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EFFECT OF SALICYLIC ACID ON BIOCHEMICAL (DISEASE RESISTANT) COMPOUNDS IN *Brassica juncea* RLM619

Sanjula Sharma* and B.S. Sohal[a]

Department of Plant Breeding and Genetics, [a]Department of Biochemistry, Punjab Agricultural University, Ludhiana, Punjab - 141 004 (India); *e-mail: *sanjulag@gmail.com*

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ABSTRACT

Salicylic acid (SA) is a well known inducer of systemic acquired resistance (SAR) in several plants. In present study the ability of SA to induce resistance in Indian mustard (*Brassica juncea)* RLM619 was investigated. SA (10 and 50 ppm) treated seeds of *B. juncea,* germinated in petri-plates, showed maximum of 34.0, 21.7 and 28.5% increase in defense-related enzyme activities *viz.*, peroxidase (PO), phenylalanine ammonia lyase (PAL) and superoxide dismutase (SOD) in hypocotyls, respectively, as compared to water-treated control. Similar trend was observed in cotyledonary leaves of germinated seedlings. Corresponding changes in phenolic contents was also observed. SA treatment @ 50 ppm showed maximum increase in defense enzymes and phenolics on all the days of observation, except for SOD activity where a decrease was seen on 3rd day. Qualitative profiling of phenolics showed that SA treatment enhanced the number of phenolic compounds as compared to control. It may be inferred that SA promotes resistance in mustard by enhancing the level of defense-related compounds and can be exploited to limit fungicidal use through induction of elevated levels of natural resistance.

Key words: *Brassica juncea*, defense enzymes, salicylic acid, systemic acquired resistance

INTRODUCTION

Plants, like animals, have an immune system that protects the plant against further pathogen attack, if they survive earlier infections. The readiness of a plant to ward off the subsequent pathogenic attacks through systemic acquired resistance (SAR) appears to be triggered by some mobile signals in phloem which carry the message from infected zone to non-infected zone (healthy). Evidences suggest that salicylic acid (SA) is a SAR mobile signal (Mölders *et al.,* 1996). SAR can be induced in plants by incompatible pathogens or by pathogen-derived extracts or by certain elicitors such as benzothiadiazole (BTH), SA, potassium salts, etc. The use of elicitors lacks environmental and toxicological side-effects and is effective against a variety of plant pathogens.

SA is synthesized *via* phenyl propanoid metabolic pathway from cinnamonic acid and benzoic acid. It accumulates intracellularly at a specific receptor or binding protein, identified as catalase. Normally catalase protects plant cells against the oxidative stress exerted by active oxygen species (AOS). However, catalase activity is blocked by the binding of SA (Conrath *et al.,* 1995), thereby alters the amount of SA inside the cell. The AOS level may be regulated if only a small amount of SA is present in cell so that catalase activity remains high and AOS level low. However, in reverse case the defense-related genes imparting disease resistance are activated perhaps by acting as secondary messenger. The use of SA reduced disease incidence in pear fruit caused by *Alternaria alternata* by inducing the activities of β-1,3-glucanase, peroxidase (PO), polyphenol oxidase (PPO), superoxide dismutase (SOD) and phenylalanine ammonia lyase (PAL) enzymes which are involved in the production or metabolism of phenolics (Harm *et al*., 2011). The present study was aimed to analyze the biochemical basis of disease resistance, by measuring the levels of defense compounds *viz.,* PO, SOD, PAL, phenolics and qualitative profile of phenolics in hypocotyls and cotyledonary leaves of germinating seeds of (*B. juncea)*, treated with SA.

MATERIALS AND METHODS

The experiment was conducted in the year 2008-09. The healthy seeds of *B. juncea* var. 'RLM619', procured from the Department of Plant Breeding and Genetics, PAU, Ludhiana, were soaked in 0, 10 and 50 ppm of SA for 24 h and germinated in petri-plates in an incubator maintained at 25±2ºC under 16/8 h light/dark period. The random samples of hypocotyls were collected on 3rd, 5th and 7th days after germination (DAG) and cotyledonary leaf samples on 7th DAG and kept at 4ºC. Each experiment was replicated three times with 25 seeds used for germination per plate.

