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VOLATILE PROFILES AS AFFECTED BY RICE BROWN PLANT HOPPER AND YELLOW STEM BORER IN RICE LAND RACES

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ABSTRACT

Rice (*Oryza sativa* L.,) plants release a complex profile of volatile organic compounds. Present study investigates the differences in volatile compounds from four rice landraces viz., Karuthakar, Norungan, Thavala Kanan and Varappu Kudaichan each under four conditions like healthy, mechanically damaged, and the ones infested by the brown plant hopper *Nilaparvata lugens* Stal and yellow stem borer *Scirpophaga incertulas* (Wlk.). The volatiles were collected using air entrainment method and characterized by the GCMS. Statistical analysis tools like clustering, principal component analysis and partial least square discriminant analysis were applied. Clear differences among the treatments were observed and certain volatile compound groups like terpenoids (squalene), unsaturated fatty acids (n-hexadecanoic, tetradecanoic and pentadecanoic acids), alkanes (heptacosane, tetracosane) were found. The statistical test of Partial Least Square Discriminant Analysis was found to be satisfactory in determining the compounds responsible for variations in treatments.

Key words: Rice landraces, *Nilaparvata lugens*, *Scirpophaga incertulas*, secondary metabolites, herbivore induced plant volatiles, terpenoids, fatty acids, esters, GCMS, Clustering analysis, multivariate analysis

Plants communicate to the environment by releasing certain organic volatile compounds. These act as chemical signals for tritrophic interaction. Healthy plants maintain a baseline level of volatile metabolites which tend to differ from those that are mechanically damaged or infested by pests (Pare and Tumilson, 1999). This phenomenon makes the field of chemical ecology more interesting as it gives a better insight into the compounds playing role in tritrophic interaction. Apart from these, it is quite noteworthy to observe a quantitative and qualitative difference in volatiles among the varieties of a plant (Krips et al., 2001; Hoballah et al., 2002). This might be the reason behind the varietal difference in attraction of insect pests and natural enemies. So, it creates a need in the exploration of volatile profiles between varieties of plant. Even though there are some studies on the difference in the HIPV's among the plant varieties, very little is known like that of Lou et al. (2006), focused on the variations in the induced volatiles between rice varieties. There is practically no work on the variations in the traditional rice landraces. Since landraces are rich in the diverse gene pool, characterizing their volatile profiles under both herbivore induced and controlled conditions is essential. This has been attempted in the present study. The multivariate data analysis has now become a powerful tool in data analysis to estimate the interactions and the data obtained in this study have been subjected to such analyses.

MATERIALS AND METHODS

Popular and stress tolerant rice landraces- Karuthakar (K), Norungan (N), Thavala Kanan (T) and Varappu Kudaichan (V) were used and their seeds were obtained from the bank of plant genetic resources, TNAU. The seeds were soaked in the water for 24 hr and then incubated in dark condition before sowing. The pre-germinated seeds were sown in clay pots kept in cages. After 14 days, the seedlings were transplanted in separate clay pots (12cm dia x 10 cm height) @ 2 seedlings/ pot and watered daily. Urea was applied 15 days after transplanting @ 0.3 g/ pot. The pots were then placed in netted cages to maintain healthy seedlings free from the attack of insects. Plants were used for experiment at 35 to 45 days after transplanting. Mechanically damaged plants were obtained by individually damaging the plants with needle at the lower and upper portion of the rice stems each with approximately 200 pricks to

simulate the feeding behaviour of brown plant hopper (BPH) *Nilaparvata lugens* (Stal).

Nymphs and adults of BPH were collected from the Tamil Nadu Agricultural University rice fields and released into the cages where TN1 (susceptible) potted plants were maintained. The BPH was allowed to multiply and then their nymphs were selected for the experiment. Three second/ third instar nymphs/ seedling were allowed to feed after starving for 2 hr. Similarly, rice yellow stem borer (YSB) *Scirpophaga incertulas* (Walker) females were collected from the field and released into the cages with TN1 variety for oviposition. The eggs were allowed to hatch and the 1st instar larvae were collected using the camel hair brush and released on to the 35 to 45-days old Karuthakar, Norungan, Thavala Kanan and Varappu Kudaichan seedlings. Each tiller was released with five to six larvae, and these used. Two replications were maintained for each, with a total of 16 treatments used in volatile collection.

