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Identification of Changes in Total Volatilome of Tomato Plant Roots in Response to Phosphorous Availability

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Summary

The volatilome are bioactive volatile organic compounds. They respond to changes in growing conditions and can work as signalling molecules within or between plants. This paper describes changes in volatile organic compounds in plants in response to changes in phosphorus availability.

INTRODUCTION

Volatilome is a term used to describe the total bioactive compounds such as volatile organic compounds (VOCs) produced by plants through its biosynthetic pathways (**Fig. 1**). These wide range of chemical compounds are produced under specific conditions and in response to changing growing conditions such as nutrient deficiencies, environmental stress conditions, pollination, defence strategies, signalling and communication strategies intra / inter plants and organisms. In a nutshell, the plant volatilome is considered as an extended metabolome, reflecting the plant's

physiological status (Lee Díaz et al. 2022). This research will use an untargeted approach to identify changes in the whole plant volatilome or VOC profile in response to changes in phosphorus nutrition.

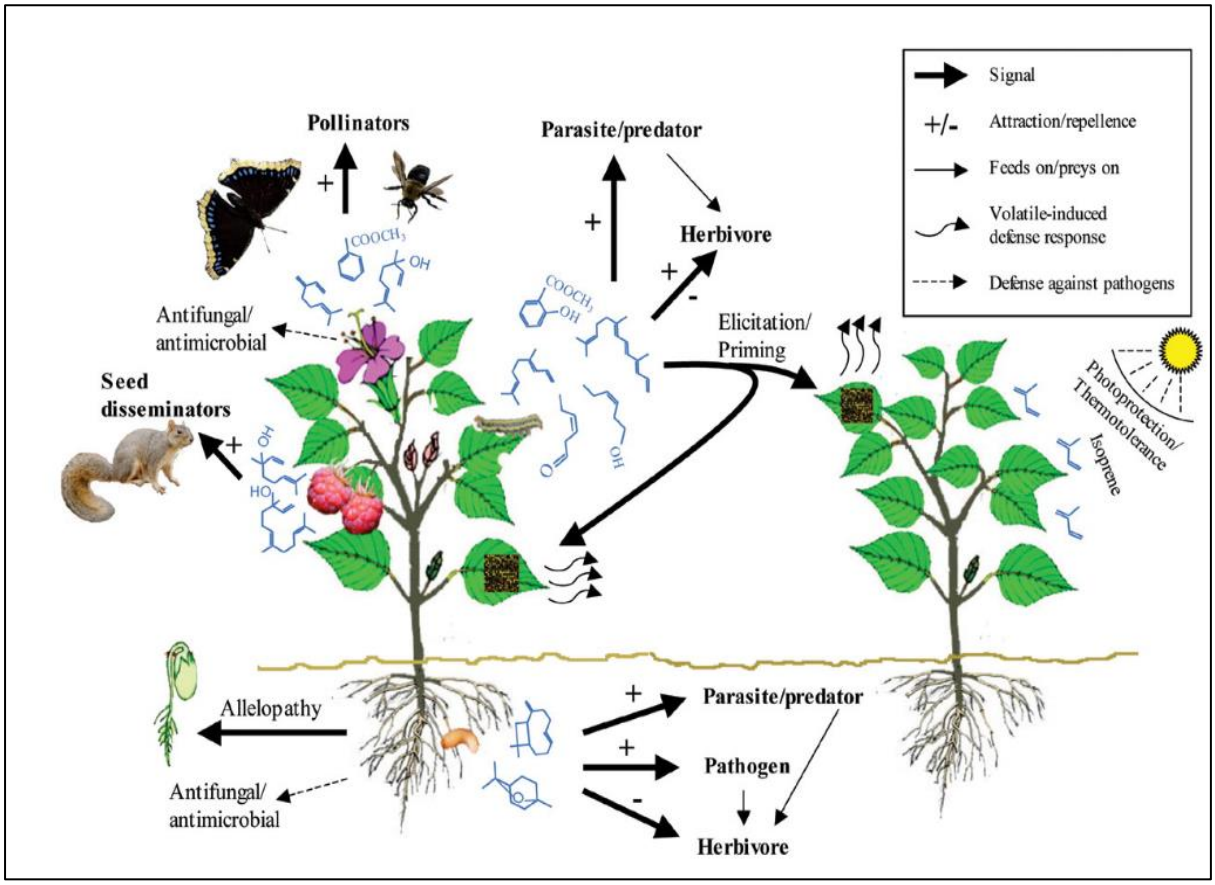

Figure 1. overview of volatile-mediated plant interactions with the surrounding environment (Dudareva et al. 2006).

