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Effect of salicylic acid on morphological and biochemical attributes in cowpea

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Abstract: *Effects of salicylic acid (SA) on seed germination, seedling growth, flowering and biochemical activities were studied out in four cowpea (Vigna unguiculata) genotypes in control environments. The results revealed that both germination and seedling growth were negatively affected by 0.02%. SA application, however did not affect the size of full expanded buds, time of 50 % flowering and date of flower initiation. A maximum increase in peroxidase (EC1.11.1.7) activity was observed in UPC 4200 over other genotypes. No significant change in the content of total soluble and intercellular fluid proteins was observed except in UPC 4200 genotype. SA induced accumulation of total soluble sugars more at flowering stage than at seed setting stage. It is evident from the present study that UPC 4200 genotype was more responsive to salicylic acid both in terms of increased peroxidase activity and less negative effect on morphological attributes, thus suggesting its wider use without negative impact on environment as salicylic acid has been reported in plants.*

Key words: *Flower initiation, Intercellular fluid proteins, Peroxidase activity, Salicylic acid, Seed germination, Sugars*

Introduction

Salicylic acid (SA) has been identified as one of the important phenolic compound in plants and also reported as allelopathic chemical (Einhelling, 1986). Maximum level of SA (19-37 mg g^{-1} fresh mass) has been reported in rice seedlings (Silvermann *et al.,* 1995). Apart from its role in diverse processes such as flowering, stomatal closure, seed germination, early seedling growth (Kumar and Tayal, 1982), presently it is most importantly considered as a signal molecule in the induction of systemic acquired resistance (Cutt and Klessig, 1992; Klessig and Malamy, 1994). Though present in plants, the exogenous application of it has shown promise as it influences the yield performance, stomatal closure, seedling growth and seed germination (Raskin, 1992). Additionally, it also affect the nitrate/ nitrite reductases and other enzyme activities involved in nitrogen metabolism without deleterious effects on environment (Jain and Srivastava, 1981; Negi and Prasad, 2001) . Some of the effects of SA may have been caused by its general chemical properties (as an iron chelator or acid) (Raskin, 1992). For these reasons, the significance of SA was not realized from such early studies and reports. Only recently there has been evidence that SA has unique and specific regulatory roles (Klessig *et al.,* 1999). In this direction the role of SA as a signal molecule in disease resistance has been reported with strong evidence (Klessig and Malamy, 1994). This has been an extremely active area of investigation during the past several years. The enhanced activity of peroxidase and increased synthesis of pathogenesis related proteins are some of the response of SA which correlate with the fundamental responses involved in hypersensitive reaction that restrict the pathogen growth and caused tissue necrosis. Decline in catalase activity along with the significant reduction in diseased area in moderately resistance to resistance genotypes over susceptible has been reported in cowpea (Chandra *et al.,* 2001).

In order to ascertain the role of salicylic acid in induction of systemic acquired resistance the activity of peroxidase and protein of intercellular leaf fluids (where most of the pathogenesis related proteins are located) (Flemming *et al*., 1991) were analyzed in the present investigation using four cowpea genotypes differing in resistance to root rot disease. Along with, the influence of SA on physiological and morphological attributes was also carried out to visualize the changes caused by it as cowpea is one of the important protein rich rainfed cultivated fodder legume.

Materials and Methods

Plant materials and application of salicylic acid: Four genotypes of cowpea (*Vigna unguiculata* L. Walp) *viz.*, Bundel 1, Bundel 2, IGFRI 450 and UPC 4200 were sown in (5 x 5 m) plots following the RBD in three replications at research farm of Indian Grassland and Fodder Research Institute, Jhansi (25°N and 78°E). All genotypes were sprayed with 0.02% (2.5 mM) solution of salicylic acid (pH 6.5) at flowering and seed setting stages. This concentration of SA found most effective as observed earlier with cowpea (Chandra *et al.,* 2001) and reported elsewhere also (White, 1979). Observations were recorded in triplicates by harvesting the plant after ten days of each spray. Roots were excised and above ground portion were considered for biomass measurement. The green leaves were kept in an oven to complete dryness, which was later utilised for the estimation of total free sugar following the method of Hedge and Hofrieter (1962).