Plant volatile collection was made using the air entrainment method. The volatile collection system basically consists of a vertically placed cylindrical glass tube (62 cm height, 6 cm internal dia). The bottom part of the cylinder was left open in order to fit the plant inside. The top of the cylinder has two raised ports (2 cm height x 0.8 cm internal dia) of which air was passed through one port and the plant volatile was collected through the other. Aquarium pump (Champion, CX-0088) was used to provide air @ 1.0 l/ min. purified and humidified air was passed by the means of activated charcoal and humidifier. The purified air after passing through the plant was pulled (0.8 l/ min) through a super Q-absorbent trap (volatile collection trap) in order to collect the volatiles. The bottom of the cylinder around the base of the plant was covered with aluminium foil to prevent the contamination of soil volatiles. The entire system was sealed air tight. Volatile collection was carried out for 24 hr and the collected volatiles were extracted from the collection trap with 700 µl of hexane in GC vials before stored at -20°C until further use.

The Clarus SQ 8C Gas Chromatography- Mass Spectrometer instrument was set as follows: Injector port temperature set to 220°C, Interface temperature as 250°C, and source kept at 220°C. The oven temperature programmed as available, 75°C for 2 min, 150°C @ 10°C/ min, up to 250°C @ 10°C/ min. Split ratio set as 1:12. The DB-5 MS capillary standard non- polar column was used. Helium was used as the carrier gas at 1 ml/ min. The MS data system has inbuilt libraries for searching and matching the spectrum. Interpretation of mass spectrum of GC-MS was done using the database of National Institute of Standard and Technology (NIST14). The spectrum of the known component was compared with the spectrum of the known components stored in the inbuilt library. The raw data of peak areas of volatile compounds were tested for normality and proceeded with the non-parametric Kruskal-Wallis test. Heat map analysis was performed using the package "d3.heatmap". K means clustering was done using the "stats" package and kmeans function. The number of clusters for kmeans clustering is found by silhouette method. Hierarchical clustering analysis using ward method was done. In order to reduce the dimensionality of the multivariate data, Principal Component Analysis (PCA) was performed using the prcromp function with the data centered and scaled before analysis. Partial Least Square Discriminant Analysis (PLS-DA) was done using "mixOmics package". All the statistical analysis was performed using the R statistical software (R version 4.0).

RESULTS AND DISCUSSION

Among the headspace volatiles released by the rice landraces Karuthakar, Norungan, Thavala Kanan and Varappu Kudaichan (healthy, mechanically damaged, BPH infested and YSB infested), 45 volatile organic compounds (VOC's) were identified (Table 1). These 45 volatile compounds were selected from the total observed compounds based on their repeated occurrence in replications. Nineteen compounds were found to be significantly different ($p<0.05$) among the treatments.

Heat map analysis as given in Fig. 1 provides the distribution of important volatiles among the sixteen treatments. Similar colour in the heatmap indicates the similar level of compound- darker the colour, higher is the concentration, and lightest blue colour indicates those undetected compounds. The results reveals qualitative and quantitative differences among the 45 volatile compounds. *K* means clustering yielded two clusters by silhouette method (Fig. 3) whereas hierarchical clustering resulted in three clusters (Fig. 2). The size of the clusters are 4 (all the treatments of Varappu Kudaichan) and 12 (Fig. 4). In hierarchical clustering, all the four treatments of Varappu Kudaichan were grouped under a cluster like *k*-means clustering. Compared to *k* means clustering hierarchical clustering was more consisted with those of PCA and PLSDA. Both the clustering analysis performed in the present study had separated the treatments of Varappu Kudaichan into