Defense enzymes and phenolics were extracted as per the method of Zhang and Kirkham (1994) and Torti *et al.* (1995), respectively. The extracts were used for the determination of PO, SOD, PAL and phenolic contents *viz.*, total phenols, o-dihydroxyphenols and flavanols. PO was assayed as per Shannon *et al*. (1996) and enzyme activity expressed as the change in absorbance at 470 nm min^{-1} g^{-1} fresh weight. SOD was assayed as per Marklund and Marklund (1974) and enzyme activity, defined as the amount of enzyme causing 50% inhibition of auto-oxidation of pyragallol, was observed in terms of increase in absorbance at 420 nm at 30 seconds interval upto 3 min. PAL was assayed as per Burrell and Rees (1974) and enzyme activity expressed as µg t-cinnamic acid formed h^{-1} g^{-1} fresh weight. Total phenols were estimated as per the method of Swain and Hillis (1959). The intensity of blue colour was measured spectrophotometrically at 760 nm against a blank and the concentration of total phenols derived from a standard curve prepared by using gallic acid (10-50 µg). For o-dihydroxyphenol assay, the method of Nair and Vaidyanathan (1964) was followed and absorbance measured after 15 min. at 540 nm against a blank. The concentration of o-dihydroxyphenols was determined from standard curve prepared by using catechol (6-40 µg). For flavanols assay the method of Balabaa *et al*. (1974) was adopted and yellow colour developed read at 420 nm against 0.1 M methanoilc solution of $AlCl_3$ as blank. The concentration of flavanols was determined from standard curve prepared by using Rutin (40-200 µg).

Thin layer chromatography (TLC) was used for qualitative profiling of phenolic compounds in hypocotyl and cotyledonary leaves as per the method of Harborne (1973). Fresh leaf samples (2.5 g each) were homogenized in a pestle and mortar using 100 ml 80 % methanol, and extract left as such for 24 h. The extract was filtered through a funnel containing silica blocked by a cotton plug and concentrated to the aqueous phase using vacuum rotary evaporator. Phenolics were then partitioned into ethyl acetate (2 x equal volume). A single spot of extract (25 µl) was applied onto silica gel TLC plates and the plates were developed in n-butanol-acetic acid-water (BAW - 4:1:5 v/v/v). Plate were then dried at room temperature and sprayed with Folin-Ciocalteaue's reagent: water (1:1), dried in hot air oven and R_f values determined (Ettre, 1993). The data was analyzed statistically using the analysis of variance and the differences between means compared by t-test (Gupta and Kapoor, 1993).

RESULTS AND DISCUSSION

The soaking seeds of *B. juncea* RLM619 in SA (10 and 50 ppm) remarkably influenced defense-related enzymes and phenolic activities. The hypocotyls and cotyledonary leaves showed increase in PO activity in SA-treated seeds as compared to control (Table 1). The hypocotyls of *Brassica* seeds soaked with 10 ppm SA showed a significant increase in PO activity on 7th DAG. Seed soaking with 50 ppm SA resulted in significant 28.7% increase on 5th DAG and 34% increase on 7th DAG. Treatment with 50 ppm SA exhibited higher PO activity than 10 ppm SA treated seeds. The PO activity in cotyledonary leaves increased by 26.3 and 34.4% in seeds soaked with 10 and 50 ppm SA, respectively, as compared to control. Ananieva *et al.* (2004) also observed increase in PO activity of barley plants on treatment with SA. PO-generated H_2O_2 inhibits pathogen directly and may also generate other reactive free radicals, toxic to several pathogens. Further, PO is involved in the oxidative polymerization of hydroxy-cinnamyl alcohols to yield lignin (Sharma *et al.*, 2012).

PAL activity increased by 18.3, 28.1 and 21.7% on 3rd, 5th and 7th DAG, respectively, in the hypocotyls of germinating seeds soaked with 50 ppm SA than control. A gradual increase in PAL activity was noticed from 3rd to 7th DAG. The increase in PAL activity due to 50 ppm SA treatment in hypocotyl

Table 1: Effect of soaking seeds with Salicylic acid on the activity of defense related enzymes in hypocotyl and cotyledonary leaves of *Brassica juncea* (var.RLM619) seedlings