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Preparation of enzyme extract and enzyme assay: Changes in peroxidase activity, total soluble and intercellular leaf fluids proteins were monitored after different days of salicylic acid treatment. The green leaves were excised, weighed (500 mg) and ground with a pestle in an ice cold mortar with 2 ml of 0.05 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0).The homogenate was centrifuged at 4 °C for 20 min at 15000 rpm. The supernatant was used for the assay of peroxidase activity and total soluble proteins. The peroxidase activity was determined by the Chance and Machly (1955) method. The oxidation of guaiacol (20 mM) used as a substrate and was measured as an increase in absorbance at 470 nm, where one enzyme unit is defined as a change of 0.1 absorbance per min. Samples of the intercellular fluid were isolated by the method of Parent and Asselin (1984). Soluble proteins in enzyme preparation and intercellular fluids was estimated according to Lowry *et al.* (1951) and expressed in [mg g^{-1}(d.m.)]. Bovine serum albumin was taken as standard protein.

Seed germination and data analysis: For germination and seedlings growth, seeds were first sterilized with 0.2% mercuric acid for 10 min and washed repeatedly with sterile water and then placed on distilled water (control) and 2.5 mM salicylic acid (treated) soaked filter paper and allowed to germinate at 25+2°C in seed germinator. There were six replications with thirty six seeds for each treatments. All petridishes were kept wet by supplying water and salicylic acid solution daily. Emergence of radicle was taken as a criterion for the outset of seed germination and represented as germination percentage after three days of salicylic acid treatment. All statistical analyses were performed by following the standard statistical method (Gomez and Gomez, 1984).

Results and Discussion

Germination of cowpea seeds was adversely affected by 2.5mM salicylic acid. Out of four genotypes, UPC 4200 and IGFRI 450 showed 75% germination on third day of treatment whereas all seeds germinated in control conditions. Bundel 1 and Bundel 2 experienced less effect on germination by SA application (Table 1). The seedling length was also less in stress as compared to control, however Bundel 2 initially indicated more growth but after three days the length of seedlings was less to that of control. After three days the length of Bundel 1 seedlings was more than that of other three genotypes (Table 1). Asthana and Srivastava (1978); Anandhi and Ramanujam (1997); Negi and Prasad (2001), have also reported an inhibitory effect of SA on germination in maize, black gram and soybean respectively. Though the level of inhibition in cowpea genotypes is less in comparison to black gram but this level of inhibition may trigger to less germination as SA has been reported to be present in the rhizosphere of soil (Einhelling, 1986). At 2.5 mM concentration SA did not stimulate significantly the flower size, time of 50% flowering as well as date of flower initiation. The leaf length increased in general but overall plant height was same (Table 2). Though stimulatory effect of SA on flowering has been demonstrated maximally than to the plant growth and development characters (Raskin, 1992), high dose of SA did not stimulate flowering in black gram (Anandhi and Ramanujam, 1997).

Two days after salicylic acid treatment all four genotypes used for investigation showed a significant change in peroxidase activity. Of the four genotypes, Bundel 2, IGFRI 450 and UPC 4200 showed significant increase in activity over control whereas Bundel 1 showed reverse trend (Table 3). After four days, peroxidase activity increased in all genotypes. The maximum increase in peroxidase activity was noticed in UPC 4200.

