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Characterization of Herbivore Induced Plant Volatiles from Rice Leaf Folder Infested Rice Plants and their Attraction to Egg Parasitoids

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Abstract

The rice leaf folder (RLF) (Cnaphalocrocis medinalis Guenee) has become a major pest in recent decades, raising concerns in rice-growing areas. Current management strategies primarily involve the use of insecticides, along with some biocontrol agents. This study focused on the chemical ecological aspect of herbivore induced plant volatiles (HIPVs) that play a crucial role as signals in tritrophic interactions. The collected HIPVs were analyzed using gas chromatography coupled with mass spectrometry (GC-MS). The differences in volatile composition between healthy plants and those damaged by RLF were examined, revealing variations based on rice variety. Both quantitative and qualitative differences were observed between healthy and infested plants. Specifically, healthy ASD 16 and CO 51 rice varieties exhibited only nine and seven volatile compounds, respectively. In contrast, RLF infested plants produced 22 volatile compounds for ASD 16 and 16 for CO 51. Additionally, the study investigated the orientation of Trichogramma chilonis (Ishii) (Ashmead) adults toward the crude volatiles released by both healthy and RLF damaged plants. The results indicated a stronger orientation towards volatiles from infested plants than from healthy plants.

Key words: Rice leaf folder, Herbivore Induced Plant Volatiles, parasitoid orientation

INTRODUCTION

Rice (*Oryza sativa*, Linnaeus) is a staple food for more than 65% of the global population and accounts for over two thirds of the Indian population. Insect pests remain significant limiting factors in rice production, causing annual losses of 20% to 30% (Visalakshmi et al., 2014). The Rice Leaf Folder (RLF, *Cnaphalocrocis medinalis*, Guenee) is a destructive foliage feeder that affects rice ecosystems throughout Asia (Luo, 2010). Severe RLF infestations can result in leaf damage as high as 60% to 70% (Kushwaha and Singh, 1988). Continuous and often imprudent

use of chemical pesticides has led to toxic effects on non-target organisms causing undesirable changes in the environment and can be prohibitively expensive for resource poor rice farmers. HIPVs play a crucial role in plant defence by either attracting natural enemies of the herbivores or serving as feeding and/or oviposition deterrents to the pests. The release of these volatile compounds significantly alters plant interactions with their environment. Recent advances in metabolomics, proteomics and genomics studies highlight the need for detailed information on plant volatiles. In recent decades, chemoecological approaches, particularly concerning tritrophic interactions, have garnered more attention as alternatives to synthetic pesticides, especially given the public and environmental concerns (Lou et al., 2006). The volatiles emitted by plants play a vital role as signals in tritrophic interactions viz., plants (1st trophic level), herbivores (2nd trophic level), and their natural enemies, which include predators and parasitoids (3rd trophic level). The significance of the third trophic level—natural enemies—was initially emphasized by Price et al. (1980), who considered it an integral part of a plant's defence mechanisms against herbivores. This study investigated the chemical profile of rice plants and the orientation behavior of natural enemies in response to HIPVs.

MATERIALS AND METHODS

The HIPVs were collected using a volatile collection unit. The chamber was cleaned with hexane HPLC grade before starting the process. Air was pumped into the airtight glass chamber for 45 minutes, after which a vacuum was created. RLF larvae were introduced to potted CO 51 and ASD 16 rice plants cultured in cages within the chamber. The HIPV air was pumped through activated charcoal at 100 ml/min, trapping HIPVs from damaged rice plants in a volatile trap (adsorption tube) attached to the jar. Volatiles were collected for 3 hours, then extracted using 20 ml of HPLC grade hexane. The tubes were cleaned with acetone, dried, and covered with aluminum foil to prevent contamination, allowing for reuse. Headspace samples were analyzed using a GC-MS (GC 2010 plus, GCMS – TQ 8040 SHIMADZU) to characterize the compounds. One ml of collected volatiles was loaded into 20 ml glass vials. The GC used a 30 m fused silica capillary column (Rxi® - 624 Sil MS), with a constant helium gas flow of 1 ml/min. The oven temperature was programmed from 80°C to 240°C with specific ramp rates and holds. Each sample analysis lasted 30 minutes.

