickpea upon treatment with pathogen by histochemical staining

The histological observations, pertaining to invasion of *F. oxysporum* in root tissue sections (10 µm) of the susceptible cultivar (L-550) and resistant (ICCV10) cultivars of different treatments (control, pathogen inoculation, SA treatment, pathogen and SA treatment) showed that SA treatment (Fig. 5B and 5F) causes cell walls lignification as confirmed by phloroglucinol /HCl method, in the form of dark lesions encircling the cells (Fig. 6). The above effect induced by SA may be a defense response, which further provides a conditioning effect that triggers the defense in case of pathogen. The lesions developed due to lignification causes with drawl of essential nutrients required supporting its growth and proliferation and it also restrict the aggressor as it is exposed to a suit of antimicrobial proteins and small molecules. The lignified cells were also observed in SA-treated susceptible cultivar (SSA) (Fig. 5B), but the extent of lignification was not as prominent as in the resistant plant (Fig. 5F) rather it was found to be extending throughout the tissues. In resistant cultivar, cotton blue stained root section revealed that the existence of lignified ring, which could prevent intercellular invasion of the fungal mycelium and was unable to colonize the entire tissue. In contrast, susceptible cultivar (L-550) shows profuse invasion of mycelia even inside the cells. Compared to the plants inoculated with pathogen alone, plants of resistant cultivar (RSAP) first treated with SA followed by inoculation with pathogen showed more lignification; this is supposed to prevent mycelial invasions (Fig. 5H) Contrary to this, susceptible (SSAP) cultivar also showed lignification but the invasion was more as compared to resistant cultivar (Fig. 5D). Arrest of mycelial growth of *F. oxysporum* pathogen under the influence of SA indicates its behavior more like a necrotroph.

Effect of SA, Spm and SA+Spm on chickpea seedlings treated F. oxysporum f. sp. ciceri (race 1)

Results reveal that the seeds of susceptible cultivar L-550 pretreated with SA, Spm and SA+Spm delay the onset of disease symptoms, whereas the seeds treated with water showed complete collapse within 25 days of treatment (Fig. 7A). In contrast, the seedlings of ICCV10 pre-treated with SA, Spm SA+Spm showed highest level of protection and remained healthy throughout the course of experiment (Fig. 7B).

Discussion

Inducing the plants own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy. Recent studies imply that prior application of fluorescent *Pseudomonas* strengthen host cell wall structures resulting in restriction of plant pathogen invasion in plant tissue (Chen et al., 2000). The chickpea seedlings exposed to cell wall protein of *F. oxysporum* f. sp. *ciceri*, showed enhanced synthesis of phenols, pathogenesis-related proteins and activities of PAL and peroxidase relative to water treated controls (Sakia et al., 2006). Stadnik and Buchenauer (2000) also reported the enhancement of PAL activity and accumulation of cell wall-bound phenolic compounds in wheat plants treated with BTH, a novel systemic acquired resistance (SAR) inducer in response to powdery mildew infection. Our study also indicates a rapid increase of PAL activity in chemical and pathogen treated chickpea seedlings. The phenolic compounds may contribute to enhance the mechanical strength of host cell wall and may also inhibit the fungal growth, as

Table 4. Effect of *F. oxysporum* f.sp c*iceri,* on polyphenol oxidase activity in roots and shoots of susceptible and resistant cultivars at different time intervals