Overall the percentage of increase in activity decreased with time but after four days of treatment Bundel 1 genotype also showed increase in activity over control. On the basis of percentage of increase /decrease in the peroxidase activity at both days of observations, four genotypes used for investigation was grouped into two categories, i) where percentage activity increases from two days of treatment to four days *i.e.* Bundel 1 and IGFRI 450, ii) genotypes where activity decreases *i.e.*, Bundel 2 and UPC 4200. The Bundel 2 and UPC 4200 genotypes showed more activity initially but declined gradually with time indicating the early responsiveness whereas Bundel 1 and IGFRI 450 showed reverse trend. Earlier report indicated the reduction in spread of banding mosaic virus of cowpea by salicylic acid, protocatechuic acid and gallic acid and maximum inhibition (92.0%) of viral infectivity was reported with six sprays of gallic acid (Prakash and Joshi, 1979). The involvement of peroxidase in these processes can not be ruled out as the increase in activity have been reported in cabbage on infection by *Plasmodiophora brassicae* (Ludwig Muller *et al.,* 1994) and in cluster bean by *Alternaria* (Saharan *et al.,* 1999). The distinct behaviour showed by these genotypes clearly indicated that different genotypes may have specific inbuilt nature to show the different level of resistance as depicted by difference in peroxidase activity.

Table- 1: Effect of SA on germination and seedlings growth of four genotypes of cowpea. Mean ± SE, n = 3

Genotypes	Germination (%)		Seedlings growth					
	Control	0.02% SA	One day		Two day		Three day	
			Control	0.02% SA	Control	0.02% SA	Control	0.02% SA
UPC 4200	100	75	2.24±0.21	1.57±0.27	4.92±0.78	3.41± 0.75	7.05±0.98	5.48±0.85
Bundel 2	100	84	3.02±0.24	2.48 ±0.26	4.82±0.68	4.31± 0.50	5.86±1.14	5.60±0.52
Bundel 1	100	94	1.56±0.32	1.73 ±0.57	5.67±0.43	5.70± 0.71	7.13±1.42	6.81±1.33
IGFRI 450	100	75	2.40±0.35	2.16 ±0.33	5.15±0.57	4.00± 0.52	7.06±0.57	5.68± 0.51

Table - 2: Effect of SA on different morphological characters in cowpea genotypes. (C- control; T- treated; NS – non significant)

Genotypes	Plant height at 50% flowering (m)		Leaf length at 50% flowering (cm)		Days of flower initiation from sowing		Size of full expanded buds l x w (cm^2)		Period to 50% flowering		Dry weight/ Plant (g)	
	C	T	C	T	C	T	C	T	C	T	C	T
UPC 4200	2.53	2.40	9.40	9.45	54	54	6.00	6.00	69	69	123.0	125.0
Bundel 2	2.69	2.42	8.56	9.45	54	53	6.24	7.28	69	69	51.2	71.5
Bundel 1	2.28	2.20	9.10	10.35	39	39	5.98	6.24	58	58	130.5	79.5
IGFRI 450	2.07	2.04	8.65	10.10	53	53	7.28	5.98	69	69	40.0	48.3
CD<0.05%	0.342		0.561		NS		0.854		NS		18.65	

Table - 3: Effect of salicylic acid on biochemical attributes in cowpea genotypes. (DAT- days after treatment; C- control; T- treated; d.m.- dry mass)

Genotypes	Peroxidas activity (unit mg^{-1} protein)				Protein content [(mg g^{-1} (d.m.)]		Total sugar at flowering stage [mg g-1 (d.m.)]		Total sugar at seed setting stage	
	2DAT		4DAT		2DAT					
	C	T	C	T	C	T	C	T	C	T
UPC 4200	15.95	35.10	27.72	39.15	110.75	134.05	19.48	71.64	17.57	24.17
Bundel 2	19.72	40.57	31.32	51.84	103.85	104.70	15.30	50.30	37.18	24.30
Bundel 1	48.01	43.91	45.90	58.15	118.90	117.75	18.58	71.78	17.51	39.18
IGFRI 450	27.47	31.39	28.07	45.59	106.90	95.05	32.19	92.30	19.81	28.60
$LSD_{0.05}$	G x T = 3.23		G x T = 6.79		G x T = 1.69		G x T = 3.48		G x T = 9.09	

