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Effect of foliar spray of elicitors on status of defense proteins in relation to mustard aphid infestation in crop *Brassica* cultivars

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Abstract: Mustard aphid, *Lipaphis erysimi* Kalt., is the key insect pest of crop *Brassicas* causing significant reduction in crop yield. In the present study, widely grown *Brassica* cultivars RLC-1 (*Brassica juncea*) and GSC-6 (*Brassica napus*) were treated with elicitors salicylic acid (SA) and jasmonic acid (JA) at 0.5mM and 1mM concentration via foliar spray (given at 40 and 60 days after planting (DAP). Their effect was evaluated in terms of total soluble protein content and activities of defense proteins (peroxidase, protease inhibitor, polyphenol oxidase, amylase inhibitor and lectins) in relation to aphid infestation in leaf tissue. SA and JA application caused significant increase in activities of defense proteins as well as total soluble proteins. JA at 1mM concentration was most effective in both *Brassica* cultivars. The 2nd foliar spray gave a booster response. The aphid population/plant reduced significantly in both the cultivars with JA as well as SA. POD and PPO registered negative correlation with aphid population count. SA and JA foliar applications seemed effective against mustard aphid through positive modulation in activities of defense proteins.

Keywords: *Brassica*, Defense proteins, Jasmonic acid, *Lipaphis erysimi*, Salicylic acid

INTRODUCTION

Brassica is second important oilseed crop after groundnut in India (Shekhawat *et al.,* 2012). The productivity of this crop is greatly impeded by mustard aphid, *Lipaphis erysimi* Kalt, which feeds exclusively on *Brassica* phloem sap (Sharma *et al.,* 2014). Aphid infestation leads to retarded growth, poor seed formation and low oil content. As a crucifer specialist, aphids have developed mechanisms to withstand or even alter plant defense chemicals that normally act as feeding deterrents for generalist herbivores. Management of aphid requires use of resistant cultivars. In the absence of resistant cultivars, insecticidal sprays are the viable option. But use of insecticides has ecological, health and economic issues (Dogimont *et al.,* 2010; Atri *et al.,* 2012). Plants defend themselves from biotic stresses by a variety of mechanisms that are either local or systemic and constitutive or inducible (War *et al*., 2012). One particular inducible defence response is Systemic Acquired Resistance (SAR). SAR is characterized by alteration of the gene expression profile, which leads to accumulation of newly synthesized proteins. Several elicitors of plant resistance have been used in controlling insect pests in agriculture (Alkahtani *et al.,* 2011; War *et al.,* 2012). Elicitors are applied as foliar spray, seed treatment, or soil drench (Gordy *et al.,* 2015). Jasmonates and salicylic acid elicitors, are signal molecules that play role against abiotic and biotic stresses as well as in plant development (War *et al.,* 2012). They regulate induced defense mechanisms in plants after insect attack and wounding. Most of the salicylate and jasmonate-induced proteins (JIPs) (Vandenborre *et al.,* 2009) have direct defense function against insects. Peroxidases (PODs) are pathogenesis-related proteins (PRs) with role in interaction between plants and insects (Kehr, 2006). Polyphenol oxidases (PPOs) regulate feeding, growth, and development of insect pests (He *et al.,* 2011; Bhonwong *et al.,* 2009). Ethephon and MeJ increased leaf peroxidase (POD) levels but MeJ alone increased polyphenol oxidase (PPO) levels in tomato (Boughton *et al.,* 2006). Plant proteinase inhibitors (PIs) and Alpha-amylase inhibitors (αAIs) have been shown to be insecticidal towards many economically important insect pests by direct assay or by expression in transgenic plants (Kaur *et al.,* 2015). Lectins are carbohydrate binding proteins that act on sap sucking insects by binding to glycoproteins of insect gut-epithelium, eventually causing death by inhibiting absorption of nutrients (Van Holle and Van Damme, 2015). Literature survey revealed that though mustard aphid is the key pest of crop *Brassicas*, area of elicitor application and role of defence proteins has not been explored much (Koramutla *et al.,* 2014; Deeksha, 2013) against this pest. The present study was thus undertaken to see

the effect of foliar spray of salicylic acid (SA) and jasmonic acid (JA) on status of defence proteins in relation to aphid infestation in two cultivars of crop *Brassicas*.