Days after germination (DAG)	Salicylic acid (ppm)		
	0	10	50
	Peroxidase ($\Delta A\ min^{-1}\ g^{-1}$ FW)		
Hypocotyls 3rd day	42.8±5.8	46.5±1.9	50.3±4.5
5th day	51.6±1.4	55.8±19.1	93.2±17.2[b]
7th day	57.5±5.4	93.7±5.4[a]	116.7±3.1[a]
Cotyledonary leaves 7th day	122.9±7.1	210.8±3.6[a]	252.0±21.4[a]
	Phenylalanine ammonia lyase (µg-t-cinnamic acid $h^{-1}\ g^{-1}$ FW)		
Hypocotyls 3rd day	243.8±63.5	252.2±43.7	353.1±75.7
5th day	269.1±38.5	370.0±63.5	479.3±25.2[a]
7th day	378.4±25.2	479.3±43.4[b]	588.6±29.1[a]
Cotyledonary leaves 7th day	487.7±38.5	605.4±25.2[b]	706.3±25.2[a]
	Superoxide dismutase ($\Delta A\ min^{-1} g^{-1}$ FW)		
Hypocotyls 3rd day	98.5±39.1	176.3±23.8[b]	57.0±23.8
5th day	103.3±7.6	191.2±6.6[a]	186.1±11.7[a]
7th day	131.9±20.5	136.1±34.7	173.6±31.3
Cotyledonary leaves 7th day	98.6±19.7	34.7±33.4[b]	8.3±11.0[b]

Each value is mean ±SD of 3 replicates; a = significant at $P_{0.01}$ difference between control and treatments (student's t- test) ; b = Significant at $P_{0.05}$ difference between control and treatments (student's t - test)

hypocotyls was higher than 10 ppm SA treatment. PAL is reportedly associated with the synthesis of phenolic compounds via phenyl-propanoid pathway (Vogt, 2010) and is a key enzyme in the biosynthesis of phenyl propane unit, which is a component of phenolic acids, flavanoids and lignins. PAL catalyses deamination of phenylalanine to trans-cinnamic acid and ammonia which is also linked with disease resistance. In present study PAL enzyme activity was more in cotyledonary leaves than in hypocotyls.

The hypocotyls of seeds soaked in 10 ppm SA showed significant increase in SOD activity on 3rd day, whereas the seeds soaked in 50 ppm SA depicted non-significant decrease as compared to control. The SA treatment significantly enhanced SOD activity (about 28.5%) on 5th DAG. SOD activity decreased significantly in the cotyledonary leaves of germinating seeds treated with 10 and 50 ppm SA. Ananieva *et al.* (2004) observed an increase in the activities of anti-oxidant enzymes viz., SOD, PO, catalase, glutathione reductase and dehydro-ascorbate reductase in leaves of barley plants on treatment of SA. Decrease in SOD activity from 5th to 7th DAG due to SA treatment may be ascribed to the fact that the effect of elicitor is not long-lasting on cell metabolism. The H_2O_2 produced by SOD helps in lignin formation and expressed antifungal effect on *S. tritici* (Shetty *et al.,* 2003). Our study suggest that the increase in PO, PAL and SOD activities in hypocotyls and cotyledonary leaves of mustard by SA treatment helps in eliciting defense mechanism by enhancing phenol and lignin accumulation.

The hypocotyls of germinated seeds treated with SA showed increase in phenolic contents *viz.,* total phenols, o-dihydroxyphenols and flavanols over control on all the days of observation (Table 2). On 5th day, the increase in

Table 2:Effect of soaking seeds with salicylic acid on phenolic content in hypocotyl and cotyledonary leaves of *Brassica juncea* seedlings

Days after germination (DAG)		Salicylic acid (ppm)		
		0	10	50
		Total phenols (mg g^{-1} DW)		
Hypocotyls 3rd day		7.7±0.5	7.8±0.5	8.4±0.5
	5th day	8.4±0.1	8.7±0.1[b]	9.2±0.2[a]
	7th day	8.7±0.1	9.2±0.3[b]	8.9±0.3
CL*	7th day	11.2±0.3	10.6±0.7	12.3±1.0
		o-dihydroxyphenol (mg g^{-1} DW)		
Hypocotyls 3rd day		1.8±0.3	2.1±0.3	2.8±0.2[a]
	5th day	2.6±0.3	3.0±0.2	3.6±0.2 [a]
	7th day	3.1±0.3	3.2±0.5	3.2±0.4
CL*	7th day	4.9±0.3	4.1±0.3[b]	5.1±0.3
		Flavanols (mg g^{-1} DW)		
Hypocotyls 3rd day		1.1±0.2	1.4±0.1	1.9±0.1[a]
	5th day	1.6±0.2	1.9±0.2	2.2±0.5
	7th day	1.9±0.3	2.1±0.2	2.0±0.2
CL*	7th day	2.6±0.2	2.3±0.4	2.4±0.4