Table 1. Volatile compounds (head space) obtained from the rice landraces

S. No.	Compound	Karuthakar (mean relative peak area ± Std. error)				Norungan (mean relative peak area ± Std. error)				Thavala kanan (mean relative peak area ± Std. error)				Varappu Kudaichan (mean relative peak area ± Std. error)				P value
		KH	KMD	KBPH	KYSB	NH	NMD	NBPH	NYSB	TH	TMD	TBPH	TYSB	VH	VMD	VBPH	VYSB	
1.	(1S,14S)-Bicyclo (12.10.0)-3,6,9,12,15,18,21,24-octaoxatetracosane	0	0	0	3.41± 1.31	0	3.47± 2.96	0	0	0	0	0	0	0	0	0	0	0.0926
2.	(2S,2'S)-2,2'-Bis (1,4,7,10,13-penta oxacyclopentadecane)	1.24± 1.05	0.71± 0.31	0	2.54± 1.20	2.14± 0.01	1.53± 0.02	0	0.49± 0.01	0	0	0	0.95± 0.05	0	0	0	0	0.1493
3.	1,2-Diamino-2-methylpropane	0	0	0.87± 0.02	0	0	0.29± 0.01	0.84± 0.04	0	0.32± 0.06	0	1.11± 0.01	0.45± 0.01	0	0	0	0	0.0227*
4.	1,4,7,10,13,16,19-Heptaoxa-2-cyclo heneicosanone	5.04± 0.48	10.34± 5.04	0	1.57± 0.98	0.77± 0.50	0.59± 0.04	0	0.85± 0.08	0	1.76± 0.01	0	0.72± 0.12	0	0	0	0	0.0229*
5.	17-Pentatriacontene	0	0	0	0	0.61± 0.01	0.36± 0.01	0	0	0	0	0	0.46± 0.03	0	0	0.42± .0.01	0	0.0563
6.	1-Hexadecanol, 2-methyl-	0	0.10± 0.01	0	0	0	0	0	0	0	0	0	0.36± 0.04	0	0	0	0	0.0382*
7.	1-Penten-3-ol	0	0	0	0	0	0	0	0	0.27± 0.01	0	0	0.36± 0.01	0	0	0	0	0.0561
8.	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	0	0	0.99± 0.01	0	0	0	0.75± 0.01	5.98± 0.51	0.38± .02	0	0	1.45± 0.01	4.19± 0.23	1.82± 0.17	0.51± 0.04	3.40± 0.18	0.0944
9.	2-Aminocyano-acetamide	0	0	0	0	0	0	0	0	0.24± 0.02	0	0	0.37± 0.01	0	0	0	0	0.0382*
10.	2-Decanol	0	0	0.91± 0.05	0	0	0	0	0	0	0	1.27± 0.03	0.30± 0.05	0	0	0	0	0.1053
11.	2-Ethyl-oxetane	0	0	1.56± 0.01	0	0	0	0	0	0.26± 0.01	0.41± 0.01	0	0	0	0	0	0	0.0793
12.	2-Ketobutyric acid	0	0	1.02± 0.01	0	0	0	1.07± 0.44	0	0	0	0.63± 0.03	0.31± 0.01	0	0	0	0	0.0518
13.	2-Myristynoyl pantetheine	0	0.36± 0.01	0	0.56± 0.06	0.65± 0.01	0	0	0.32± 0.01	0.69± 0.01	0.39± 0.01	0	0	0	0.25± 0.01	0.38± 0.03	0	0.0782
14.	2-Nonadecanone 2,4-dinitrophenyl hydrazine	0	0	0	0	0	0	0	0	0	0	0	0.44± 0.12	0.41± 0.08	0	0	0	0.0926

(contd.)