	Susceptible cultivar L-550 phenols (µg /h/ gfw)				Resistant cultivar ICCV-10 phenols (µg /h/ gfw)			
Time (d)	Roots		Shoots		Roots		Shoots	
	Control	Pathogen treated	Control	Pathogen treated	Control	Pathogen treated	Control	Pathogen treated
1	18.40±1.18	19.55±0.65	11.18±0.11	14.20±0.28**	28.18±0.52	38.14±2.00	13.17±9.81	26.23±1.13
2	21.47±0.33	22.42±0.56	12.37±0.51	19.34±0.17**	32.29±2.28	47.39±2.26**	16.42±0.33	30.42±0.57**
3	26.39±0.46	28.41±0.51*	17.40±2.19	23.48±0.67*	38.25±0.51	53.29±0.63*	22.43±0.60	35.47±3.43***
4	31.22±1.16	39.28±2.39**	22.21±0.58	29.21±0.52**	45.29±0.51	65.34±2.26**	41.42±1.14	62.42±2.34**
5	25.26±2.30	49.21±2.29**	28.26±0.54	43.41±2.22**	52.34±2.98	89.32±0.61**	55.43±2.34	105.44±4.07**

Activity is expressed as Unit min^{-1} mg^{-1} protein. Each value in the table represents the mean ± S.E from three independent experiments. Differences from control values were significant at $^{*}_{P<}0.05$; $^{**}_{P<}0.01$ $^{***}_{P<}0.001$, according to ANOVA variance for regression.

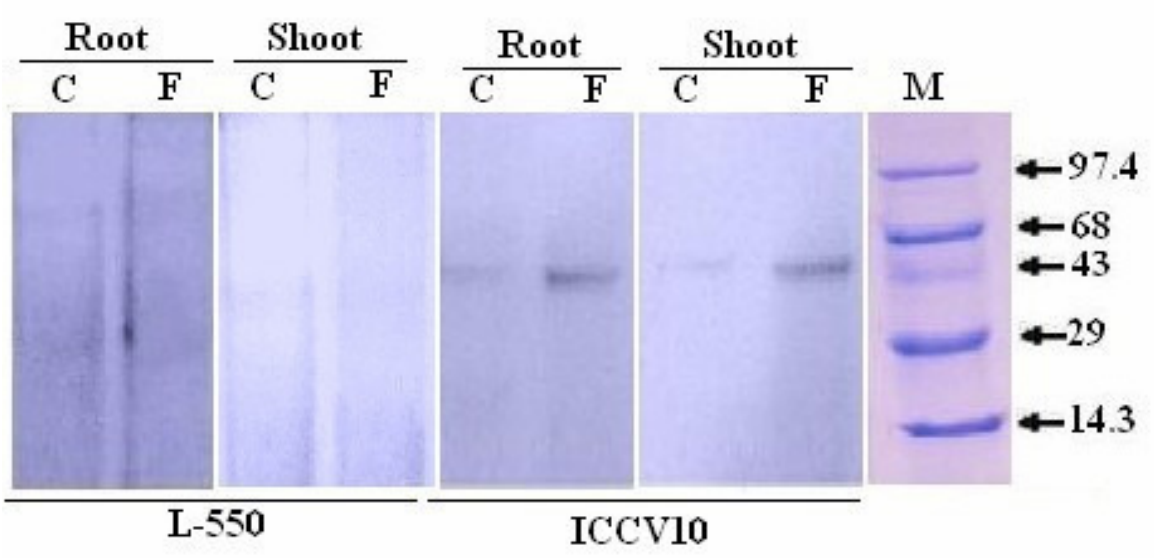

Fig 4. Immunoblot for β-1, 3-glucanase in roots and shoots of susceptible cultivar (L-550), and roots and shoots of resistant cultivar (ICCV10). Lane C: control, lane F pathogen treated roots and shoots.