Phosphorus (P) is an essential microelement required by all plant cells yet, it is both the least mobile and least available of the major plant nutrients (Kovar & Claassen 2005). Owing to its presence in major organic molecules, such as nucleic acids, (RNA, DNA,) ATP and membranes (Péret et al. 2011), plant productivity can be greatly limited by P starvation in the growing environment. In most soils, inorganic P is available at low levels in solution because of its strong adhesion to soil minerals hence, affecting phosphorus chemical mobility and bioavailability (Hinsinger 2001). This makes this essential element almost inaccessible to plants thus, a major limiting factor for plant growth and development (Massalha et al. 2017).

The uptake of P usually in the form of orthophosphate (Pi) from soil solution is made possible through symbiotic associations with arbuscular mycorrhizal (AM) fungi (Chiu & Paszkowski 2019). There is research evidence that suggests that plant roots and AM fungi perceive each other prior to their physical interaction. However, the identities of the diffusible signals are currently unknown, but the plant signal is most abundant in the root exudates of phosphate deprived plants.

Through a combination of growth, developmental and metabolic responses, plants have developed strategies to sense, cope, and respond to P changes in their growing environment. These strategies aim to reduce usage, and increase uptake and recycling (Rouached, Arpat & Poirier 2010). A strategy utilized by plants for acquiring P in the soil is through symbiotic relationships with arbuscular mycorrhizal (AM) fungi. Smith and Waters (2012), reported that the deployment of this strategy in response to P limitation is mediated in part by strigolactone signalling. In general, strigolactones are carotenoid-derived plant hormones involved in the regulation of plant development i.e., aerial shoot branching, and rhizosphere signalling to stimulate root-AM interactions.

Plants exude strigolactones to attract AM in the rhizosphere to increase P uptake through the root. In turn, AM fungi obtain photosynthates from the host plants (Yoneyama et al., 2012). Considering the complexity of the soil microbiome, there is an enhanced competition for limited available nutrients. As a result of this complexity, it is highly unlikely that strigolactones are the only communication mechanism for plants in response to P starvation.

Plants are top emitters of Volatile Organic Compounds (VOCs). An estimated 1,700 chemical compounds out of over 100,000 chemical products known to be produced by plants are volatiles (Spinelli et al., 2011). These compounds are involved in a range of ecological functions including responding to stress conditions (Holopainen & Gershenzon 2010), defence mechanism (Farnier et al., 2012; Penuelas et al., 2014; Vaughan et al., 2013), signalling and communicating with other organisms in the rhizosphere (Wenke, Kai & Piechulla 2010). Therefore, VOCs are viable options for alternative signals that accumulate in response to P deficiency to ensure plant survival. Therefore, the non-targeted approach adopted in this project will attempt to discover the changes in the whole volatilome profile at different P levels which may be key mediators in biotic interactions, signalling and communication belowground.

Tomato (*Solanum lycopersicum*) is an important vegetable crop worldwide and are known to be high emitters of VOCs. Tomato plant responses to stress conditions such as nutrition, drought, salt, insect pests and the influence of AM fungi on its growth and yield has been well documented (Asensio, Rapparini & Peñuelas 2012; Bai et al. 2018; Catola et al. 2018; Chitarra et al. 2016; Rivero et al. 2018b). Also, the characterization of the volatilome under other conditions have been reported but to the best of our findings, there has been no work done to identify the collective volatilome in response to increasing levels of Phosphorous.

It is our expectation that through this project we will be able to identify changes in the total Volatilome of tomato plant roots at increasing concentrations of P compared to controlled nutrient composition over 7 days of application using Headspace, Solid phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME-GC/MS) technique as described by Rivers et al. (2019).

