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Extraction and Analysis of Volatiles from Flowers of the Orchid Genus *Pleurothallis*

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1. Abstract

Previous research conducted on the micromorphology of *Pleurothallis* in the family Orchidaceae suggests that some *Pleurothallis* species may be pollinated by pseudocopulation. These species' labellar structure is similar to that of another orchid genus pollinated by pseudocopulation, *Ophrys.* We hypothesize that flowers of these species produce floral volatiles similar to insect pheromones to attract the pollinator. The aim of our study was to utilize pre-determined optimized sample preparation and GC-MS analysis parameters for analyzing floral volatiles (March 2020), to analyze unknown volatile samples from *Pleurothallis* flowers. Chromatograph data was inputted to MiniTab and used to generate dendrograms of different species based on their volatile composition. Most notably, hypothesized deceptive species grouped separately from hypothesized rewarding species, and samples from different plants of the same species grouped together. An analysis of chromatograph peaks was also done to identify possible allomones. Promising volatiles that are known insect attractants were discovered and confirmed. These volatiles were: (Z)-9-Tricosene, 4-hydroxy-4-methyl-2-pentanone, Hexadecanoic acid ethyl ester, and (Z)-7-Pentacosene.

2. Introduction

The family Orchidaceae is composed of flowering plants whose methods of reproduction include both reward and deceit pollination. In the first method, the pollinator is rewarded by nectar or oils produced by the flower. The plants that utilize the second method do not produce any reward, but rather attract pollinators through mimicry of reward flowers or potential mates.[1] This is evolutionarily beneficial for the plant as it does not expend extra energy producing nectar or oils. *Ophrys*, a genus of flower within the family Orchidaceae, has been discovered to attract pollinators by a tactic called pseudocopulation.[2] Pseudocopulation pollination is a form of deceit pollination in which the pollinator is sexually attracted to and tries to mate with the flower. The flower's morphology and the pheromone-like chemicals, or allomones, it releases to mimic a potential mate attract the pollinator, and as it attempts to mate with the flower, pollinia become attached to the insect.[1] These pollinia are then transferred to other *Ophrys* plants as the insect travels from flower to flower. The modifications to the appearance and volatiles of a species of flower are often attractant to a specific species of insect. Some *Ophrys* species attract bees, others attract flies, and some even attract spiders.[2] *Pleurothallis* is another genus of Orchidaceae we hypothesize to include species that are pollinated by pseudocopulation. Dr. Mark Wilson et al. previously conducted research in which they hypothesized that the labellar micromorphology of the *Pleurothallis* flower is consistent with that of *Ophrys*.[3] They analyzed the labella of multiple species of *Pleurothallis* for secretory tissue or cavities that may be involved in pseudocopulation. The presence or absence of these tissues was used to infer possible pollination mechanisms.[1] We aim to test the hypothesis that some species of *Pleurothallis* emit allomones consistent with those excreted by pseudocopulatory flowers. Floral volatiles containing known allomones, together with labellar micromorphology, would be further support for the hypothesized pollination by pseudocopulation.

3. Method or Experimental

Sample Collection Procedure:
Flowers were sampled after blooming and submerged in approximately 2 mL of ethanol. Samples in ethanol were stored at -80ºC before being prepared for GC-MS analysis. To prepare samples for the GC-MS, between 23.63 mg and 31.97 mg anhydrous magnesium sulfate was added to 2 mL of ethanol collected from the flower sample vial. Ethanol samples were allowed to settle at room temperature in 2 mL screw-top vials with the anhydrous salt for 48 hours. At least 0.5 mL of the ethanol was then transferred to a crimp-top GC-MS vial and placed in the auto-sampler of the GC-MS. One mg/mL ethanol solutions of standards of promising volatile findings related to insect pheromones were also prepared.
Gas Chromatography-Mass Spectrometry Procedure:
An Agilent 5975 C Mass Spectrometer and Agilent 7890 A Gas Chromatograph with 30 m x .25 mm and .25 µm diameter column and helium carrier gas were used to analyze volatile samples. An optimized ORCHID method previously determined in March of 2020 was run on various experimental samples as well as chemical standards.[4]
ORCHID Method:
The GC-MS temperature ramp was programmed from 60ºC (2-minute holding) to 290ºC at a rate of 10ºC/minute, with an injector port temperature of 250ºC. A 5 µL injection was run in splitless mode. There were washes of 3 µL Solvent A isooctane and 3 µL Solvent B acetone with a 3.5 min solvent delay.
Minitab Procedure:
Minitab Statistical Software was used to compare chromatograph data collected from experimental samples by generating correlation matrices, cluster analyses of variables, and principle component analyses. For each desired comparison, raw chromatograph data from the samples was compiled in an excel sheet and combined. The combined data were then opened in Minitab and each chromatogram was standardized by subtracting the mean and dividing by the standard deviation. This controlled for any differences in peak abundance, assuring that any differences found would be from peak pattern. This was done as abundance may differ as a result of variables we are not interested in exploring yet such as sample collection time and time stored after collection. Next, to make the data easier to work with in Minitab, the standardized data was transferred back to excel and any values that were at or below the baseline of the chromatographs were manually deleted. This was only done for a few comparisons as it proved to be time consuming and did not impact our results significantly. Finally, standardized data were used to produce our three modes of comparison: correlation matrices, cluster analyses of variables, and principle component analyses.