phenolics are fungi toxic in nature. Altering the level of phenolic compounds in plants has been demonstrated to change disease susceptibility (Yao et al., 1995). Similarly, reduction of phenylpropanoid levels by co-suppression of PAL increases disease susceptibility (Maher et al., 1994). In the present study, the roots and shoots of chickpea treated with SA, Spm, SA+Spm and pathogen, have shown to accumulate high levels of phenolics, compared to water treated controls (Table 1). However, the accumulation of phenolics was high in the resistant cultivar than that of the susceptible cultivar. Increased in phenolic content after chemical treatments may be due to increase of PAL activity, as PAL has been reported to be associated with the synthesis of phenolic compounds via phenylpropanoid pathway (Hahlbrock and Scheel, 1989). PAL is a key enzyme in the biosynthesis of phenyl propane unit, which is a component of phenolic acids, flavonoids and lignins. In our studies PAL activity increased in response to chemical and pathogen treatments. Increased PAL activity level in response to pathogen or elicitor spray has been reported (Song et al., 1993). Similarly, chickpea seedlings treated with *Rhizobium* Pch43 followed by *F. oxysporum* f. sp. *ciceri* race 0, resulted in increased accumulation of phenolics and PAL was observed by Arfaoui et al. (2007). Increase of PAL activity in rice seedling after SA spray has also been reported (Cai and Zheng, 1997). Invasion of root tissues by the pathogen might have resulted in decreased activity of PAL in susceptible cultivar. Whereas, increased activity of PAL in resistant cultivar might have prevented the fungal invasion and thus the activity maintained at higher levels during the experimental period. Therefore, these results suggests that increased accumulation of PAL with pathogen could re-established the notion that in response to invasion of pathogen, PAL is synthesized more rapidly in resistant cultivar ICCV10 than that in susceptible cultivar L550.

The PPO over expressing transgenic tomato plants exhibited high resistance to *Pseudomonas syringae*, the causative agent of speck disease compared with control plants (Li and Stiffens, 2002). Localized inoculation of tomato leaflets with *P. syringae* induces a significant increase in PPO activity and leads to systemic resistance to the subsequent infection by *P. syringae* (Stout et al., 1999). In the present study it was observed that higher activities of PPO in the resistant cultivar ICCV10 than that of susceptible cultivar L550, in all the treatments (Table 3 and 4). The induced PPO-2 isoform in roots and shoots treated with SA, Spm as well as SA+Spm and with pathogen might have also been implicated in induced defense responses against the pathogen

Table 5. Effect of SA, Spm and SA+Spm on phenylalanine ammonia-lyase activities of roots and shoots of susceptible and resistant cultivars at different time intervals

	Susceptible cultivar L-550 phenols (µg /h/ gfw)				Resistant cultivar ICCV-10 phenols (µg /h/ gfw)			
Time (h)	Control	SA	Spm	SA+Spm	Control	SA	Spm	SA+Spm
48	24.82±0.55	48.78±2.31*	45.63±2.44*	53.18±2.26*	39.44±0.91	79.15±1.14**	70.84±1.66 **	84.74±2.33**
96	45.36±3.42	83.78±0.28**	69.21±2.25**	88.59±1.16**	66.13±7.51	112.11±1.16**	99.15±2.30**	122.60±2.25**
144	59.56±2.27	99.06±0.54**	87.13±1.09**	125.12±1.68**	80.45±1.60	144.52±2.14**	126.10±2.3*	168.08±1.73*
192	83.11±2.36	114.66±2.80	105.14±2.91	133.62±1.13**	113.27±1.09	196.61±1.66**	174.82±2.06*	213.14±1.03**
240	88.54±2.17	135.55±0.57*	125.50±0.55*	168.24±1.14*	122.19±1.17	248.16±1.11**	238.09±2.39*	299.44±3.51*
Shoots (240)	(17.19±2.79)	(29.56±4.08)**	(26.69±1.17)**	(33.69±1.17)**	(37.22±1.14)	(86.68±1.13)**	(76.15±1.74)**	(125.74±2.77)**

Activity is expressed as µg of trans-cinnamic acid $h^{-1}mg^{-1}$protein. Values in parentheses indicate shoots and in the table represent the mean ± S.E from three independent experiments. Differences from control values were significant at$^{*}_{P<}0.05$; $^{**}_{P<}0.01$ $^{***}_{P<}0.001$, according to ANOVA variance for regression.