MATERIALS AND METHODS

We subjected tomato plants to three levels of P nutrition over a times series and adopted an optimized Headspace, Solid phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME-GC/MS) (Fig 2) to identify and quantify VOCs that provided a competitive advantage in P acquisition.

Figure 2. Single quadrupole GC/MSD instrument (Agilent Technologies, Palo Alto, CA, USA) retrofitted with a MPS 2 Gerstel Multipurpose sampler (Gerstel GmbH & Co. KG, Germany).

Briefly the HS-SPME-GC/M is an 8-step process as shown in the schematics (**Fig. 3**). the HS-SPME involves the use of a fibre that is chemically coated with either a solid (sorbent) or a liquid (polymer) adsorption phase to extract both volatile and non-volatile analytes from various liquid or gas phase media. If equilibrium is reached at ambient conditions, the amount of analyte that can be extracted by the fibre will be proportional to analyte concentration in the sample. However, to speed up the extraction time, heat and/or agitation is applied to the sample to induce faster release of the analytes. After extraction, the SPME fibre is transferred to the heated inlet of the Gas Chromatography/Mass Spectrometry for the desorption of the analytes and then the subsequent analysis.

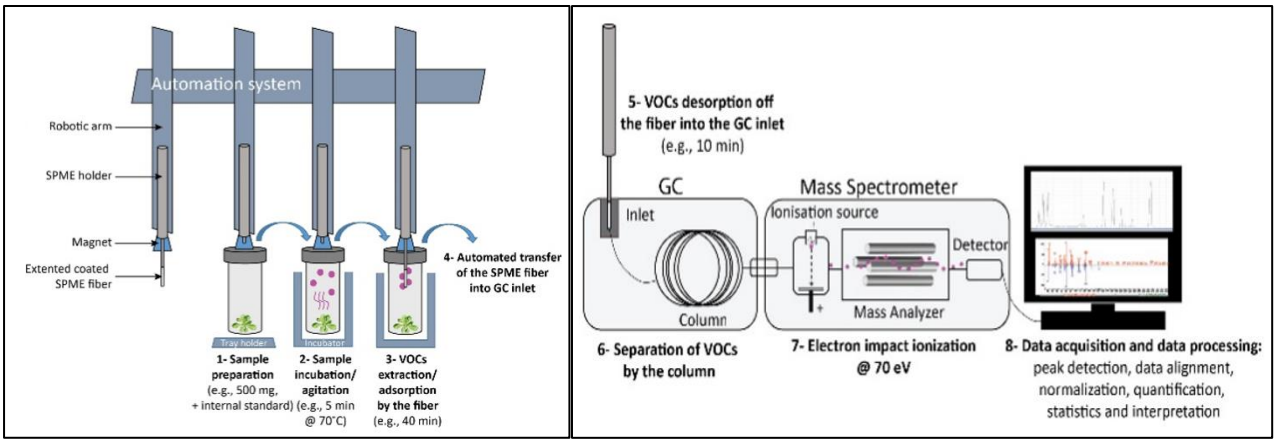

Figure 3. Schematics of HS-SPME – GC/MS technique (Julie Leroux, 2022).

The major advantage of HS-SPME is that the extraction is fast, simple, reproducible, and can often be done without solvents. For certain analytes, the limits of detection (LODs) can reach parts per trillion (ppt or ng/L) trace levels. For this research we adopted an automated HS-SPME sample handling system as shown in B of the image 2 on the right of the screen, the prepared fresh/frozen sample is placed in a sealed HS vial and arranged in the sample tray holder (**Fig. 2B**). The robotic arm of the system grabs the sample vial into the incubator/agitator holder where heat induces the plant to release the volatiles into the headspace of the vial. The SPME fiber is then inserted into the GC inlet for conditioning to release any residual contaminants for 10min at 250°C.

The analytes moving through the GC column, aided by the carrier gas, undergo an optimized temperature program to help separate the analytes according to boiling point and polarity (**Fig. 2C**, step 6). As the analytes enter the ion source of the mass spectrometer, the molecules are captured and undergo the standard GC/MS hard ionization technique known as electron impact ionization (EI). EI involves the production of free electrons from the filament at a constant 70eV (electron volts) to bombard each molecule (molecular ion) to produce characteristic fragmentation ions of low mass-to-charge ratios (m/z). The product ions and molecular ions are then converted and detected as electrical signal by the detector (usually an electron multiplier) (**Fig. 2C**, step 7).