A spearman correlation was used to measure the linear relationship between the samples. This was done with two samples at a time, generating a number between negative one and one. The closer to negative one or one the value was, the more correlated the two samples. Values generated for all the samples were compiled into a table.
A cluster analysis of variables uses the numerical distance between two variables to calculate their similarity. All the variables can then be grouped on a dendrogram based on their similarity to each other. In our case, the variables were our different *Pleurothallis* samples. The closer together these samples were grouped, the more similar they were calculated to be.
Lastly, a principle component analysis assigns the largest amount of variance in the data to the smallest number of principal components possible. Each principle component represents a differentiating variable in the data. In our case, the first three principal components usually accounted for over 90% of the variance in the data. Each principle component generated a column of values associated with every inputted retention time. The more important a retention time was for differentiating between data sets, the higher the absolute numerical value of the principle component. After the retention times responsible for the most variance were identified, they were then used to identify important differentiating peaks in the GC-MS spectra. These peaks were analyzed to determine possible chemical compounds important for differentiating between deceptive and rewarding *Pleurothallis* species.

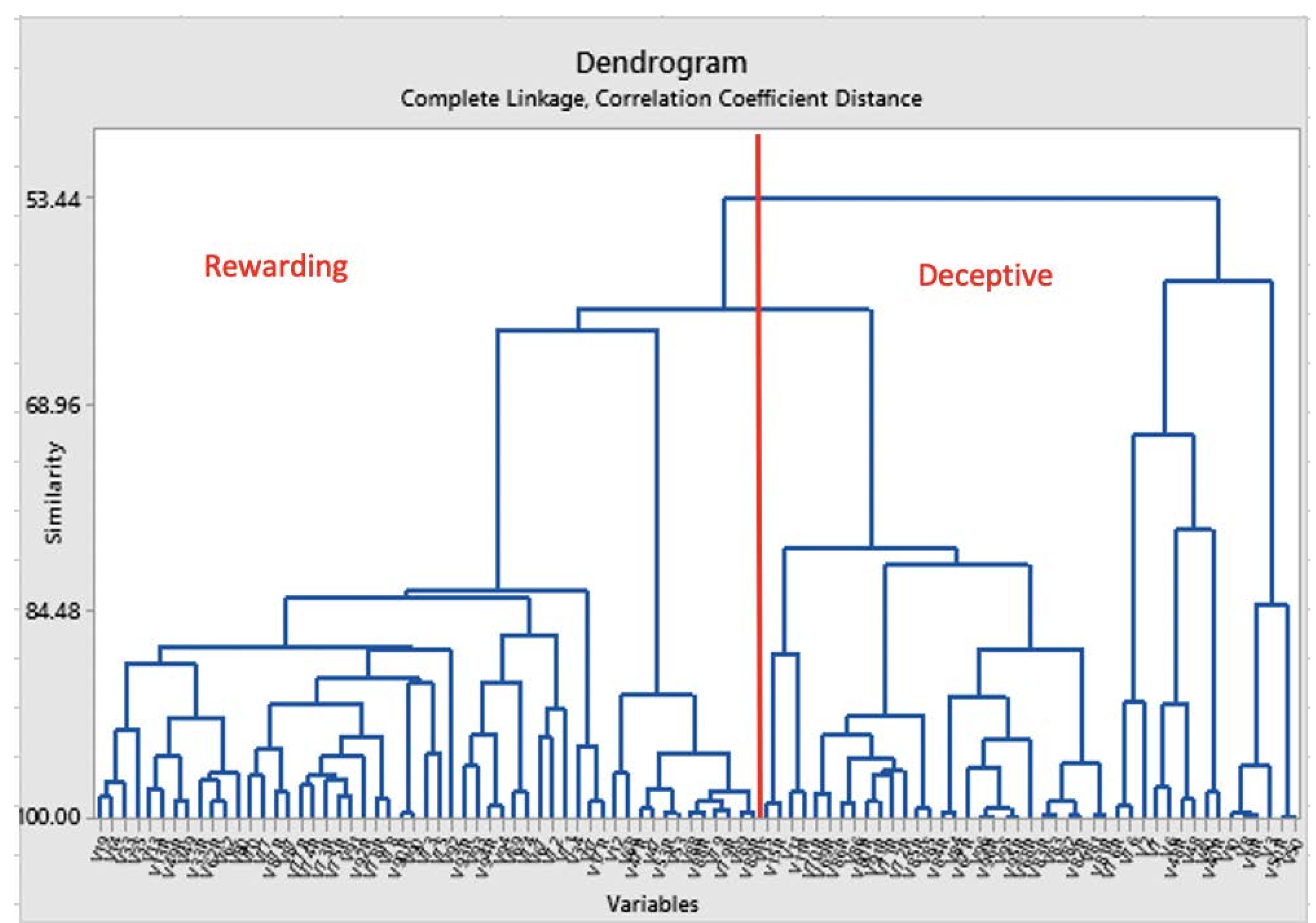

Figure 1. Dendrogram of all experimental samples run summer of 2020.

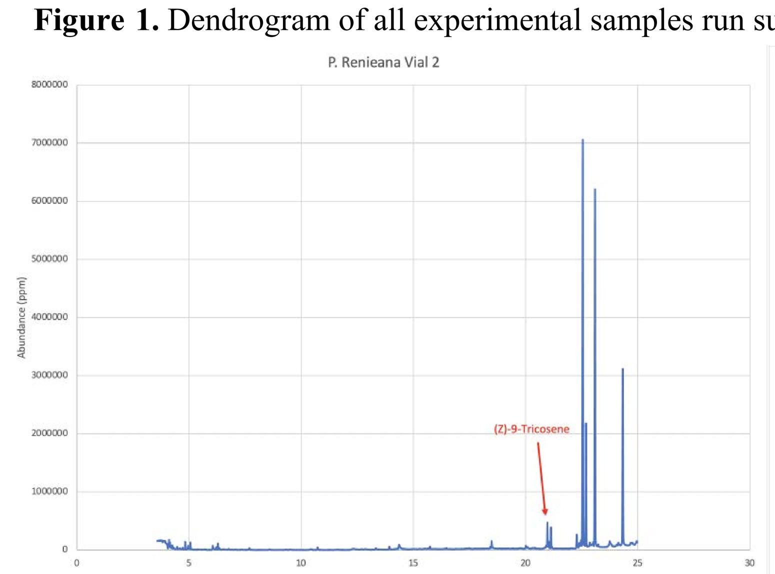

Figure 2. Chromatogram from hypothesized deceptive species *P. renieana*.