S. No.	Compound	Karuthakar (mean relative peak area ± Std. error)				Norungan (mean relative peak area ± Std. error)				Thavala kanan (mean relative peak area ± Std. error)				Varappu Kudaichan (mean relative peak area ± Std. error)				P value
		KH	KMD	KBPH	KYSB	NH	NMD	NBPH	NYSB	TH	TMD	TBPH	TYSB	VH	VMD	VBPH	VYSB	
15.	3',8,8'-Trimethoxy-3-piperidyl-2,2'-bi naphthalene -1,1',4,4'-tetrone	1.27± 0.35	0	0	1.67± 0.11	1.95± 0.01	0	0	0	0	0.23± 0.01	0	0.26± 0.01	0	0	0	0	0.2504
16.	3-Hydroxy-2-butanone (Acetoin)	0	0	1.29± 0.07	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0088*
17.	3-tert-Butylsulfanyl-3-fluoro-2-trifluoro methyl acrylicacid methyl ester	0	0	0	0	0	0	0	0	0.27± 0.01	0.27± 0.01	0	0	0	0	0	0	0.0561
18.	à-D-Glucofuranose, 6-O-(trimethylsilyl)-, cyclic 1	0	0	0	0	0	0	0	0.31± 0.01	0	0.57± 0.02	0	0	0	0	0	0	0.0561
19.	Azetidine	0	0	2.03± 1.24	0	0	0	0	0.25± 0.01	0	0.24± 0.04	1.39± 0.01	0.24± 0.02	0	0	0	0	0.0474*
20.	Borinic acid, diethyl-	0	0	1.10± 0.30	0	0	0	0	0	0	0	1.23± 0.01	0.34± 0.01	0	0	0	0	0.0210*
21.	Butoxyacetic acid	0	0	0.83± 0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0088*
22.	Dasycarpidan-1-methanol, acetate (ester)	0	0	0	0	0.36± 0.01	0.18± 0.01	0	0	0	0.54± 0.01	0	0	0	0	0.58± 0.03	0	0.04472*
23.	Diisooctyl phthalate	0	0	0	0	5.20± 0.14	1.83± 0.01	1.26± 0.01	8.58± 0.03	0.77± 0.51	0	1.18± 0.20	3.74± 2.43	4.38± 0.02	0	4.78± 0.13	5.13± 0.04	0.1388
24.	Dodecanoic acid	0	0	1.45± 0.01	0	0	0	0	0	0.30± 0.01	0	2.15± 0.01	0	0	0	0	0	0.0793
25.	Heptacosane	0	0	0	0	0	0	0.65± 0.26	0	0	0	0	0	0	0	0	0	0.0088*
26.	Heptaethylene glycol	1.46± 0.98	0	0	0	0.69± 0.35	2.25± 0.01	0	0.40± 0.01	0	0	0	0	0	0	0	0	0.0577
27.	Heptaethylene glycol monododecyl ether	0	0	0	2.29± 1.34	0	0	1.28± 0.03	0.91± 0.01	0	20.78± 0.02	0	0	0	0	0	0	0.1755
28.	Hexacosane	0	0	1.44± 0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4514
29.	Methane, isocyanato-	0	0	1.08± 0.01	0	0	0.36± 0.01	0	0	0	0	0.68± 0.02	0.28± 0.01	0	0	0	0	0.0362*
30.	Methyl 21-methyldocosanoate	0.28± 0.11	0	0	0	0	0	0	0	0	0	2.35± 0.21	0	0	0	0	0	0.0088*