VOC Identification

Untargeted volatilomics aims to identify the whole volatilome (both novel and known VOCs including VAs) in plant samples hence the deconvolution algorithm in the Agilent Masshunter Qulatitative and Quantitative software (**Fig. 4**) was utilized for the separation of overlapping peaks and their respective MS spectrum in the total ion chromatogram (TIC). Each peak MS spectrum is then matched against the NIST mass spectral reference library (**Fig. 5**) and Kovats non-isothermal RI matching (≥70% confidence).

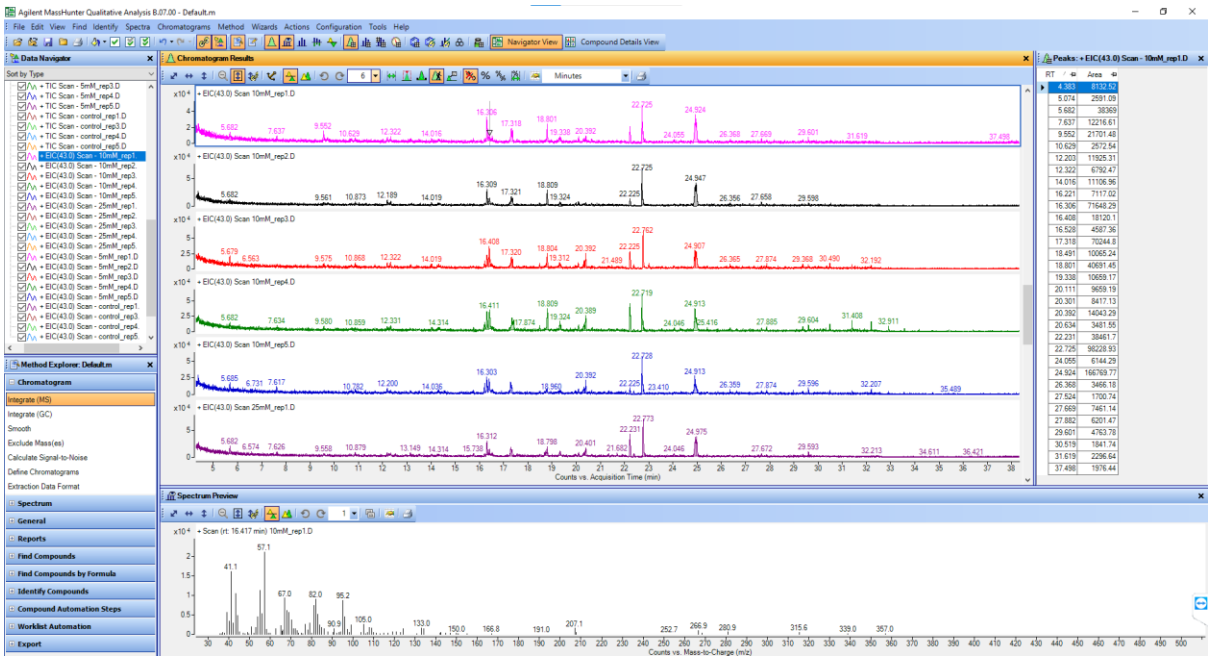

Figure 4. An example of output from Agilent MassHunter Qualitative Analysis Software.