invasion. However, no induction of PPO was observed in susceptible cultivar L-550. According to Kosuge (1969), PPO as catechol oxidase was increased following infection by virus, bacteria, fungi, nematode or mechanical injury. Hence these results indicate that the oxidative enzyme converts phenolic compounds of plants in to polyphenols and quinones, the toxic substances for the extracellular enzymes produced by the pathogens. Thipyapong and Stiffens (1997), also reported the PPO-catalyzed phenolic oxidation in limiting disease development. Therefore, induction of PPO is quite likely to govern some mechanism of biochemical resistance in ICCV10 and other resistant cultivars that arose from the interaction between *F. oxysporum* and host.

Expression of defense related genes such as those encoding pathogenesis-related proteins are used as markers for the establishment of SAR (Du and Klessig, 1997). PR-proteins are host-coded proteins induced by different types of pathogens and abiotic agents. Synthesis and accumulation of PR-proteins have been reported to play an important role in plant disease resistance (Van Loon, 1997). In pea, seed treatment with *P. fluorescens* induced the accumulation of β-1, 3-glucanase and chitinases at the site of penetration of fungal hyphae of *F. oxysporum* f. sp. *ciceri*. These enzymes act up on the fungal cell wall resulting in degradation and loss of inner contents of cells (Benhamou et al., 1996). Maurhofer et al. (1994) reported that induction of systemic resistance by *P. fluorescens* was correlated with accumulation of β-1,3-glucanase and chitinase. Ineffective isolates of *P. fluorescens* did not trigger accumulation of β-1, 3-glucanase and did not induce systemic resistance in tobacco against tobacco mosaic virus. In our study, 43-kDa β-1, 3-glucanase has been induced by *F. oxysporum* f. sp. *ciceri*, only in roots and shoots of resistant cultivar, but not in susceptible cultivar. Therefore, these results indicate the possibility of involvement of β -1, 3-glucanase in defense of chickpea against wilt. A direct role for β-1, 3-glucanase in defense of plants against pathogen has been proposed because the substrates for these enzymes are major components of cell walls of many fungi (Fridlender et al., 1993). Spm induces TMV resistance by inducing PR-1, PR-2, PR-3 and PR-5 proteins in tobacco leaves infected with tobacco mosaic virus (Yamakawa et al., 1998). In the present study it is interesting to note that the induction of PAL, PPO, β-1, 3-glucanase and phenolics by Spm were similar to that of SA. Earlier we have shown