(contd.)

(Table 1 contd.)

S. No.	Compound	Karuthakar (mean relative peak area ± Std. error)				Norungan (mean relative peak area ± Std. error)				Thavala kanan (mean relative peak area ± Std. error)				Varappu Kudaichan (mean relative peak area ± Std. error)				P value
		KH	KMD	KBPH	KYSB	NH	NMD	NBPH	NYSB	TH	TMD	TBPH	TYSB	VH	VMD	VBPH	VYSB	
31.	n-Hexadecanoic acid	0.25± 0.10	0	0	0.31± 0.07	0.28± 0.02	0.50± 0.01	0.58± 0.02	0.69± 0.13	0.52± 0.08	1.21± 0.01	0	0.51± 0.13	3.58± 0.38	2.63± 0.03	5.26± 0.13	4.95± 0.26	0.03995*
32.	Nonanal	0.56± 0.01	0	1.21± 0.18	0	0	0	0	0	0	0	0	0	0	0	0	0	0.4904
33.	octacosane	0	0	0	0.84± 0.27	0	0	0	0	0	0	0	0	0	0	0	0	0.0088*
34.	Octadecanal, 2-bromo-	0.22± 0.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0088*
35.	Octadecane	1.28± 0.01	0	0	0	0	0	0.43± 0.05	0	0	0	0	0	0	0	0	0	0.0561
36.	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	0.48± 0.22	0.27± 0.01	0	0.86± 0.06	0.68± 0.01	0.59± 0.22	0.45± 0.01	0.48± 0.01	0.34± 0.06	0	0	0.25± 0.01	0	0.74± 0.23	0.87± 0.10	0.44± 0.03	0.1547
37.	Octaethylene glycol monododecyl ether	0.13± 0.01	0.25± 0.01	0	2.22± 0.12	0	0	0.88± 0.01	0.58± 0.03	0.60± 0.12	0.85± 0.03	0	0	0	0	0	0	0.0985
38.	Pentacosane	0	0	6.05± 5.01	2.07± 1.83	0.27± 0.03	0	0	0	0	1.21± 0.01	0	0.50± 0.02	0	0	0	0	0.2579
39.	Pentadecanoic acid	0	0	0	0	0	0	0.32± 0.01	0	0	1.75± 0.03	0	0.75± 0.03	0.61± 0.02	0.42± 0.04	0.58± 0.02	0.63± 0.04	0.0810
40.	Pentane, 2,2,3,4-tetramethyl-	0	0	0.85± 0.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0088*
41.	Pentane, 3-methyl-	0	0	1.22± 0.25	0	0	0	0	0	0	0.25± 0.01	0	0	0	0	0	0	0.0382*
42.	Squalene	0	0	0	0	0	0	0	0	0	0	0	0	6.74± 4.44	7.45± 1.95	4.30± 0.07	10.55± 0.33	0.0102*
43.	Tetracosane	0	0	0	3.49± 0.98	0	0	0.36± 0.01	0	28.88± 0.13	0	0	0	0	0	0	0	0.1053
44.	Tetradecanoic acid	0	0	0	0	0	0	0	0	0	0	0	0	2.49± 0.01	1.62± 0.06	2.42± 0.03	3.11± 0.09	0.0088*
45.	Tetratetracontane	0	0	0	0	0	0.31± 0.07	4.40± 0.10	0	0	0	0	0	0	0	0	0	0.0561