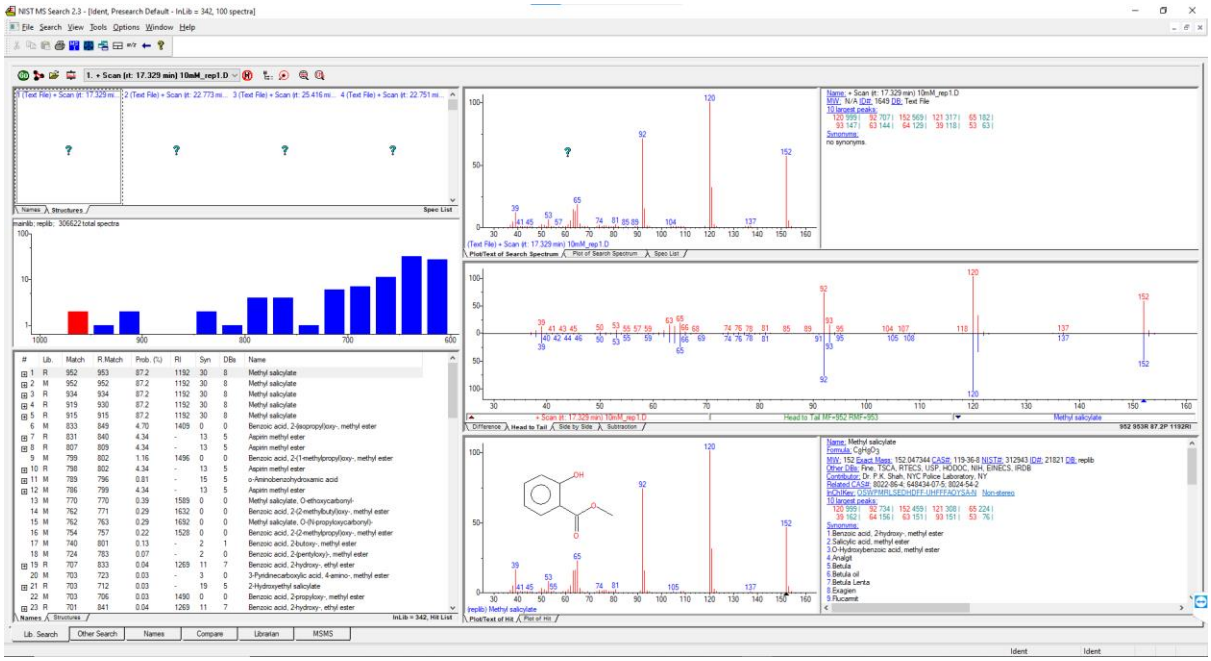

Figure 5. NIST Mass Spectral Library (version 2014).

Quality Control (QC) and Quality Assurance (QA)

QA/QC standards were used as described by Rivers et al. (2019) to maintain the consistency across sequence rounds. The randomisation of samples and standards within statistical 'blocks' were adopted to account for any systemic fluctuations during the SPMS-GC/MS sequence analysis. QC laboratory blanks (empty HS vials exposed to the laboratory environment during sample and standard preparation prior to capping) were also interspersed to check for carry-over contamination between samples, fibre bleed, and to account for other laboratory VOCs.

Statistical Analysis

The relative content of each VOCs obtained directly from GC peak areas and appear as percentage composition (Palá-Paúl et al. 2004). Agilent MSD Chemstation software (version E.02) was used for data acquisition; Agilent MassHunter software (version B.07) was used for data analysis. Statistical analyses were performed using R and Microsoft excel for ANOVA and T-test of significance. For the box plot, the stats were performed using Agricolae package in R where we ran an ANOVA to determine the presence of a significant difference among the treatments and the control followed by an LSD (Least Significant Difference) post hoc test to determine which treatments were significantly different. Identification of VOCs was based on mass spectrometry and the NIST Mass Spectral Library (version 201) was used for mass spectral matching (≥ 70% confidence) and peak annotation. Kovats nonisothermal RIs were calculated for all identified peaks using n-alkanes C9-C22 and compared against scientific literature RIs from the NIST, PubChem and Adams Essential Oils databases was used to determine the chemical composition of the VOCs (Quan & Ding, 2017).

RESULTS AND DISCUSSION

VOCs Changes with P Treatments

We plotted the relative concentrations of each compound eluted from the roots and five distinct regions differentiating the treatments can be seen in the heat map (**Fig. 6**). VOCs such as Eucalyptol, 2-Oxo-1 methyl-3-isopropylapyrazine, Benzene - acetaldehyde, Pyrazine, 2-methoxy-3-(2-methylpropyl), and Butane, 1-chloro-3-methyl has a stronger change or reduction with increasing P levels.