MATERIALS AND METHODS

Seeds of two widely grown cultivars of Brassica crop; RLC-1 (*Brassica juncea*) and GSC-6 (*Brassica napus*) were were procured from Oilseed section, Department of Plant Breeding and Genetics, PAU, Ludhiana. The seeds were sown on 1st November 2014 in pots in triplicate and pots were kept in glass house. The foliar application of elicitors; SA and JA at 0.5mM and 1mM concentrations, was done with atomizer at 40 and 60 days after planting (DAP). The leaf samples were collected for seven consecutive days after each spray and analysed for total protein content and enzyme activities.

Enzyme assays: Peroxidase (POD) and Polyphenol oxidase (PPO) were estimated by the method of Claiborne and Fridovic (1979) and Mayer and Harel (1979) respectively. PI activity was estimated by the method of Benjakul *et al.* (1999) using N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate. α-AI activity was estimated by the method of Bernfeld (1955). Lectins were assayed by the serial dilution method of Liener and Hill (1953). Total soluble proteins (TSP) were estimated by the method of Lowry *et al.* (1951) using BSA (20-200 mg) as standard.

Assessment of aphid population per plant: The aphid population was monitored under natural conditions, 14 days after second sample collection.

Statistical analysis: The data of seven days was pooled and analyzed using two-way analysis of variance (ANOVA) by CPCS software version 1.0. (A program package for the analysis of commonly used experimental designs by Dr. Harjinder Singh Cheema and Dr. Balwant Singh, Punjab Agricultural University, Ludhiana). Correlation analysis was also done using same software.

RESULTS AND DISCUSSION

Foliar elicitor application resulted in significant increase (CD 5%) in total soluble proteins (Table 1).

Table 1. Effect of foliar spray of SA and JA on total soluble protein (mg/g fresh weight) content in leaves of *Brassica* cultivars.

		TSP			
Cultivars		**RLC-1**		**GSC-6**	
Elicitor (mM)	**DAP**	**40**	**60**	**40**	**60**
C	ddw	10.48	8.44	7.95	11.30
SA	0.5	15.44	15.41	15.76	15.86
	1	13.33	14.79	14.42	15.64
JA	0.5	13.07	14.06	14.48	15.62
	1	13.45	14.10	12.27	13.79

Each value is mean of seven days, SA; Salicylic acid, JA; Jasmonic acid, DAP; Days after planting, ddw; double distilled water, C; Control.

Table 2. Effect of foliar spray of SA and JA on activities of defense proteins in leaves of *Brassica* cultivars.

		POD (µmoles of o-dianisidine oxidised/ min/g fresh weight)				PPO (µmoles of benzoquinone produced/ min/g fresh weight)				PI (IU/g fresh weight)				α-AI (% inhibibition/min/g fresh weight)			
Cultivars		**RLC-1**		**GSC-6**		**RLC-1**		**GSC-6**		**RLC-1**		**GSC-6**		**RLC-1**		**GSC-6**	
Elicitor (mM)	**DAP**	**40**	**60**	**40**	**60**	**40**	**60**	**40**	**60**	**40**	**60**	**40**	**60**	**40**	**60**	**40**	**60**
C	ddw	0.58	0.27	0.16	0.18	0.97	1.75	0.73	1.49	21.36	16.09	23.24	14.06	80.14	79.61	79.41	79.76
SA	0.5	1.35	1.89	0.68	1.66	2.71	1.82	1.70	2.35	14.89	11.04	13.81	11.49	86.29	80.73	85.81	80.72
	1	1.20	1.72	0.61	1.59	1.78	2.91	1.81	3.24	17.82	10.10	16.13	10.51	84.39	80.48	83.93	80.48
JA	0.5	1.40	2.52	0.36	1.70	3.02	4.22	1.82	3.44	27.49	24.96	27.76	28.28	83.56	80.31	83.85	81.47
	1	1.92	3.18	1.30	2.17	3.49	4.44	2.50	5.09	29.03	30.85	30.74	28.33	81.68	80.65	85.02	80.35