*Indicates the significant p value of the non-parametric Kruskal Wallis test; Healthy (H), Mechanically damaged (MD), *N.lugens* infested (BPH) and *S. incertulas* infested (YSB)

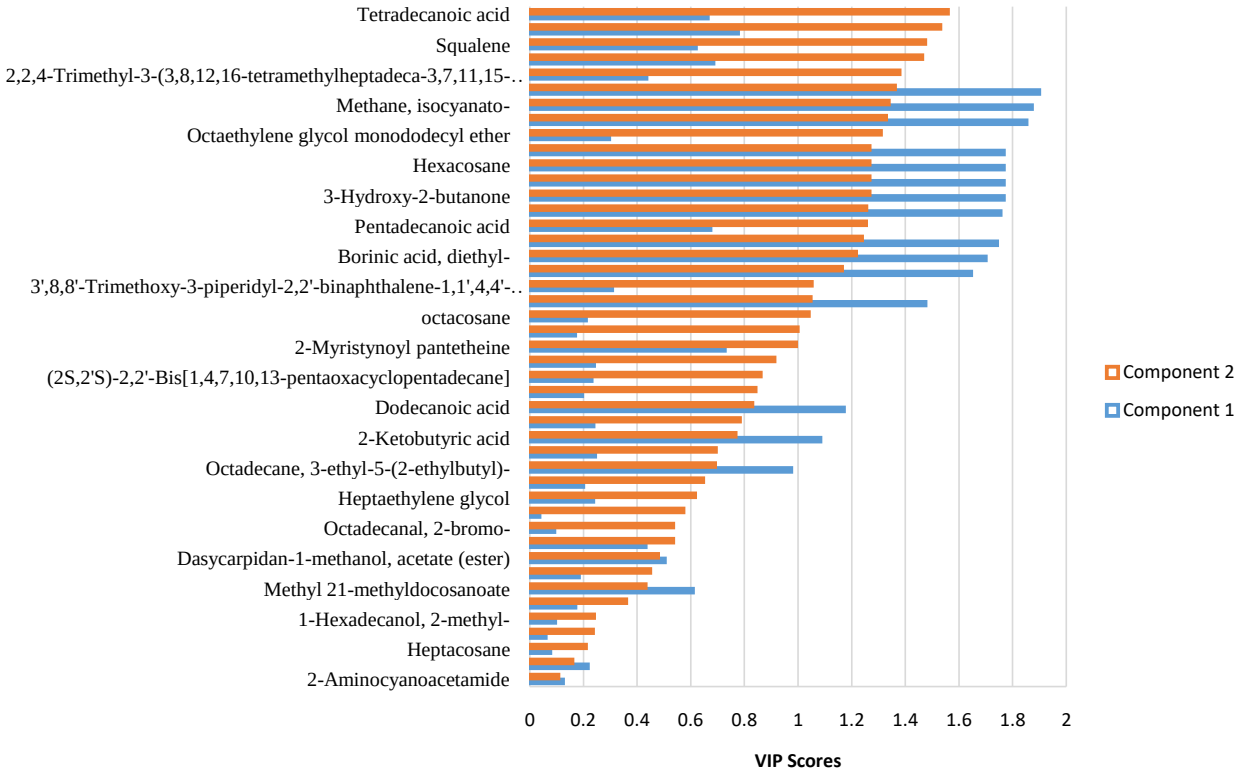
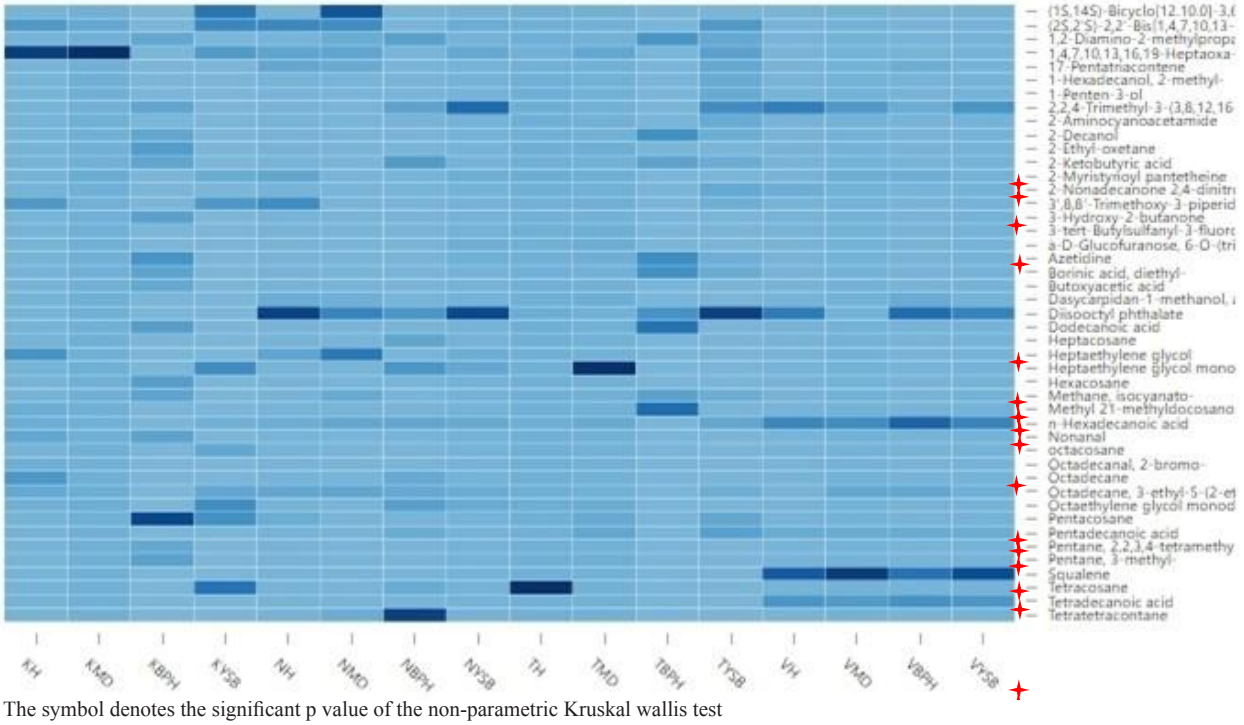