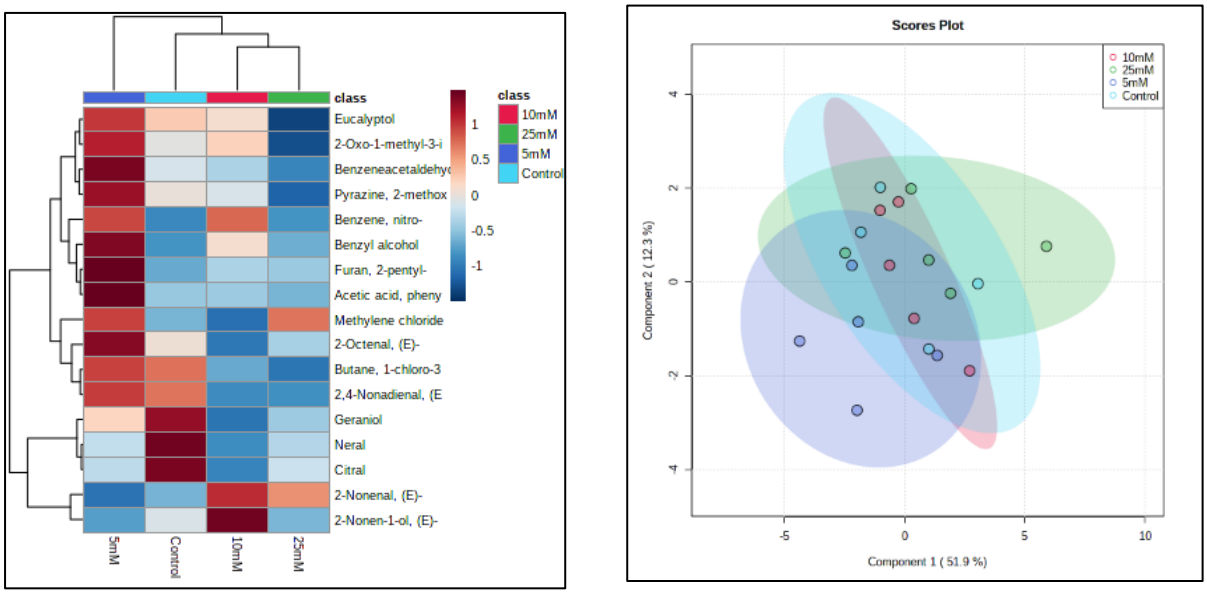

Figure 6. Clustering result shown as heatmap (distance measure using Euclidean, and clustering algorithm using ward.D) (left) and PCA Scores plot between the selected PCs. The explained variances are shown in brackets

It is noteworhty to mention that 2-Nonenal, (E)- and Methylene chloride were eluted in more abundance with increasing P levels. The PCA scores plot were plotted to help identify how the different treatments created a spread in of the VOC compound eluted from the root samples. From our study, we have identified that there are clear differences in the volatilomic profile of the treatments, but when comparing specific VOCs, there were no significant differences to be found for the duration of the treatment. As depicted in the heat map things are very different, the profile is changing however, there is no significant differences between within 4 of the specific volatiles.

Plants have a unique ability to adapt and cope with changing environmental conditions We have potentially looked at the VOCs produced by plants in the soil as a means of determining the efficiency of P fertilization and changes in plant metabolisms to adapt to different levels of availability. The understanding of signalling and communications involved in the intensive exchange of nutrients and metabolites in the rhizosphere is an added layer of information that could be deployed in precision agriculture for site-specific management of production. In this study we have used a broad and untargeted approach to identify those VOCs that have been eluted in P stress conditions and how these would induce a reorganisation of the volatilome to ensure plant survival.

Table 1.Full data for all VOCs identified, including names, Chemical Abstracting Service (CAS) number, molecular formula, molecular weight, observed and NIST Kovats non-isothermal RIs, and NIST forward and reverse matching scores.