Compound identification was conducted using NIST14 by comparing spectra of unknown compounds with those in the library, and results were tabulated with peak areas and retention

times. Identification of active chemical compounds responsible for the orientation of natural enemies of RLF was made by fractionation of the collected crude volatiles was carried out by column chromatography with silica gel as adsorbents. The collected elutant were concentrated to 1 ml using a nitro evaporator and were loaded onto the silica column. Primarily, it was done using 100 per cent hexane HPLC grade (300 ml). This was followed by hexane and ethyl acetate mixture in different proportion viz., 92:8, 90:10 and 80:20 each of 300 ml. These proportions are of increasing polarity and resulted in the collection of F1, F2, F3 and F4 the first, second third and fourth fractions respectively. The collected fractions were then evaporated using a rotary vacuum evaporator to 1 ml. The concentrated fractions were subjected to GCMS for chemical characterization and olfactory assay.

The response of *T. chilonis* adults to HIPVs was analyzed using a four-way olfactometer, a square box with four horizontal side tubes supported by stands. The design allowed for the introduction of test insects through a detachable top. Each arm connected to an odour container that housed volatile samples, with an air pumping system to attract the parasitoids and a suction pump to create a vacuum. The corners of the box were closed to direct insect movement towards the volatiles. Experiments were conducted under controlled laboratory conditions (26±2°C, 65±5% humidity, 160 lux light). Each run involved 70 adult *T. chilonis* parasitoids exposed to plant volatiles induced by RLF. Filter paper strips soaked with 0.2 ml of test fractions from infested rice plants were placed in the odour container, alongside controls. Adult parasitoids were released through the top opening, and preferences were recorded two times at two hour intervals and mean was derived. The experiment was replicated seven times, with the olfactometer cleaned with ethanol beforehand and purified air pumped through the arms for 45 minutes to prevent volatile mixing.

RESULT AND DISCUSSION

The study of volatile compounds released by the rice leaf folder indicated that damaged rice plants emitted more volatiles than healthy plants (Table 1). The chemical profiles of healthy rice plants for ASD 16 and CO 51 identified nine and seven compounds, respectively. The healthy plants of ASD 16 released Pentadecane with the highest percent area (19.7), and Octadecane was the compound of CO 51 healthy plants with the highest peak area (22.22). The infested plants of ASD 16 released 22 compounds, but only 16 compounds were identified in both healthy and damaged plants of ASD 16 and CO 51 (Table 1). Rice plants treated with

Jasmonic acid and infested by the Brown Plant Hopper (*Nilaparvata lugens*) released more volatile organic compounds (VOCs) than their undamaged counterparts (Lou et al., 2006). Qingsong et al. (2015) reported that rice plants damaged by *Chilo suppressalis* exhibited significant differences in both the composition and quantity of volatiles when compared to undamaged plants. Rani and Sandhyarani (2012), who also noted a greater number of volatiles in rice plants damaged by yellow stem borer (YSB) compared to healthy plants, with differences observed in both quantity and quality.

Among RLF-damaged and healthy plants, eicosane was found to be common (Table 2). In F1 of infested ASD 16 rice plants, eicosane was 3.74 and 3.99 times more than dodecane and hexadecane respectively. The similar findings was observed in F2, in which eicosane was 3.50 and 3.80 times more than dodecane and hexadecane respectively. Rani and Sandhyarani (2012) reported that eicosane was one of the compounds crucial for the orientation of trichogrammatids, acting as an ovipositional stimulant and increasing parasitization rates. The Volatile composition of ASD 16 infested rice plants in different fractions was given in Table 2. Notably, several new hydrocarbons absent in healthy plants were detected in the volatiles of herbivore-damaged plants. Lou et al. (2005) noted that the quantity of volatile compounds released from herbivore-damaged plants was significantly higher, which could attract parasitoids. Major differences were found in eicosane and dodecane levels between damaged and healthy plants. Qingsong et al. (2015) documented tridecanone, nonanone, and methyl salicylate in both Bt and non-Bt plants damaged by herbivores, which were not present in healthy, undamaged plants. Michereff et al. (2019) found that different maize genotypes released various herbivore-induced plant volatiles (HIPVs) and that many new compounds lacking in healthy plants were present in those damaged by herbivores.