Fig. 1 (Supplementary). Variable Importance Projection scores of the Components 1 and 2 for the volatile compounds



The symbol denotes the significant p value of the non-parametric Kruskal wallis test

Fig. 1. Heatmap of VOCs emitted from treatments of rice landraces- Karuthakar, Norungan, Thavala Kanan and Varappu Kudaichan

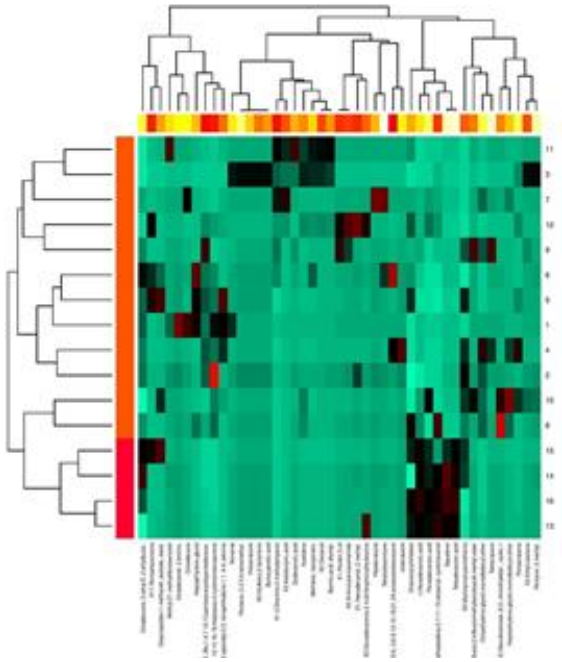


Fig. 2. *K* means clustering heat map

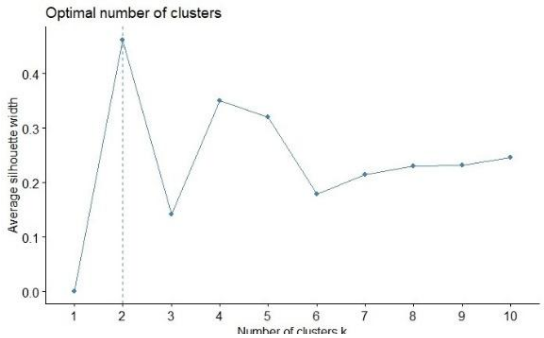


Fig. 3. Clusters selection – silhouette method

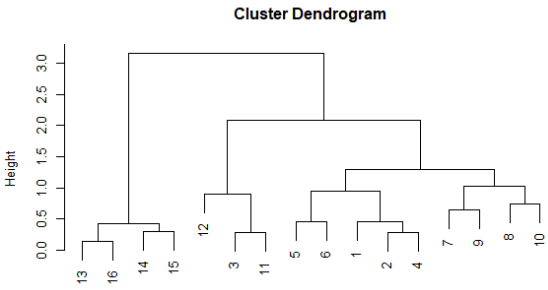


Fig. 4. Hierarchical clustering – ward method

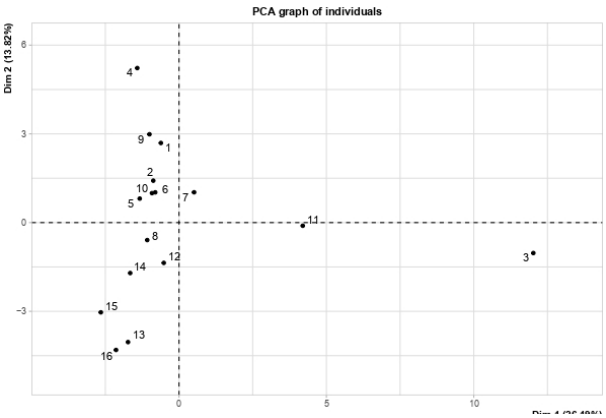


Fig. 5. PCA Score plot (1-16 indicates treatment orders)

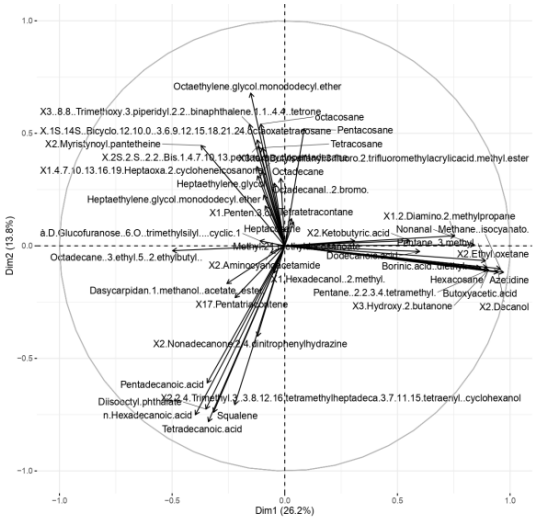


Fig. 6. Loadings plot of variables

separate cluster. This might be due to the influential role of compounds like disooctyl phthalate, n-hexadecanoic and pentadecanoic acids, squalene and tetradecanoic acid. This was also verified from the loadings plot of PCA and PLSDA. Clustering analysis also indicated the uniqueness in the volatile profiles released by each insect species irrespective of the varietal differences. Similar results had been earlier reported (Chen et al., 2020; Hoballah et al., 2002).

Principal Component Analysis was applied to the 45 volatile compounds to determine whether the samples belonging to different treatments of rice landraces can be separated based on their quantitative or qualitative differences in the emitted volatile profile. PC 1 and PC 2 explained approximately 26.19% and 13.82% of the total variation, respectively which accounts to totally of 40.01%. In Fig. 5, the treatments were represented as the matrix of scores according to the principal components. The numbers in the score plot denote the order of treatment groups as mentioned in the Table 1. Overall, from the results of PCA score plot (Fig. 5), it is evident that except Varappu Kudaichan, the landraces were found clustered according to their treatment similarities like the results of clustering analysis. Fig. 6 shows the volatile compounds responsible for the position of particular treatment in the score plot. Their project values on each principal component show how much weight they have on that principal component. For instance, treatment groups like VYSB,

VMD, VBPH, VH, TYSB and NYSB which is on the negative side of the PC1 and PC2 are strongly influenced by the compounds like 2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl heptadeca-3,7,11,15-tetraenyl)-cyclohexanol), n-hexadecanoic acid, squalene and tetradecanoic acid. These compounds were also found to be highly correlated among themselves as these vectors lie close to each other with less angle between them. Similarly, certain compounds lie close the PC1 and other compounds to the PC2. These three groups of compounds were found to be at 90° between each group indicating that they are negatively correlated with each group.