#	Compound IUPAC Name	Trivial Name	CAS	PubChem CID	Molecular formula	MW (g/mol)	average RT (min)	Observed RI	NIST RI (Semi-standard non-polar)	NIST Forward Match	NIST Reverse Match	Quantifier ion (m/z)	Biological Class	Subclass	Reference
1	1-chloro-3-methylbutane	Butane, 1-chloro-3-methyl-	107-84-6	7893	$C_5H_{11}Cl$	106	4.63	N/A	693±4 (10)	721	752	70	Amino acid metabolism	Alkane	(Nugroho et al. 2022)
2	Methyl (z)-N-hydroxybenzenecarboximidate	Oxime-methoxy-phenyl-	67160-14-9	9602999	$C_8H_9N_2O$	151	8.40	904	N/A	804	841	133		Acid	Ramani, Krishnaveni and Shalini (2018)
2	Benzaldehyde	Benzaldehyde	100-52-7	240	C_7H_6O	106	10.25	966	962±3 (416)	872	887	106	Benzenoid/ Phenylpropanoid	Aldehyde	(Giridhar, Rajasekaran & Ravishankar 2005)
3	2-pentylfuran	Furan, 2-pentyl-	3777-69-3	19602	$C_9H_{14}O$	138	10.99	991	993±2 (179)	823	873	81	Green Leaf Volatile	Furan	(Tahir et al. 2019)
4	1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane	Eucalyptol	470-82-6	2758	$C_{10}H_{18}O$	154	12.33	1035	1032±2 (580)	727	780	67	Terpenoid	Alcohol	(Sabra et al. 2018)
5	Phenylmethanol	Benzyl alcohol	100-51-6	244	C_7H_8O	108	12.48	1040	1036±4 (174)	907	924	79	Benzenoid/ Phenylpropanoid	Alcohol	(Jaitz et al. 2011)
6	2-phenylacetaldehyde	Benzeneacetaldehyde	122-78-1	998	C_8H_8O	120	12.76	1049	1045±4 (378)	696	844	91	Benzenoid/ Phenylpropanoid	Aldehyde	(Bos et al. 2002)
7	(E)-oct-2-enal	2-Octenal, (E)-	2548-87-0	5283324	$C_8H_{14}O$	126	13.14	1062	1060±3 (124)	869	910	70	Oxylipin/Fatty Acid	Alkane	(Zainol Hilmi, Idris & Mohd Azmil 2019)
8	2-Oxo-1-methyl-3-isopropylpyrazine	2-Oxo-1-methyl-3-isopropylpyrazine	78210-68-1	529437	$C_8H_{12}N_2O$	152	14.02	1090	1225±N/A (1)	808	830	137	Oxylipin/Fatty Acid	Ester	(Gu et al. 2022)
9	(E)-non-2-enal	2-Nonenal, (E)-	18829-56-6	5283335	$C_9H_{16}O$	140	16.21	1164	1162±3 (223)	827	887	41	Green Leaf Volatile	Alkane	(Petropoulos et al. 2014)
10	benzyl acetate	Acetic acid, phenylmethyl ester	140-11-4	8785	$C_9H_{10}O_2$	150	16.31	1167	1164±2 (64)	896	922	108	Benzene Derivatives	Acid	(Piesik et al. 2011)
11	(E)-non-2-en-1-ol	2-Nonen-1-ol, (E)-	31502-14-4	5364941	$C_9H_{18}O$	142	16.41	1170	1176±4 (10)	815	870	57	Green Leaf Volatile	Alcohol	(Knowles & Knowles 2012)
12	2-methoxy-3-(2-Methylpropyl)pyrazine	Pyrazine, 2-methoxy-3-(2-methylpropyl)-	24683-00-9	32594	$C_9H_{14}N_2O$	166	16.64	1178	1183±3 (41)	822	860	124	unclassified		(Ryan et al. 2005)
13	methyl 2-hydroxybenzoate	Methyl salicylate	119-36-8	4133	$C_8H_8O_3$	152	17.38	1203	1192±2 (145)	962	962	120	Benzenoid/ Phenylpropanoid	Acid	(Murungi et al. 2018)
14	(2E,4E)-nona-2,4-dienal	2,4-Nonadienal, (E,E)-	5910-87-2	5283339	$C_9H_{14}O$	138	17.87	1220	1216±4 (99)	705	916	81	Oxylipin/Fatty Acid	Alkane	(Rong et al. 2021)
15	(2E)-3,7-dimethylocta-2,6-dien-1-ol	Neral	106-24-1	637566	$C_{10}H_{18}O$	154	18.41	1239	1255±3 (343)	927	929	69	Terpenes	Alkane	(Lawo et al. 2011)
16	(2Z)-3,7-dimethylocta-2,6-dienal	Geraniol	106-26-3	643779	$C_{10}H_{16}O$	152	18.49	1242	1240±3 (168)	883	892	69	Terpenes	Alcohol	(Vasilev et al. 2014)
17	(2E)-3,7-dimethylocta-2,6-dienal	Citral	5392-40-5	638011	$C_{10}H_{16}O$	152	19.33	1272	1276±N/A (1)	878	921	69	Terpenoid	Alkane	(e Silva et al. 2021)
18	Nitrobenzene	Benzene, nitro-	98-95-3	7416	$C_6H_5NO_2$	123	14.09	1093	1080±15 (14)	934	944	77	Benzenoid/ Phenylpropanoid		(Deb, Roy & Huq 2012)