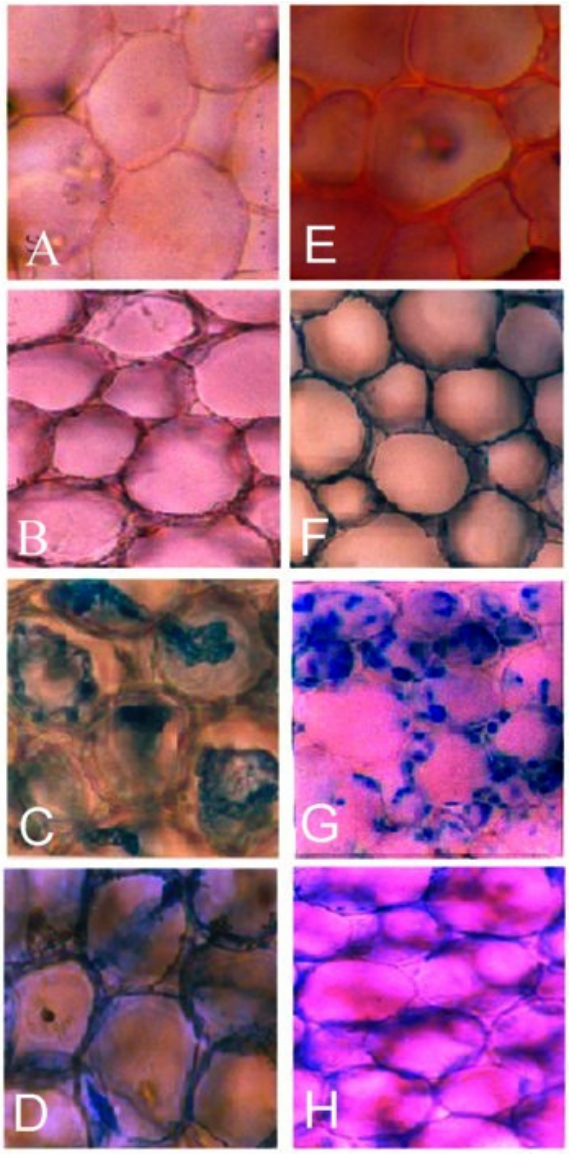

Fig 5. Influence of SA on the ultra-structural changes in susceptible and resistant cultivars of roots by histochemical staining. (**A**) susceptible cultivar control; (**B**) susceptible cultivar treated with salicylic acid; (**C**) susceptible cultivar inoculated with pathogen; and (**D**) susceptible cultivar treated with SA followed by pathogen. (**E**) resistant cultivar control; (**F**) resistant cultivar treated with salicylic acid; (**G**) resistant cultivar inoculated with pathogen; and (**H**) resistant cultivar treated with SA followed by pathogen.

Table 6. Effect of *F. oxysporum* f sp c*iceri* on phenylalanine ammonia-lyase activity in roots and shoots of susceptible and resistant cultivars at different time intervals

	Susceptible cultivar L-550 phenols (µg /h/ gfw)				Resistant cultivar ICCV-10 phenols (µg /h/ gfw)			
Time (d)	Roots		Shoots		Roots		Shoots	
	Control	Pathogen treated	Control	Pathogen treated	Control	Pathogen treated	Control	Pathogen treated
1	85.16±1.69	98.66±0.57**	14.08±0.53	21.81±2.34*	122.06±1.70	149.10±0.57**	31.12±1.21	46.17±1.14*
2	91.85±1.42	109.90±1.48*	19.24±1.11	33.96±1.15**	135.18±0.60	168.13±1.15**	38.22±2.34	69.42±1.53*
3	98.15±0.56	119.18±1.15**	26.23±1.68	38.37±0.59**	148.12±1.14	182.48±1.70**	49.14±1.22	71.77±1.48*
4	110.11±2.89	125.79±2.89*	30.15±1.15	41.12±58*	166.12±1.14	225.42±76**	51.85±2.89	88.44±2.35**
5	118.11±1.10	159.53±1.45**	42.13±2.31	65.51±3.45**	185.12±1.73	288.77±5.22**	66.16±3.46	119.68±1.14***

Activity is expressed as µg trans-cinnamic acid h^{-1} mg^{-1} protein. Each value in the table represents the mean ± S.E from three independent experiments. Differences from control values were significant at $^{*}P<0.05$; $^{**}P<0.01$ $^{***}P<0.001$, according to ANOVA of variance for regression.