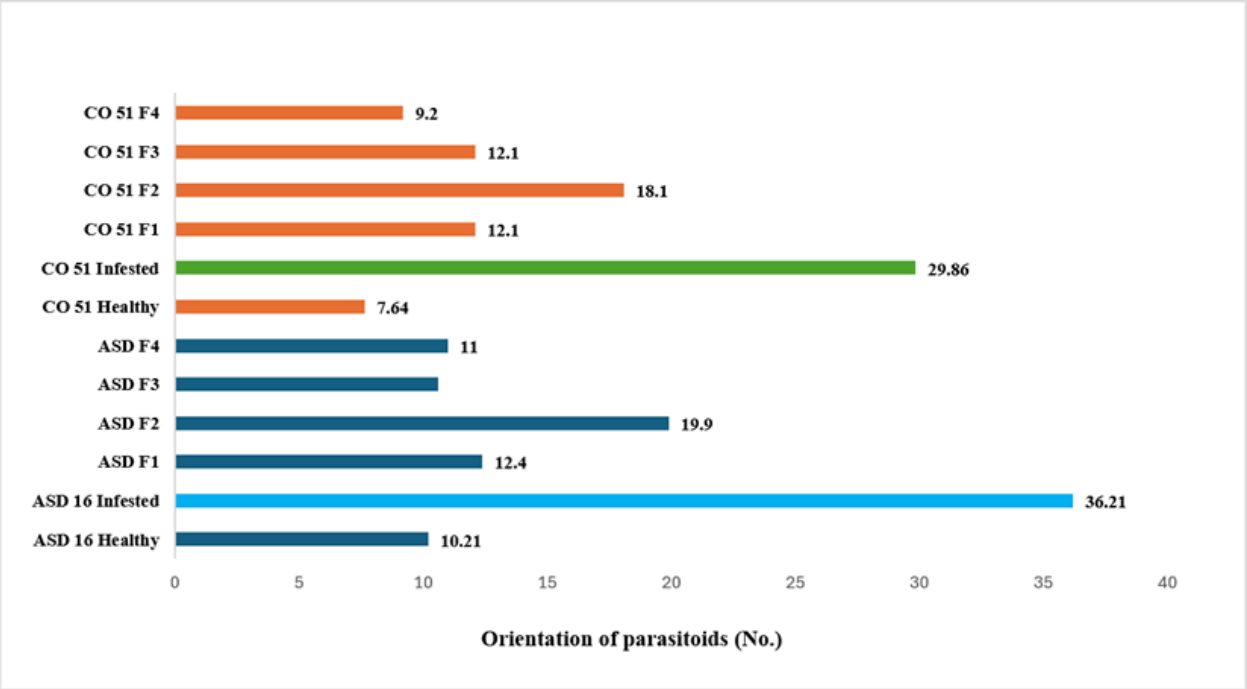


Fig. 1. Orientation of T. chilonis towards various fractions of volatiles released by RLF damaged rice plants and healthy plants (ASD 16 & CO 51)

Nonane was the compound identified only in F3. Dodecane and octane were the prominent compounds identified in F4. The similar results were observed in infested CO 51 rice plants (Table 3) in which eicosane was found in all fractions except F4. Nonane was found in F1 and F3 and octacosane was presented in F2 and F4. Wolfling and Rostas (2009) reported that octacosane was one of the compounds responsible for attracting the parasitoid *C. marginiventris* to *S. frugiperda*. Rani and Sandhyarani (2012) noted that hydrocarbons with a higher number of carbon atoms were particularly effective in attracting natural enemies. Generally, the volatiles from herbivore-damaged and undamaged plants ranged from nine to 30 carbon atoms. Octane (C8) was identified as the lowest order of volatile compound, while triacontane (C30) was the highest. Paul et al. (2008) mentioned that compounds containing 14 to 30 carbon atoms were emitted from extracts of tomato plants, while Kumar et al. (2018) found that GC-MS analysis of cruciferous volatile compounds revealed hydrocarbons ranging from heneicosane to dotriacontane.