Each value is mean of seven days, SA; Salicylic acid, JA; Jasmonic acid, DAP; Days after planting, ddw; double distilled water, C; Control

Table 3. Effect of foliar spray of SA and JA on lectin activity (HU/g fresh weight) in leaves of *Brassica* cultivars.

Cultivars	DAP	Elicitor (mM)		Days after spray (DAS)		
				1	2	3
RLC- 1 (*Brassica juncea*)	40	C	ddw	-	-	-
		SA	0.5	80	-	-
			1	-	-	-
		JA	0.5	-	-	-
			1	-	-	-
GSC- 6 (*Brassica napus*)		C	ddw	-	-	-
		SA	0.5	80	-	-
			1	-	-	-
		JA	0.5	-	-	-
			1	80	80	-
RLC- 1 (*Brassica juncea*)	60	Control	ddw	-	-	-
		SA	0.5	-		-
			1	-	-	-
		JA	0.5	-	-	-
			1	-	-	-
GSC- 6 (*Brassica napus*)		Control	ddw	-	-	-
		SA	0.5	80	80	-
			1	-	-	-
		JA	0.5	-	-	-
			1	80	80	-

Each value is mean±SD of three replication, SA: Salicylic acid, JA: Jasmonic acid, DAP: Days after planting, DAS: Days after spray, ddw: double distilled water, C: Control.

Table 4. Effect of foliar spray of SA and JA on aphid population/plant in different *Brassica* cultivars.

Cultivars	Elicitor (mM)		Aphid count
RLC- 1 (*Brassica juncea*)	SA	0.5	83.33±6.03
		1	54.67±7.51
	JA	0.5	32.67±9.02
		1	12.33±5.51
	C	ddw	105.00±6.24
GSC- 6 (*Brassica napus*)	SA	0.5	82.67±5.86
		1	42.00±4.36
	JA	0.5	58.67±4.51
		1	32.33±4.51
	C	ddw	116.00±8.19

Table 5. Correlation between defense proteins and aphid population.

Aphid population/plant	Peroxidase	Polyphenol oxidase	Protease inhibitor	α-Amylase inhibitor	Total soluble protein
	-.899	-.925	-.599	-.433	-.505

Critical Value of r at 5% = 632.

0.5mM SA resulted in highest increase followed by JA. After 1st and 2nd spray, SA treatment registered 1.5 and 1.8 fold increase in mean protein content in RLC-1. Similar trend was observed in GSC-6. Ritu Raj *et al.* (2014; 2016) reported increase in soluble protein content in cotton cultivars in response to JA which could be due to induction in PR proteins. Thakur and Sohal (2014) and Agamy *et al.* (2013) reported increase in total protein content in response to SA foliar spray in *Brassica* and potato respectively against *Alternaria* spp. Haggag *et al.* (2010) reported that Me JA application on sugarbeet plants caused accumulation of PR proteins against beet mosaic virus (Bt MV) infection. War *et al.* (2012) reported that PR proteins are involved in plant defense against insects and multiple signaling pathways including JA, SA and/or ethylene (ET) regulate their induction.