#Each value is mean ±SD of 3 replicates; a = significant difference at $P_{0.01}$ between control and treatments (student's t-test) ; b = Significant difference at $P_{0.05}$ between control and treatments (student's t - test); * CL = Cotyledonary leaves

phenolics was significant in the hypocotyls of the seeds soaked in 50 ppm SA whereas 10 ppm SA treated seeds showed significant increase in total phenolic contents only on 5th and7th day. The o-dihydroxyphenols content showed no significant change due to 10 ppm SA treatment, whereas 50 ppm SA treatment induced significant increase on 5th and 7th DAG. Flavanols content were higher in seeds soaked in 10 ppm SA. In cotyledonary leaves of germinating seeds soaked in 50 ppm SA the phenolic content increased non-significantly in comparison to control whereas 10 ppm SA showed non-significant decrease. The present study revealed significantly high level of total phenols, o-dihydroxyphenols and flavanols in the hypocotyls and cotyledonary leaves collected from the seeds soaked in SA. The increase in phenolic contents may be acribed to the elicitor-induced changes in the metabolic pool during defense response. The phenolic compounds act in chemical defense of higher plants mainly in three ways. Firstly, they occur in healthy plants in concentrations sufficient to inhibit the pathogenic growth; secondly, as a response to infection, their concentration increases imparting resistance to the invading microorganism. Thirdly, certain post-inflectional products (phytoalexins), generally not found in healthy plants, increase on infection (Lattanzio *et al.,* 2006). Higher level of phenolic content in hypocotyls and cotyledonary leaves may be due to higher activity of PO and PAL. In present study increase in PO and PAL coincided with increase in phenolic contents.

Table 3: Thin layer chromatography of extracts from hypocotyls and cotyledonary leaves of *B. juncea* RLM619 after soaking seeds in salicylic acid

R_f values	Days after germination (DAG)								
	5th (Hypocotyls)			7th (Hypocotyls)			7th (cotyledonary leaves)		
	Salicylic acid (ppm)								
	0	10	50	0	10	50	0	10	50
0.48	-	-	-	-	-	-	-	+	+
0.60	-	-	+	+	+	+	+	+	+
0.67	+	+	+	+	+	+	+	+	+
0.70	-	-	-	-	-	-	-	+	+
0.81	-	-	-	-	-	-	-	+	+

The extracts from hypocotyl and cotyledonary leaves of germinating *B. juncea* seeds when run on TLC plates showed the presence of more number of phenolic compounds in SA soaked treatment than in water treated control (Table 3). Based on R_f values, two phenolics were identified in hypocotyls collected on 5th DAG. Phenolics with R_f value 0.60 appeared only in hypocotyls sampled from germinating seeds treated with 50 ppm SA but was absent in control and 10 ppm SA. In hypocotyls, collected on 7th DAG, the phenolic compounds with R_f values 0.60 and 0.67 were observed. No new phenolics appeared on treatment with SA as compared to control in hypocotyls sampled on 7th DAG. On 7th DAG, 5 phenolics appeared in cotyledonary leaves, out of which, phenolics with R_f value 0.48, 0.70 and 0.81 are of importance as they appeared only in cotyledonary leaves sampled from germinated seeds treated with 10 and 50 ppm SA and were absent in control. Number of phenolics was more in cotyledonary leaves than in hypocotyls. In present study, mustard plants treated with SA showed differences in phenolics from control. Band with R_f value 0.60 appeared only in hypocotyls sampled from the seeds soaked with 50 ppm SA and were absent in control. The phenolic band did not get induced with water alone or 10 ppm SA but treatment with higher SA level (50 ppm) might be playing role in the induction of new phenolics. These phenolics later get induced and might be the reason that the phenolic compound was visible on 7th day. Therefore, it may be inferred that high concentration of elicitor caused early induction of some phenolics and induced change in the number and type of phenolics after elicitor treatment. Ana and Dubery (2003) also reported the accumulation of soluble and wall-bound phenolics and phenolic polymers in *Musa acuminata* roots exposed to cell wall derived elicitor from the pathogen, *Fusarium oxysporum,* f.sp. *cubense,* race 4.

Conclusion: The study emphasizes the levels of PO, PAL, SOD and phenolic contents in the hypocotyls and cotyledonary leaves of mustard by SA treatment help in eliciting the defense mechanism. Induced synthesis of phenolic compounds is a common feature of host-pathogen interaction on treatment with certain chemical elicitors and plays role in host (*B. juncea*) resistance against the pathogenic attack. Defense enzymes and phenolic levels could be used in assessing the resistance in *B. juncea* RLM619 against *A. brassicae*.

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