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Semiochemicals from ex Situ Abiotically Stressed Cactus Tissue: A Contributing Role of Fungal Spores?

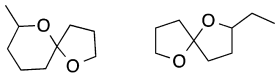
ABSTRACT: Semiochemicals play a central role in communication between plants and insects, such as signaling the location of a suitable host. Fungi on host plants can also play an influential role in communicating certain plant vulnerabilities to an insect. The spiroketal conophthorin is an important semiochemical produced by developing fungal spores. Spiroketals are also used as signals for scolytid communication. Plants and fungi are known to emit varying volatile profiles under biotic and abiotic stress. This paper reports distinctive temporal-volatile profiles from three abiotic treatments, room temperature (control), −15 °C (cold), and −15 °C to room temperature (shock), of cactus tissue plugs. Volatiles from the three treatments included monoterpenes from control plugs, compounds of varying classes and origin at later stages for cold plugs, and known semiochemicals, including spiroketals, at later stages for shock plugs. The results highlight several important findings: a unique tissue source of the spiroketals; abiotic cold-shock stress is indicated as the cause of spiroketal production; and, given previous findings of spirogenesis, fungal spore involvement is a probable biosynthetic origin of the spiroketals. These findings suggest an important role of fungal volatiles as signaling plant vulnerability to insects.

KEYWORDS: *chalcogran, conophthorin, host plant volatiles, Opuntia humifusa, plant−insect−microbe interaction, spiroketal*

INTRODUCTION

Volatiles play an important role in communication between plants, insects, and microbes. Plants, including agricultural crops, emit volatiles that insect herbivores and parasitoids use to locate food or ovipositional sites, among others.[1−3] Plants produce diverse volatile profiles in response to numerous and complex biotic or abiotic stressors, including herbivory, microbes, temperature, or drought.[4−8] In particular, the interactions between plants and microbes and the resultant volatile emissions from either the plant, the microbe, or both are of interest for the production of insect semiochemicals.[3,9,10]

Recent investigations for controlling the insect pest navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae), have identified the spiroketals conophthorin (**1**) and chalcogran (**2**) as important semiochemicals produced by fungal spores (Figure 1).[11,12] These spiroketals have also been reported as



1 **2**

Figure 1. Structures of the spiroketals conophthorin, **1**, and chalcogran, **2**.

semiochemicals of insect pests such as scolytid bark beetles (Coleoptera)[13] or more recently the scolytid coffee berry borer (Coleoptera).[14]

During a recent investigation of *Opuntia humifusa* cactus tissue, our laboratory unexpectedly detected the presence of both conophthorin (**1**) and chalcogran (**2**). The cactus tissue samples that produced the spiroketals had been shipped overnight in individual containers stored over ice. Attempts to reproduce this result from *O. humifusa* tissue from plants grown in our laboratory and analyzed at room temperature failed. Consideration of conditions between the two experiments led to investigation of the effect of temperature on the plant tissue. Freezing temperatures are an abiotic stressor that influence the cellular integrity of plant tissue matrices[15] and thus may have an impact on volatile emissions. However, the effect of freezing temperatures on fungi or fungal spores has been less studied. The results obtained and reported herein describe the unique source and conditions for the production of spiroketals. Additionally, the role of cold stress on either the tissue or fungal spores may provide insight into a unique plant−insect−microbe interaction.

MATERIALS AND METHODS

Plant Material. Plugs were taken from potted prickly pear cactus (*O. humifusa*) pads. Three plugs were removed from the center of five different cactus pads (three treatments, five replicates), approximately 112 cm^2 in area and 2 cm thickness, using a sterilized (95% EtOH and oven-dried) 9 mm cork borer. Using a sterilized glass stir rod, plugs were gently pushed from the cork borer into 4 mL scintillation vials (Wheaton, Millville, NJ, USA) with a Teflon-lined septum cap. Headspace analyses were performed immediately after the vials were sealed and then subjected to the treatment temperatures. The room temperature (control) treatment replicates were sampled on day 0; day 2 (48 h closed system), vented and resealed; day 7 (120 h closed system), vented and resealed; and day 14 (168 h closed system). The plugs to be shocked were sealed, sampled, placed in a freezer set at −15 °C for 23 h, allowed to warm to room temperature for 1 h, analyzed (24 h closed system), and then sampled on day 3 (48 h closed system), vented and resealed; day 8 (120 h closed system), vented and resealed; and day 15 (168 h closed system). Once removed from the freezer after the cold shock, the replicates for this treatment remained at room temperature. The cold plugs were placed in the freezer at −15 °C and were analyzed on the same schedule as the shock treatments, although they remained at −15 °C for the duration of the experiment.