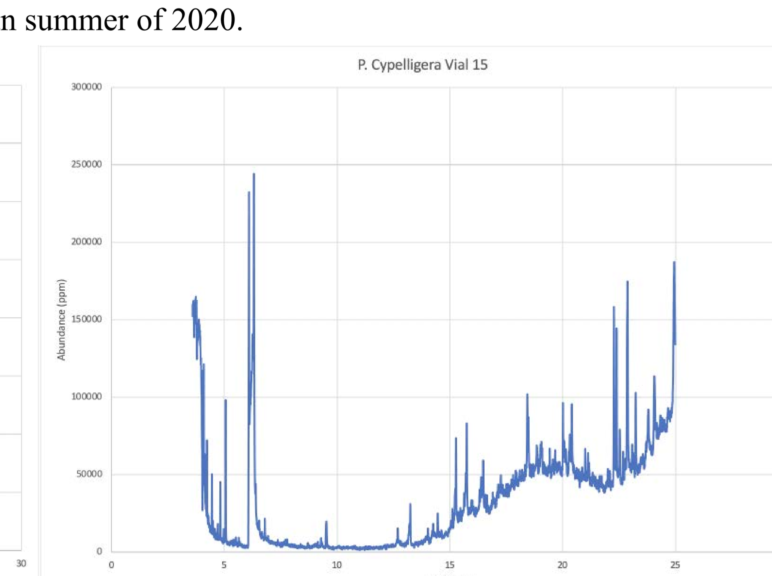

Figure 3. Chromatogram from rewarding species *P. cypelligera.*

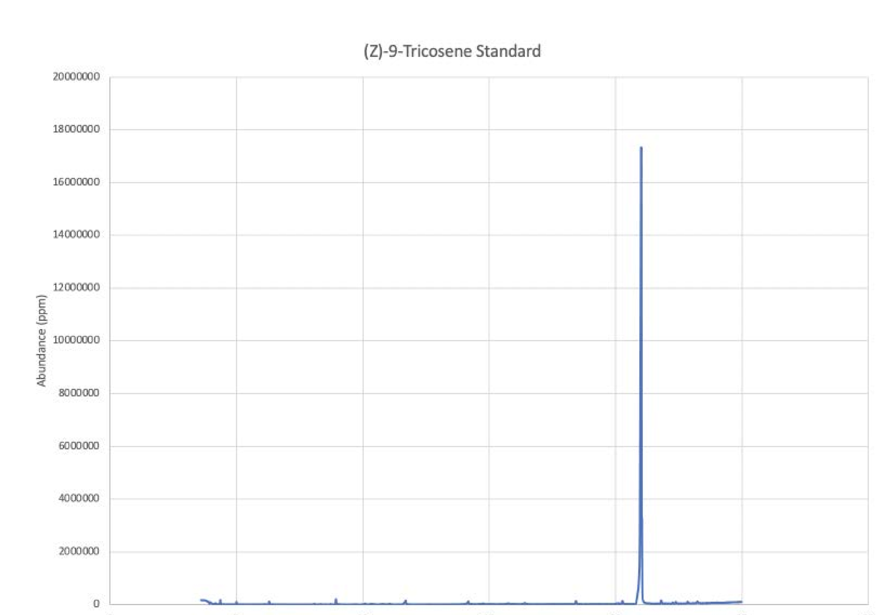

Figure 4. Chromatogram from (Z)-9-Tricosene standard.

4. Results

Dendrograms and correlation matrices generated using Minitab have shown that hypothesized deceptive species group separately from rewarding species and different flowers of the same species grouped together. Chromatogram peaks from hypothesized deceptive species were identified as known insect attractants and verified using chemical standards. The separation between rewarding and deceptive species in dendrograms and correlation matrices shows that those species are not chemically similar. Figure 1 is a dendrogram of every sample that was run, to the left of the line are rewarding species and to the right hypothesized deceptive species. The hypothesized deceptive species grouping separately from the rewarding species bolsters the argument that they do not produce a reward and may be pseudocopulatory. The fact that samples from different flowers of the same species grouped together on these dendrograms as well allows us to be confident in the reliability of these results. Chemical differences between deceptive and rewarding species are also visible when comparing chromatograms. Figure 2 shows a chromatogram from a deceptive species and Figure 3 is a chromatogram from a rewarding species. Deceptive chromatograms were always larger in scale, meaning the volatiles from those samples were present in higher abundance. This caused those chromatograms to appear smooth with a few distinct tall peaks, while rewarding chromatograms appear jagged with many peaks of similar heights. The verified insect attractants found in hypothesized deceptive samples also bolster our argument that these species may be pseudocopulatory. Chromatograms from each of the deceptive samples were analyzed using GC-MS computer software and the NIST chemical database. Any prominent peaks were identified by matching the generated mass spectra to mass spectra in the database. Next a search of all identified chemicals was conducted to determine if they had been previously identified as insect attractants. Standards of chemicals identified as known attractants were