Partial Least Squares Discriminant analysis, was applied to make an even better separation between the treatments of landraces. The 45 volatile compounds were designated as the X-matrix, while the Y matrix consisted of the details of the sixteen treatments. Figure 5 explains 26% and 14% variance of X variate (volatile compounds). Clear differentiation among the rice landrace treatments was observed as given in Fig. 7. Like PCA, the treatments like VH, VMD, VYSB, VBPH, TYSB and NYSB were grouped together. The treatments KBPH and TBPH are far from these groups and lie on the negative side. On the upper side of the plot, treatments like NH, NMD, NBPH, KMD, TMD, KH and TH are together like cluster whereas the treatment KYSB were far from these treatments. Compounds like 8 (2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl heptadeca-3,7,11,15-tetraenyl)-cyclohexanol), 31 (n-hexadecanoic acid), 39 (pentadecanoic acid), 42 (squalene) and 44 (tetradecanoic acid) were found to lie on the correlation circle of the landrace Varappu Kudaichan (Fig. 8). Among the 45 volatile compounds observed, 25 were found to have VIP scores above 1. Of these, 14 volatiles have significant difference with p value ≤ 0.05, and these are considered to be highly influential ones for each treatment groups.

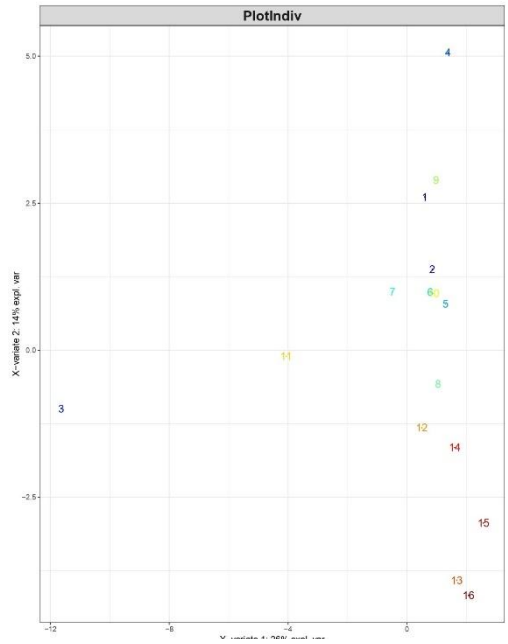


Fig. 7. PLSDA – Score plot

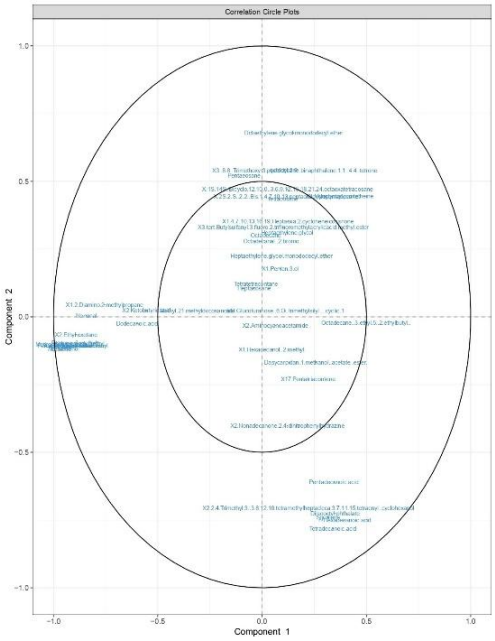


Fig. 8. PLSDA- Loadings plot

Some of the compounds reported to have significant difference in the present study are known to have influence in tritrophic interaction. The squalene which was found to influence the landrace Varappu Kudaichan is comparatively higher in the YSB and BPH infested treatments. This compound is a triterpenoid and is known to possess wound healing properties. It is also considered to be a potential compound in biological control as it attracts the natural enemies like *Chrysoperla* sp. and some parasitoids (Dutton et al., 2002; Jones et al., 2011). This might be the reason for its relatively higher amount in infested rice landrace. Compounds like n-hexadecanoic acid, pentadecanoic acid and tetradecanoic acid were also found to influence the landrace Varappu Kudaichan. These are saturated fatty acids and play important role in plants, and are known to possess oviposition deterrent activity against insects. Similarly, dodecanoic and hexadecanoic acids in *Solanum sarrachoides* were found to poorly deter the oviposition of *Tetranychus evansi*, Baker and Pritchard (Murungi et al., 2016). Compounds like octacosane and heptacosane play a role in intraguild predation

(Nakashima et al., 2006). Different multivariate analysis performed on the data provided the similarity in certain results like clustering of similar treatments from all the landraces together except Varappu Kudaichan and the volatile compounds that influence in the separation of the landrace Varappu Kudaichan from others.

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