Collection and Analysis of Volatiles. The headspace volatiles were adsorbed onto PDMS SPME fibers (Supelco, Bellefonte, PA, USA) from the sealed jars containing the cactus tissue plugs and at the intervals described above. The SPME fibers were exposed to the volatiles for 30 min at room temperature for the control and shock treatments and at −15 °C for the cold treatments. All treatments were

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Table 1. Volatiles Detected at Day 3, 8, or 15 from ex Situ Cactus Plugs by HS-SPME over the 2 Week Period and from the Three Abiotic Treatments

		detected in treatment[a]								
		day 3			day 8			day 15		
compound identity	RI[b]	Δ	∫	≈	Δ	∫	≈	Δ	∫	≈
α-pinene[c]	1019		X		X	X		X	X	
α-fenchene[c,d]	1052	X			X			X	X	
camphene[c]	1058	X		X				X	X	
hexanal	1076		X			X			X	
isobutyl alcohol[d]	1095			X			X			X
β-pinene	1106		X			X			X	
limonene	1197		X			X			X	
3-methyl-1-butanol	1209			X			X			X
3-octanone	1251			X						
p-cymene[c]	1265		X		X	X		X	X	
cyclohexanone[d]	1285					X			X	
conophthorin	1286						X			X
unknown	1300					X			X	
6-methyl-5-heptene-2-one	1334			X		X			X	
(Z)-chalcogran	1348						X			X
(E)-chalcogran	1353						X			X
unknown sesquiterpene	1356						X			X
unknown	1368						X			X
nonanal	1391					X			X	
3-hepten-1-yl acetate[d]	1435			X			X			
1-octen-3-ol	1451			X			X			
2-ethyl-1-hexanol	1491		X	X		X	X	X	X	X
benzaldehyde	1515		X	X	X				X	
guaiacol[d]	1853			X			X			X
2-phenylethanol	1907			X			X			X

[a]Key: Δ = control (room temperature); ∫ = −15 °C; ≈ = −15 °C to room temperature; control samples were monitored on days 2, 7, and 14. [b]Retention indices relative to *n*-alkanes on DB-Wax column for compound identification including comparison of retention times and mass fragmentation to authentic samples. [c]Volatiles detected at day 0 in all treatments. [d]Tentatively identified.

performed in five replicates. Collected volatiles were analyzed using a method identical to previously published protocols[9] on a DB-Wax column, 60 m × 0.32 mm i.d. × 0.25 μm (J&W Scientific, Folsom, CA, USA) installed on a 6890 gas chromatograph coupled to an HP-5973 mass selective detector (Agilent, Palo Alto, CA, USA). Retention index (RI) values were calculated using a homologous series of *n*-alkanes on the DB-Wax column. The identities of the reported volatiles were determined by comparison of fragmentation patterns to NIST and Wiley databases, as well as verified by injection of authentic samples and comparison of retention times and fragmentation patterns. Sources of compounds for GC-MS authentication were identical to those in a recent publication.[16] Compounds not verified by authentication are listed as tentative in Table 1.

Statistical Analysis. Volatiles were only considered for analysis if they were detected within three of the five replicates at any time point. Relative peak areas were log transformed. Principal component analyses (PCA) were performed on the data using BioNumerics 4.6 (Applied Maths, Austin, TX, USA) where the three treatments (five replicates per treatment, $n = 15$) were compared at each of the three time points.

RESULTS AND DISCUSSION

The volatiles α-pinene, α-fenchene, camphene, and *p*-cymene were detected from the cactus tissue samples in all treatments at day 0 (Table 1). The compounds α-pinene, β-pinene, and *p*-cymene have been reported from the headspace of intact *Opuntia stricta*;[17] however, the previously reported cymene was the meta-isomer, whereas we detected and report the para-isomer. Additionally, we report the monoterpene camphene as a component of *O. humifusa* plugs, whereas camphene was not detected in the previous study of *O. stricta*. In an additional note regarding volatiles from cacti, Flath et al.[18] report numerous compounds of various classes from their hydrodistillation of prickly pear fruit. However, the differences of method extraction and tissue studied[18] do not allow for comparison to either the present study or the headspace analysis performed by Pophof et al.[17]

PCA showed three distinct clusters representing the three treatments (control, cold, and shock) at days 3 and 8, respectively (Figure 2A,B), thus supporting the overall difference in volatile emissions from the tissues as shown in Table 1. From the PCA for day 3, the volatiles α-pinene, hexanal, β-pinene, and limonene in the cold treatment and 3-octanone, 3-hepten-1-yl acetate, and 1-octen-3-ol in the shock treatment were most responsible for the distinct clustering of the shock (red ellipse) and cold (blue ellipse) treatments (Figure 2A).