Significant (CD 5%) increase in leaf peroxidase (PR -9 protein) activity was observed in response to foliar application of SA and JA at 0.5 and 1.0 mM concentration in both the cultivars (Table 2). After 1st and 2nd spray of 0.5 mM SA, RLC -1 registered 2.3

and 7.0 fold increase in POD activity, wheresa JA caused 2.4 and 9.3 fold increase. While 1mM SA and JA registered lesser fold increase. GSC- 6 registered similar trend. Second spray, at 60th DAP gave higher increase in POD activity. POD catalyzes oxidation of compounds like phenolics, lignin or suberin resulting in reinforcement of host plant cell walls against pathogenic agents (Wang *et al.,* 2005). Phenoxy and other oxidative radicals produced directly deter insect feeding and toxins produced therein, reduce digestibility leading to nutrient deficiency with drastic effects on growth and development of insects (Chen *et al.,* 2009). Gao and Zhang (2013) demonstrated that SA treatment significantly induced POD activity in pear leaf. SA at 1.5 mM elevated POD activity in chickpea leaves (War *et al.,* 2011b). Foliar application of BABA, SA and JA resulted in higher POD activity and reduced *Heterodera avenae* population in wheat (Pokhare *et al.,* 2012). The increased POD activity with JA and SA in the present study probably resulted in decreased aphid infestation (Table 4).

JA and SA application significantly (at 5% level) induced leaf poly phenol oxidase activity as compared to controls (Table 2). RLC-1 leaf samples of 1st collection, registered 2.8 fold increase with SA whereas JA showed 3.1 and 2.4 fold increase in activity at 0.5mM concentration. SA at 1mM concentration showed 1.8 and 1.7 fold increase whereas JA showed 3.6 and 2.5 fold increase in enzyme activity in post 40 day and 60 day leaf samples. In GSC-6, SA at 0.5mM showed 2.3 and 1.6 fold increase while 1mM SA showed 2.5 and 2.2 fold increase and JA registered 3.4 fold increase in activity in samples of 1st and 2nd collection respectively. JA caused maximum induction in activity. PPO oxidize phenolic compounds to reactive quinones, which alkylate essential amino acids and thereby reduce their nutritional value. JA, SA and ethylene have been reported to induce defense enzymes (War *et al.,* 2011a; Noreen and Ashraf, 2009). Significant increase in PPO activity in wheat was observed with application of JA and SA (Pokhare *et al.,* 2012). Exogenous application of SA at 1.5 mM induced higher PPO activity in chickpea (*Cicer arietinum* L.) leaves (War *et al.,* 2011b). Treatment with 1mM MeSA increased PPO activity in poplar leaves after 24 and 48 hrs, but 10 mM concentration was inhibitory (Tang *et al.,* 2015) probably due to phytotoxicity at higher dose (Rajjou *et al.,* 2006). The defense enzymes protect plants against pathogens (Radhakrishnan and Balasubramanian, 2009; Vicent and Plasencia, 2011), and insects (Zhao *et al.,* 2009). In tomatoes, exogenous JA and MeJ induced poly phenol oxidases (PPOs) and proteinase inhibitors (PIs), and reduced the performance of herbivores (Tan *et al.,* 2012).

Salicylic acid application at 0.5 mM significantiy (CD 5%) depressed PI activity in RLC-1 as well as in GSC-6 (Table 2). In RLC-1, the PI activity declined from 21.36 IU/g FW to 14.89 IU/g FW tissue after first spray. Application of JA however significantly (CD 5%) increased the activity. Purified protease inhibitor depicted negative effects on the mean larval weight, larval mortality, pupation, and mean pupal weight of *Spodoptera littoralis* (El-Latif, 2015). Studies by Tammi and Dolatti (2013) suggested that induction of PIs in barley by Me J (1mM) treatments may have negative impacts on population growth of Russian wheat aphid (RWA; *Diuraphis noxia*). α-Amylase inhibitor activity in RLC -1 (*Brassica juncea*) registered 7.13% and 1.39% increase with 0.5mM SA whereas 5.04% and 1.08% with 1mM SA respectively after 40th and 60th day spray. JA also significantly (CD 5%) increased the activity (Table 2). The inhibitor activity increased in GSC-6 also. α-AI inhibited the digestive amylases in the midgut of bruchids and *Callosobruchus maculatus* larvae when reared on artificial diets containing α-AIs (Farias *et al.,* 2007). SA @ 0.5 mM and JA @ 1mM induced lectins in *Brassica* leaves. Maximum haemagglutination activity was observed with 1mM JA in both cultivars upto 48 hours after spray (Table 3). GSC-6 showed higher induction compared to RLC-1. JA induced expression of *NICTABA* lectin in tobacco leaves (Lannoo *et al.,* 2007; Vandenborre *et al.,* 2009) within 12 hrs of exposure, reaching a maximum after 60 hr. Jasmonic acid methyl ester (JAME) acts systemically in intact plants. After removal of JAME, the lectins progressively disappear from the leaf tissue (Lannoo *et al.,* 2007). Both SA and JA induced lectin agglutination activity in *Brassica* leaves (Deeksha, 2013). Several jasmonate-inducible lectins are expressed in leaf tissues of monocots (Jiang *et al.,* 2006). Plant lectins are reported to be toxic towards insects (Shahidi-Noghabi *et al.,* 2009). Toxicity of purified lectins from various sources against mustard aphid was studied by Deeksha (2013). War *et al.* (2012) reported that JA, SA and/or ethylene (ET) regulate induction of PRs that are involved in plant defense against insects. Coppola *et al.* (2013) reported that SA signalling pathway and stress responsive SA dependent genes played dominant role in compatible interaction between tomato and aphids. Bilgin *et al.* (2010) reported accumulation of newly synthesized proteins as part of induced defense response in plants towards pests.