Several studies have identified the changes in the plant volatilome and its effects on AM – plant interactions under different stress conditions such as salinity and drought (Aroca et al., 2013; Rivero et al. 2018a; Ruiz-Lozano et al., 2016).

Interestingly, the plant – AM association requires a finely regulated molecular dialogue, in which strigolactone (SLs) production – derived from carotenoids are shown to be essential cues for instance SLs production are increased significantly by the in order to maintain the symbiotic association to cope with the stress condition especially in P starvation (López-Ráez et al. 2008). It is important to note that the carotenoid cleavage is a common biosynthetic reaction occurring in the plant biosynthetic pathway, including the production of important plant signalling molecules and our results have identified VOCs that are derivatives from this pathway and changing to P availability.

An interesting observation from our study was the changes we saw in the terpenes Citral, Neral and Geraniol. These compounds were in general showing a decreasing trend with increasing P availability and because there have been scientific debates for these VOCs to be either monoterpenoids or apocarotenoids in their biosynthetic classes, they could very well be apocarotenoids and have been derived from the Carotenoids pathway. This is because

we are seeing a similar linear trend as other apocarotenoids such as transgeranylacetone we saw in our earlier research, regulated from the acyclic upstream carotenoids. This then could be an indication that the plants are trying to feed things through the Carotenoids biosynthetic pathways all the way to strigolactones. Its evidence their pathway could be manipulating in a way that increases the production of the strigolactones needed for the AM symbiotic association. In other words, the strigolactones pathway is being regulated in a way that will feed more carotenoids down to beta carotenes which is a precursor to strigolactones to make sure the plants are able to acquire those high levels of phosphorus nearby.

Furthermore, it seems there may be suboptimal phosphorus changes, it may be that there appears to be specific changes between 5mM and 20mM or 5mM and control treatments and once you get higher concentration you lose that change and, it returns. So, it very well could be that be there is a small steady increase of concentration of whatever P treatment we were applying. We believe the P concentration appears to be changing the pathway or manipulating metabolism in a way that once we go above 5mM it then returns and shifts to another extreme. The heat map maybe suggesting there are optimal levels of phosphorus and once you exceed those optimal levels of P, you will see a different change, this needs to be addressed in future experiment, this potential, and this hypothesis needs to be tested in future trials.

From our results, we have clearly seen that the adoption of the optimised HS-SPME GC-MS provides a high-throughput, sensitive means of identifying and quantifying VOCs and is applicable in detecting the changes in the total volatilome of tomato plants. In our study, we observed a change in the entire volatilome of the plants under different treatments although no significant differences were observed within the specific VOCs identified. We believe that this in part, could be due to the age of the tissues in our experiment. McQuinn and Leroux et al (unpublished) have reported an elevated levels of volatiles and a much more dynamic profile in developing buds versus mature or fully developed tissues. Therefore, we believe future studies would be improved by observing the changes in younger tissues and collected at different growth stages.

CONCLUSION

As P is a limited resource the ability to use VOCs as a component to understand the interaction in the rhizosphere whilst providing important information on changes in the biosynthetic pathway that may enhance or decrease the signalling and communication systems. We have seen that the adoption of a fast, sensitive, and high throughput technique such as the optimised HS-SPME GC-MS technique has provided with an insight of the manipulation of volatilomic pathways by plants in adapting to changes in their environment. This is the first time the technique has been adopted for Tomato roots volatilomic studies, but we believe it is applicable to other tissues and species. In summary, we have seen that the plant is making sacrifices to improve the required signals for AM symbiotic associations in limited P conditions.

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