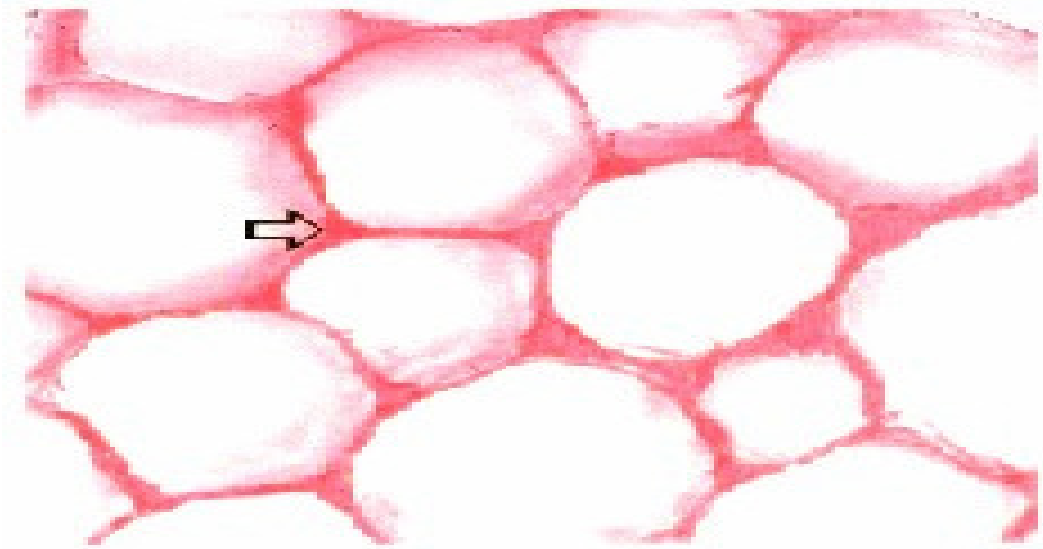

Fig 6. Detection of lignin in SA treated root of resistant cultivar ICCV10.

that the susceptible cultivar L-550 expressed multiple catalases and were insensitive to SA, and did not express SAR, whereas, the resistant genotype (ICCV10) did not express multiple catalases and the native catalase isoforms were sensitive to SA, followed by expression of SAR (Raju et al., 2007). In the present study it was observed that the susceptible cultivar L550 contained lower levels of phenolics, PAL, PPO and β-1, 3-glucanase (PR-2) and is unable to establish systemic acquired resistance (SAR). In contrast, the resistant cultivar ICCV10 contained high levels of phenolics, PAL, PPO and induction and β-1, 3-glucanase (PR-2), as a result the cultivar able to establish (SAR) on treatment with SA, Spm and *F. oxysporum* f. sp. *ciceri.* Hence the accumulation of phenolics, PAL and β-1, 3-glucanase and induction of PPO-2 isoforms by SA, Spm and SA+Spm in roots and shoots of resistant cultivar might have collectively contributed to the induced resistance in chickpea plants against *F. oxysporum* f. sp. *ciceri*. Results of infectivity tests (Fig. 5D), shows that infection was higher in susceptible cultivar inoculated with pathogen compared to resistant cultivar. The data indicate that the application of SA in both cultivars induces defense against the pathogen. However, defense was more pronounced in resistant cultivar than the susceptible one (Fig. 5H). It has been reported that jasmonate mediate induced responses to necrotrophic pathogens in some other plant species (Coego et al., 2005). Jasmonic acid has been reported to strengthen the defense response by enhancing lignifications of cell walls in spikes of wheat cultivars (Mandal et al., 2006). These results further support our conclusion that the resistant cultivar ICCV10 is able to establish ISR against chemicals and pathogen but not by susceptible cultivar L-550. In this study, it was found that the induction of enzyme activities and isozymes by SA and Spm were similar to that of *F. oxysporum* f. sp. *ciceri* infection and can be correlated with the defense against pathogen invasion in chickpea. We have screened different genotypes of chickpea for the induction of PPO isoforms and found that the resistant genotypes (ICCV10, GBS-963, GBC-2 and A-1) for wilt disease expressed multiple isoforms of PPO, while the susceptible genotypes (L550, GBC-6 GCP-101, and GCP-107) did not. Earlier we have reported that the induction of POD isoforms, chitinase (PR-3), and β-1, 3-glucanase (PR-2) were observed in the resistant cultivar ICCV10 but not in the susceptible cultivar L-550 on treatment with SA, Spm and SA+Spm (Raju et al., 2007). Based on our previous and present results, conclude that the chickpea recognizes SA and Spm through a