Aphid infestation: Aphid count/plant significantly (CD 5%) reduced at 1mM JA compared to control (Table 4). Foliar spray of 0.5mM and 1mM SA decreased the population to 83 ± 6.03 and 54.67 ± 7.51 respectively. Likewise the population reduced to 32.67 ± 9.02 and 12.33 ± 5.51 with 0.5mM JA and 1mM JA respectively. GSC-6 also depicted decrease in aphid population. The results clearly depicted the effectiveness of the elicitors against aphids. 1mM JA was most effective followed by 0.5mM JA. Peroxidase and polyphenol oxidase registered negative correlation with

aphid population/plant (Table 5). Correlation between induced PPO activity and insect resistance has been reported in many plants (Bhonwong *et al.,* 2009; Usha Rani and Jyothsna, 2010; War *et al.,* 2011a). The activities of POD and PPO enzymes increased in rice in response to small brown planthopper (SBPH) infestation (Duan *et al.,* 2014). External application of natural or synthetic compounds enhanced the tolerance of crops to environmental stresses (Tanou *et al.,* 2012). Artificial induction of JA-regulated defense had a negative impact on phloem-feeding insects (Zarate *et al.,* 2007). Jasmonic acid treatment slowed the development and rate of population growth of *Lipaphis erysimi* (Bergen, 2008). The induced activity of POD, PPO, PAL and LOX with foliar application of elicitors (BABA, JA and SA) was correlated with the reduced *H. avenae* (nematode) penetration, population and fecundity (Pokhare *et al.,* 2010). BTH and SA caused reduction in *Tuta absoluta* count on tomato plants (Hussein *et al.,* 2014). Tammi and Dolatti (2013) showed that 1mM MeJ significantly decreased nymphal development time and pre-fecundity time of Russian wheat aphid (*Diuraphis noxia*), possibly, due to induction of PIs in barley plants. Brunissen *et al.,* (2009) showed that MeJ application lengthened the pre -reproductive period of potato aphid (*Macrosiphum euphorbiae*)**.** Mahmoud and Mahfouz (2015) reported that SA application significantly decreased aphid number in wheat.

Conclusion

In the present study JA and SA foliar spray increased activities of various defence enzymes. JA proved more efficacious than SA. Its application caused maximum 9 fold increase in POD, 3.6 fold in PPO. It also increased PI and AI activity. The aphid population declined to 32.67 ± 9.02 and 12.33 ± 5.51 with 0.5mM JA and 1mM JA. The study thus indicated that foliar spray of JA and SA resulted in decreasing the aphid population build up in crop *Brassicas* probably via increasing activities of various defense proteins. This pilot study holds scope for future, in terms of validation of the results, on large scale in fields.

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