In the study of orientation behaviour of parasitoids towards volatiles, a higher number of adult parasitoids were attracted to volatiles released from RLF damaged plants of both the varieties. The number of adults oriented towards volatiles from damaged plants were 36.21 and

29.86 for ASD 16 and CO 51 respectively. Only 10.21 and 7.64 adults oriented in the vicinity of volatiles from healthy plants of ASD 16 and Co 50 respectively (Fig. 1). Maximum mean parasitoid attraction (18.1) was recorded in fraction 2 of the volatiles from CO 51 RLF damaged plants. Both F1 and F3 showed 12.1 mean number of parasitoid attractions. The least orientation was noted in fraction 4. Higher parasitoid attraction was noticed in fraction 2 (19.90 no.) followed by fraction 1of ASD 16. The least attraction was observed towards fraction 3 (10.60 no.) The GC- MS studies carried out by Singh *et al.* (2017) revealed that, the volatile hydrocarbon of higher range *i.e.,* compounds with carbon atoms ranging from 22 to 34 were responsible for the attraction of trichogrammatids and also for the enhancement of foraging activities of the parasitoids. The studies on the parasitic efficiency of *T. chilonis* due to the volatiles emitted by the crucifers resulted in the conclusion that, the volatile compounds containing 21 to 32 carbon atoms *viz.,* heneicosane (C21), docosane (C22), tricosane (C23), pentacosane (C25), heptacosane (C27), nonacosane (C29) and triacontane (C30) were responsible for the attraction *T. chilonis* (Kumar et al., 2018).

CONCLUSION

From this study, it is concluded that the compounds having higher number of hydrocarbons (20 to 30) may be responsible for the higher orientation of the natural enemies in the rice ecosystem. The exploration of chemical mediated tritrophic interaction as induced by RLF in the rice ecosystem requires elicitor based studies for field application.

Declarations and Statements

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NN did investigation, writing of original Draft; KP conceptualised the research and assisted writing of original draft and supervised it. **SV involved in conducting, reviewing** and editing the draft and MD validated data and edited.

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Table 1. Volatile composition of healthy and RLF damaged rice plants (ASD 16 and CO 51)

S. No.	ASD 16				CO 51			
	Healthy plants		Damaged plants		Healthy plants		Damaged plants	
	Name of the compound	Percent Area	Name of the compound	Percent Area	Name of the compound	Percent Area	Name of the compound	Percent Area

1.	Undecane	19.7	Undecane, 2,4-dimethyl	1.63	Nonane, 2,5-dimethyl	9.28	Heptadecane	2.64
2.	Decane, 5,6-dimethyl	7.94	Tridecane, 5-methyl	2.28	Octane, 6-ethyl-2-methyl	10.06	Dodecane, 2,6,10-trimethyl	12.83
3.	Docosane	6.41	2,6,10-Trimethyltridecane	5.74	Eicosane	11.64	1-Nonadecanol	4.45
4.	Octadecane	13.91	Octadecane, 5-methyl	1.97	Octadecane	22.22	Dodecane, 2,6,11-trimethyl	5.6
5.	Dodecane, 4,6-dimethyl	10.76	Heptadecane, 2,6,10,15-tetramethyl	13.88	3-Buten-2-ol	6.98	Pentadecane	3.69
6.	Eicosane, 2-methyl-	6.02	Nonane, 5-(1-methylpropyl)	3.08	Dodecane, 2,6,10-trimethyl	11.1	Hexadecane	4.04
7.	1-Undecene, 5-methyl	10.68	Dodecane, 2,6,11-trimethyl	7.73	Tridecanol, 2-ethyl-2-methyl	18.63	Tetracosane	8.71
8.	Nonane, 4,5-dimethyl-	4.6	Octane, 2,3,6,7-tetramethyl	1.48			Undecane, 2,4-dimethyl	4.23
9.	Pentadecane, 2,6,10,14-tetramethyl	19.98	Tetradecane	1.95			Octacosane, 1-iodo	18.33
10.			Tetracosane	4.25			Nonadecane	3.12
11.			Nonadecane	2.8			Tetradecane	3.27
12			Pentadecane	2.49			Heneicosane	9.83
13.			2,3-Dimethyldodecane	1.79			Octadecane	3.28
14.			Tetradecane, 5-methyl	5.62			Nonane	2.67
15.			2,6,10-Trimethyltridecane	1.31			Tridecanol, 2-ethyl-2-methyl	3.24
16.			Octacosane	17.34			Eicosane	10.09
17.			Hexacosane	3.93				
18.			Dodecane, 4,6-dimethyl	1.3				
19.			Heneicosane	10.13				
20.			10-Methylnonadecane	1.11				
21.			Eicosane	6.56				
22.			Triacontane	1.63				