The volatiles 3-octanone and 1-octen-3-ol on day 3 from the shock tissue are characteristic of fungal metabolism of fatty acids,[9,19] as well as the volatiles 3-hepten-1-yl acetate and 2-ethyl-1-hexanol. Both of these latter volatiles have reported plant origins,[20,21] but the compound 2-ethyl-1-hexanol is also thought to be a contaminant.[22] Whereas we found no reported semiochemical activity of either 3-hepten-1-yl acetate or 2-ethyl-1-hexanol, it should be noted that 3-octanone and 1-octen-3-ol elicited high electrophysiological responses from both male and female *Amyelois transitella* (Lepidoptera: Pyralidae) moth antennae.[16]

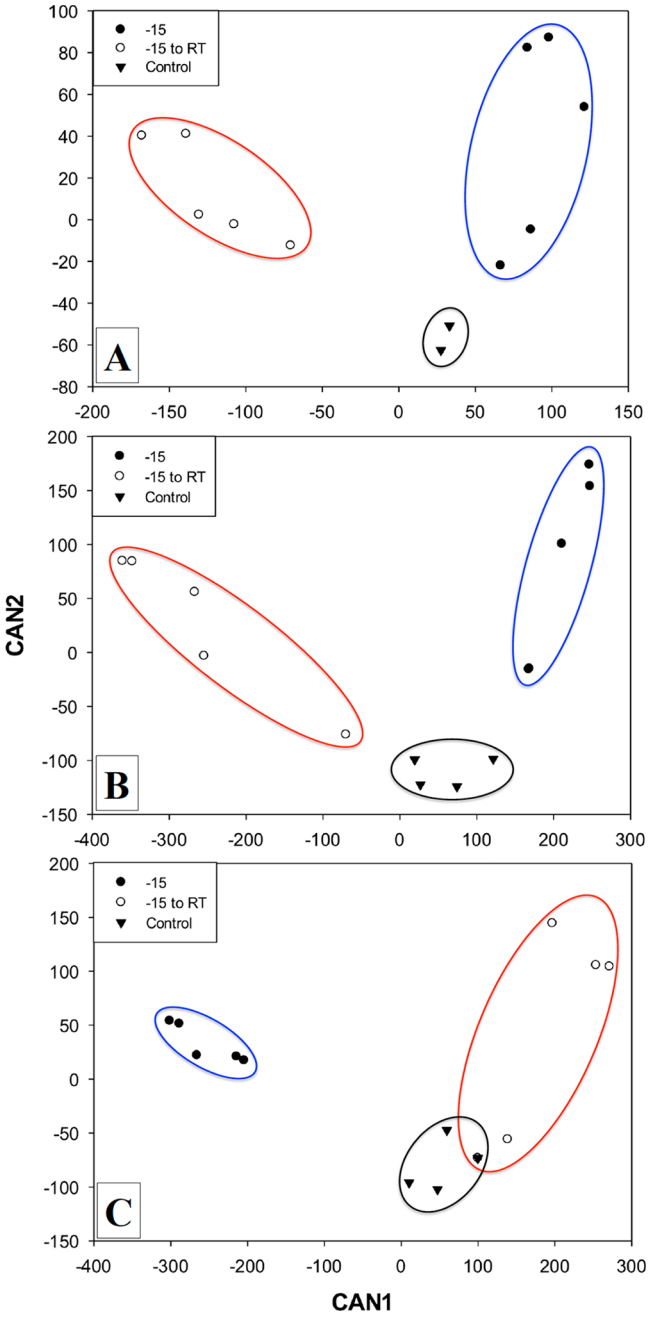

Figure 2. Principal component analysis of data from the five replicates of all treatments at (A) day 3, (B) day 8, and (C) day 15. Control treatments are shown in black ellipses, cold treatments in blue ellipses, and shock treatments in red ellipses.