The peroxidase activity increases in response to infection of plants by pathogens, and higher rate of increase has been related with resistance of the plants (Andreev and Shaw, 1965). At the same time it has also been reported that exogenously applied salicylic acid induces an increase in peroxidase activity within 24 hr over the water injected control (Rasmussen *et al.,* 1991). Additionally, many peroxidases were found to be induced during infection with pathogens and its role with respect to the deposition of lignin like compounds was observed (Kerby and Somerville, 1992). In this study Bundel 2, IGFRI 450 and UPC 4200 showed a significant increase in peroxidase activity within short period of salicylic acid treatment (Table 3), this indicates that these genotypes can be identified as early responsive genotypes while Bundel 1 showed increase in activity after initial depression indicating late responsiveness genotype. Additionally, UPC 4200 genotype has shown an increase in soluble protein content signifying the importance of this genotype as one of the better suitable line for further study. Our earlier report also indicated the usefulness of UPC 4200 as it showed significant decrease in catalase activity and least area under disease progress caused by *Rhizoctonia solani* (Chandra *et al*., 2001). The total soluble protein content did not show major changes except UPC 4200 where it increased significantly (Table 3). This further supports the view that UPC 4200 is a more responsive genotype. As the increase in expression of many pathogenesis related proteins including H_2O_2 binding proteins by SA have been reported (Chen *et al.,* 1993, 1995).

Table 3 indicated that SA at flowering stage causes an increase in the level of total soluble sugar in all genotypes. The maximum increase was noticed in IGFRI 450 genotype but did not affect the biomass in terms of fresh and dry weight. At flowering stage Bundel 2 and Bundel 1 genotypes showed increase in soluble sugar after salicylic acid treatment. The decrease in biomass after salicylic acid treatment was reported by Anandhi and Ramanujam (1997) in a black gram genotype T-9, where total soluble sugar has increased significantly after salicylic acid treatment. Overall increase in soluble sugar content in Bundel 1, IGFRI 450 and UPC 4200 was relatively less at seed setting stage than that of flowering stage after salicylic acid treatment. Our earlier reports also indicated that Bundel 1 and IGFRI 450 genotypes showed increase in the level of soluble sugar even at the early stage of plant after salicylic acid treatment (Chandra and Bhatt, 1998). Since sugars play a major role in diseasea resistance by suppressing the proteolytic and cellulolytic enzymes essential for pathogenesis (Horsfall and Dimond, 1957), it may be argued that the genotypes showing increase in sugar level after SA treatment might show better induced resistance.

The protein observed in intercellular fluids indicated maximum increase in UPC 4200 over other genotypes after SA treatment (Table 4). Since most of the pathogenesis related proteins reside in the leaf intercellular fluids (Parent and Asselin, 1984), the increase in level of proteins is presumably because of better resistance mechanism possessed by this particular

Table - 4: Protein content (mg protein μl^{-1}) in intercellular leaf fluid of cowpea

Genotypes	Protein content in intercellular fluid	
	Control	0.02 % SA treated
UPC 4200	1.327+ 0.029	2.688+ 0.071
Bundel 2	1.352± 0.006	2.020 ± 0.153
Bundel 1	0.898± 0.012	1.348 ± 0.017
IGFRI 450	1.213± 0.015	1.633 ± 0.050

genotype. Though, it has been reported that the accumulation of a protein with an Rf value of 0.57 in the intercellular fluid of cowpea after SA application causes more than 90% reduction in formation of AIMV lesions (Hooft Van Huijsduijnen *et al.,* 1986), the study at different genotypic levels will provide better understanding especially when other parameters related to resistance are found different. In conclusion, the genotype UPC 4200 depicted distinctiveness in terms of increase in peroxidase activity and intercellular fluid protein indicated most-responsive genotype can be used to demonstrate the response of other phenolics reported to be environmentally safe as most of these are plant products in one or other forms.

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