Table 2. Volatile composition of from RLF damaged rice plants of ASD 16 variety (Fractionated analysis)

S. No.	Fraction 1 (100% Hexane)			Fraction 2 (Hexane: Ethyl acetate- 92:8)			Fraction 3 (Hexane: Ethyl acetate- 90:10)			Fraction 4 (Hexane: Ethyl acetate- 80:20)		
	Compound	**Area**	**Multiple**	**Compound**	**Area**	**Multiple**	**Compound**	**Area**	**Multiple**	**Compound**	**Area**	**Multiple**
	Undecane, 4,7-dimethyl	353923	1.05	Heptadecane, 2,6,10,15-tetramethyl	478591	1.28	Hexadecane, 1-iodo	209390	1.04	Undecane, 4,8-dimethyl	184924	1.29
	Decane, 3,8-dimethyl	138406	2.69	Dodecane, 4-methyl	180574	3.38	Nonane	88209	2.47	Dodecane	91293	2.62
	Octane, 2,3,3-trimethyl	151614	2.45	Tetracosane	163992	3.73	Octane, 2-methyl	99303	2.19	Decane, 2,9-dimethyl	135465	1.76
	Tridecanol, 2-ethyl-2-methyl	206794	1.80	Hexadecane	160741	3.80	Tridecanol, 2-ethyl-2-methyl	222558	0.98	Tridecane, 2-methyl	49877	4.79
	Dodecane, 2,6,10-trimethyl	99471	3.74	Docosane, 1-iodo	398896	1.53	Undecane, 3,8-dimethyl	119771	1.82	Octane, 3,4,5,6-tetramethyl	117130	2.04
	Hexadecane	93184	3.99	Octacosane, 1-iodo	735720	0.83	Heptane, 2,4-dimethyl	60101	3.62	Eicosane	238998	1.00
	Eicosane	372015	1.00	Hexacosane	156236	3.91	Eicosane	217737	1.00			
				Dodecane, 2,6,10-trimethyl	174756	3.50	Octadecane, 5-methyl	71987	3.02			
				Heneicosane	617936	0.99						
				Nonadecane	165496	3.69						

				Eicosane	610956	1.00						

Table 3. Volatile composition of from RLF damaged rice plants of CO 51 variety (Fractionated analysis)

S. No.	Fraction 1 (100% Hexane)			Fraction 2 (Hexane: Ethyl acetate- 92:8)			Fraction 3 (Hexane: Ethyl acetate- 90:10)			Fraction 4 (Hexane: Ethyl acetate- 80:20)		
	Compound	**Area**	**Multiple**	**Compound**	**Area**	**Multiple**	**Compound**	**Area**	**Multiple**	**Compound**	**Area**	**Multiple**
	Dodecane, 2,6,10-trimethyl	239157	1.08	Heptadecane	294466	0.93	Dodecane, 2,6,10-trimethyl	94566	1.18	1-Nonadecanol	339050	1.00
	1-Nonadecanol	144708	1.78	Undecane, 2,4-dimethyl	54070	5.09	1-Nonadecanol	164901	0.67	Octacosane, 1-iodo	48200	7.03
	Nonane	76019	3.39	Dodecane, 2,6,11-trimethyl	273635	1.01	Undecane, 3,7-dimethyl-	71320	1.56	Octadecane	92483	3.67
	Undecane, 2,4-dimethyl	91994	2.80	1-Nonadecanol	125771	2.19	Tetracosane	83079	1.34			
	Nonadecane	320602	0.80	Hexadecane	63264	4.35	Octadecane	58516	1.90			
	Tetradecane	65553	3.93	Tetracosane	105995	2.60	Nonadecane	102192	1.09			
	Heneicosane	145222	1.77	Octacosane	90059	3.06	Octane, 2-methyl-	38636	2.88			
	Eicosane	257345	1.00	4,4-Dimethyl octane	114350	2.41	Nonane	40797	2.73			
				Hexacosane, 1-iodo	411529	0.67	Eicosane	111207	1.00			
				Heneicosane	189178	1.45						

				Tridecanol, 2-ethyl-2-methyl	86742	3.17						
				Eicosane	275170	1.00						