The shock and cold treatments at day 8 produced a number of new compounds not found in the control samples (Figure 2B and Table 1). The volatile composition of the cold treatment cactus tissue (blue ellipse) is suggestive of tissue stress with detection of hexanal, a fatty acid breakdown product,[5,23] as well as a sudden increase in monoterpenes, such as β-pinene, limonene, and *p*-cymene, which are not seen in the control or shock treatments. This latter phenomenon may contribute to the plants' production of certain terpenoids as a defense mechanism against oxidation,[4] as well as to the inhibition of fungal germination.[24] An interesting finding was the detection of benzaldehyde (days 8 and 15) and nonanal (day 15) in the later days of cold treatment. These aldehydes, among other compounds, have been reported as fungistatics produced by plant bacteria,[25] although the reason for their presence in this tissue and this particular treatment is presently unknown. Therefore, the increase in volatiles with these notable activities in the cold treatment at day 8 warrants further investigation regarding the role of extended cold on plant tissue volatile emissions.

The shock tissue at day 8 produced a diverse range of compounds (Table 1) not detected in the control and cold tissue treatment and resulted in the separation of the shock treatment (red ellipse) relative to the other treatments as seen in the PCA (Figure 2B). The most striking and a primary reason for this paper was the production of a number of compounds that have elicited noteworthy electrophysiological and behavioral responses in other lepidopteran[11,16] and coleopteran[13,14] insects. Most notable among these were the spiroketals conophthorin (**1**) and chalcogran (**2**), the origin of which has been directly associated with developing fungal spores.[9]

Aside from guaiacol, the volatiles from the day 8 shock treatment have associations with fungi and damaged tissue,[9,26] semiochemical activity in Coleoptera,[27] and elicition of electrophysiological responses from navel orangeworm moth antennae.[16] In particular, the volatile 2-phenylethanol elicited a very strong response from male navel orangeworm antennae (Lepidoptera).[16] Additionally, 2-phenylethanol has been reported as a yeast-produced volatile that inhibited aflatoxin-producing genes in the fungus *Aspergillus flavus*,[28] thus raising a possible secondary role for signaling among fungi. Furthermore, a recent report[29] described the semiochemical activity of conophthorin for the ambrosia beetle (Coleoptera) and the emission of conophthorin from stressed and weakened deciduous tree hosts. Given our findings[9,10] concerning the apparent spirogenesis from fungal spores, it would be of interest to determine if the condition of the tree and the beetle's attraction to it are a result of fungal emissions.

Conspicuously absent from the day 8 and 15 shock treatment volatiles were the monoterpenes, which have been implicated in antifungal activity.[24,25] The differences in volatile profiles of the shock and cold treatments raise numerous questions regarding the effect of cold on ex situ plant tissue, either as a preservation and defensive mechanism (sustained cold) or as brief instance (tissue shock). These differences also suggest a subsequent effect on the microbial community within the plant tissue and a consequential effect toward possible insect−microbe mutualisms.

The results described in this paper address a number of important issues in several ongoing investigations: (1) the identification of potential semiochemicals from *O. humifusa* of the insect pest cactus moth (*Cactoblastis cactorum*), a highly invasive pest that has spread across the southeastern portions of the United States, threatening native *Opuntia* species that are important agricultural, ecological, and ornamental plants;[30] (2) the role of abiotic stress on plant tissue, its resident microbes, and the resultant volatiles in either insect−microbe or microbe−microbe interactions; and (3) the expanding role of the spiroketals conophthorin and chalcogran in the possible mutualism between certain insects and fungal spores. The results also highlight several important new findings and include a unique tissue source of the spiroketals; the implication of abiotic cold-shock stress as a trigger for spiroketal production; and, given our previous findings of spirogenesis,[9,10] the possibility of spore involvement as a

biosynthetic origin of the spiroketals. Likewise, these findings have important implications regarding fungal volatile emissions when plants may be vulnerable to insect pests.

John J. Beck*[,†]
Nausheena Baig[†]
Daniel Cook[‡]
Noreen E. Mahoney[†]
Travis D. Marsico[§]

[†]Foodborne Toxin Detection and Prevention, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710, United States

[‡]Poisonous Plant Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 1150 East 1400 North, Logan, Utah 84341, United States

[§]Department of Biological Sciences, Arkansas State University, State University, Arkansas 72467, United States

AUTHOR INFORMATION

Corresponding Author

*(J.J.B.) Phone: (510) 559-6154. Fax: (510) 559-6493. E-mail: john.beck@ars.usda.gov.

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Notes

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