then purchased to be run in the GC-MS. If the retention times and mass spectra of these standards matched the peaks from the deceptive sample chromatograms, we were able to say with certainty that that chemical was present. Identified volatiles include: (Z)-9-Tricosene, 4-hydroxy-4-methyl-2-pentanone, Hexadecanoic acid ethyl ester, and (Z)-7-Pentacosene. (Z)-9-Tricosene was the first volatile identified and the most intriguing as it is better known as muscalure, a common chemical used in fly traps as it is a sex pheromone released by female house flies.[5] Figure 2 shows a (Z)-9-Tricosene peak present in a hypothesized deceptive species, and Figure 4 shows the chromatogram of the (Z)-9-Tricosene standard that was used to verify that peak.

5. Conclusions

We are unable to state with certainty that the hypothesized deceptive flowers are pseudocopulatory until pseudocopulation has been observed in nature and recorded. However, the presence of known insect attractants in these flowers and their chemical dissimilarity to known rewarding species bolsters our argument that some species in the genus *Pleurothallis* may be pseudocopulatory.

6. Possible Future Direction

Further in-depth data analysis must be performed to establish more patterns amongst the species. Preliminary patterns that should be explored further include: chemical dissimilarity amongst hypothesized deceptive species and the impact of time after blooming on chemical composition of the flower. The first pattern if established would not be surprising as the modifications to the volatiles of a species of flower are often attractant to a specific species of insect. Based on geography, it is not unlikely that these species attract different insect species. The second pattern, if established, could impact our sampling method as we would attempt to establish a time after blooming at which the volatile composition of the flower is optimized for attracting the insect. Lastly, other possible insect attractants have been identified from deceptive sample chromatogram data. Standards of these volatiles should be run in order to verify those matches.

7. Acknowledgements

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8. References

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