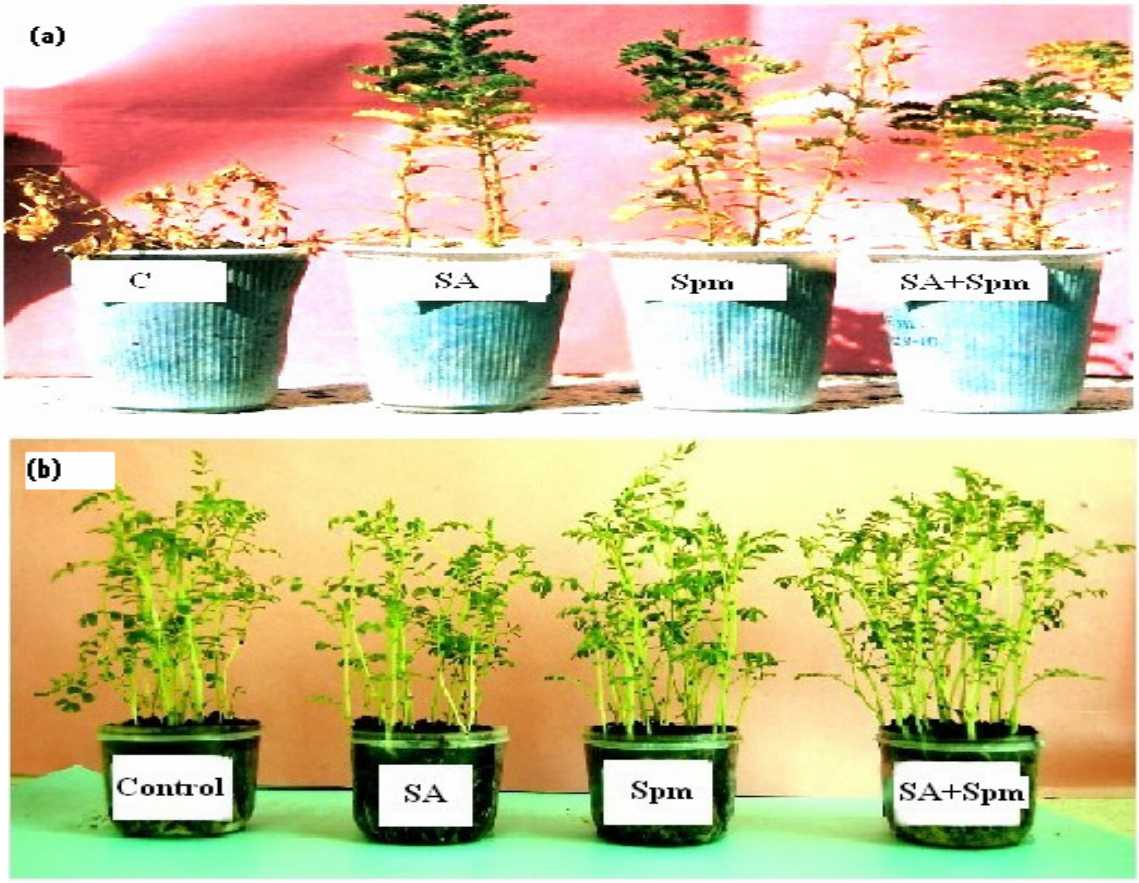

Fig 7. Effect of SA, Spm and SA+Spm on seedlings inoculated with *Fusarium oxysporum* f sp *ciceri* race 1. (C) Seeds soaked in water and inoculated with *Fusarium oxysporum* (control); (SA)- SA presoaked seeds inoculated with *Fusarium oxysporum*; (Spm)- Spm presoaked seeds inoculated with *Fusarium oxysporum*; (SA+Spm) SA+Spm presoaked seeds inoculated with *Fusarium oxysporum*. (A) susceptible cultivar L-550, and (B) resistant cultivar

mechanism identical with those used to detect pathogen infection. Overall our findings suggest that SA and Spm systemically induced resistance in resistant cultivar ICCV10 accompanied by accumulation of phenolics and other PR-proteins. Based on the above studies it is possible to distinguish different genotypes expressing SAR.

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