Volatiles from *Fusarium verticillioides* (Sacc.) Nirenb. and Their Attractiveness to Nitidulid Beetles

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It is known that sap beetles (Coleoptera: Nitidulidae) can vector the fungus *Fusarium verticillioides* (Sacc.) Nirenb. (= *F. moniliforme* Sheldon), which causes an important ear-rot disease in corn and also produces fumonisin mycotoxins. The volatiles produced by this fungus were studied to establish whether they could attract sap beetles. Such an association would suggest more than just an incidental role in transmission of the fungus by the beetles. *F. verticillioides* consistently produces a blend of five alcohols (ethanol, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol), acetaldehyde, and ethyl acetate. Ethanol is the most abundant alcohol. The fungus also produces four phenolic compounds (the most abundant of which is ethylguaiacol), a series of presently unidentified sesquiterpene hydrocarbons, and an unidentified compound that is probably a 10-carbon ketone. Solid-phase microextraction was the key technique used in volatile analysis. The volatile profiles change over time and differ somewhat among fungal strains: The alcohols, aldehyde, and ester always appeared first and were present for each strain. Production of the phenolics lagged by several days, and in some strains these compounds were barely detectable. Volatile production eventually diminished in all strains. All strains were attractive to the sap beetle, *Carpophilus humeralis* (F.), in wind-tunnel bioassays. Attraction was correlated primarily to the presence of the alcohols, acetaldehyde, and ethyl acetate, rather than to the phenolics. To verify that the identified culture volatiles were responsible for beetle attraction, cultures were quantitatively simulated with synthetic chemicals, and the cultures and corresponding synthetic mixtures were then compared by bioassay. The comparisons were favorable. Volatile emission patterns from cultures were fairly robust with respect to inoculum level or incubation temperature, but some manipulation was possible. For example, after freeze-drying and rehydrating (a rapid simulation of winter/spring conditions), *F. verticillioides* produced ethyl acetate and other esters at unusually high levels. The fungus produced attractive volatiles following ear inoculation of milk-stage field corn as well as on sterile, mature kernels in the laboratory.

Keywords: *Fusarium verticillioides; F. moniliforme; Coleoptera; Nitidulidae; Carpophilus humeralis; solid-phase microextraction; SPME; attraction; bioassay; volatile compounds*

INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenb. (synonym = *Fusarium moniliforme* Sheldon) causes a widespread disease of maize ears known as "*Fusarium* kernel or ear rot" in which a powdery or cottony-pink mold growth develops on kernels damaged by corn insects or on individual infected kernels scattered over the ear (Shurtleff, 1984). In their study of the molecular systematics of the *Gibberella fujikuroi* complex, O'Donnell et al. (1998) rejected the name *F. moniliforme* because it has always been applied to a multitude of phylogenetically distinct species (Booth, 1971; Nelson et al., 1983) and because it is a later synonym of *F. verticillioides*. Importantly, corn isolates of *F. verticillioides* have been shown to produce the mycotoxin fumonisin B_1 (Thiel et al., 1991; Leslie et al., 1992). The fumonisins are a complex of compounds that have been linked to diseases in certain animals and may play a role in human cancer etiology [reviewed by Munkvold and Desjardins (1997)]. Infection of developing kernels by *Fusarium* can occur in a number of ways (Munkvold et al., 1997), including systemically from the seed and by infection through the silks. Insects can be involved in the infection process, and sap beetles (Coleoptera: Nitidulidae) have been implicated as vectors (Windels et al., 1976).

In certain fungi, the likelihood of being vectored by insects is enhanced by the production of chemicals that are attractive to the potential vectors, and a number of fungi are known to emit volatiles that are attractive to sap beetles (Lin and Phelan, 1992). However, relatively little has been published about volatiles emitted from *Fusarium* species. Volatile emissions were studied from *F. culmorum* (W. G. Smith) Sacc. with the goal of being able to detect and identify fungi in stored grain simply by analyzing grain-bin volatiles (Börjesson et al., 1989). Volatiles identified from *F. culmorum* were ethyl acetate, 2-methyl-1-propanol, and various mono- and sesquiterpenes. Sesquiterpenes were analyzed from various strains of *F. sambucinum* Fuckel to better understand trichothecene biosynthesis (Jeleń et al., 1995). Gas chromatographic "fingerprints" were used to recognize various pathogenic strains of *F. oxysporum* Schlecht (Moore et al., 1991).

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The purpose of this study was to analyze the volatiles produced by corn isolates of *F. verticillioides*, both qualitatively and quantitatively, and to determine whether the types and levels of compounds detected could be consistent with sap beetle attraction. The nitidulid used for these studies was *Carpophilus humeralis* (F.), which attacks a variety of crops in the southern United States, including pineapples, dates, figs, and corn (Connell, 1981). The use of solid-phase microextraction (SPME), a relatively new, solvent-free analytical technique (Pawliszyn, 1997), greatly facilitated the analysis of the volatiles of lower molecular weight.

MATERIALS AND METHODS

Fungal Strains. All *F. verticillioides* cultures were obtained from the ARS Culture Collection, Peoria, IL. The accession numbers and original sources include NRRL 13586 *Zea mays*, Iowa, USA; NRRL 13993 *Zea mays*, Germany; NRRL 20956, *Zea mays*, California, USA; NRRL 20960, *Zea mays*, South Africa; NRRL 20984, *Zea mays*, Indiana, USA; NRRL 22055, *Zea mays*, Nepal; NRRL 22172, *Zea mays*, Germany; NRRL 25102, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on *Zea mays*, Mexico; and NRRL 25457, *Zea mays*, South Carolina, USA.

Culture Conditions. Conidia were harvested from 10-day-old slant cultures on potato dextrose agar that had been incubated at 25 °C, suspended in sterile distilled water, and diluted to give a final concentration of 10^6 spores/mL. Except where otherwise stated, 1 mL of the resulting spore suspension was used to inoculate whole autoclaved corn kernels (10 g of corn, dry weight, wetted to have ~50% moisture content) in individual 50 mL flasks (described below) that were both capped and wrapped in aluminum foil, to exclude light, and incubated for at least 8 days at 25 °C. Light is known to stimulate sporulation in *Fusarium* (Booth, 1971) and could conceivably impact volatile production. Our experimental protocol required that we intermittently remove the *Fusarium* cultures from the incubator to monitor volatile production and sap beetle behavior, and therefore we wanted to exclude exposure to light as an uncontrolled variable.

Unless otherwise indicated, incubation was at 25 °C and there was a constant flow of air through the flasks at a rate of 5 mL/min. The source was a tank of compressed air (of a grade suitable for GC-FID use). The air was first passed through a filter of Super Q porous polymer (Alltech Associates, Deerfield, IL) to remove organic impurities, then through a bubbler containing distilled water to increase humidity, and finally through the culture flasks. Each of the 50-mL flasks was equipped with a Pyrex inlet/outlet adapter (Ace Glass, Vineland, NJ); the flasks and adapters had 24/40 ground glass joints. A plug of silanized glass wool was placed in each inlet and outlet as a spore filter (Börjesson et al., 1989) to help maintain sterility. A short length (~5 cm) of Teflon tubing (4.5 mm i.d.) was connected to each outlet, beyond the spore filter, in which SPME chemical sampling took place.

SPME Sampling. SPME sampling was done using a fiber with a 100-μm poly(dimethylsiloxane) coating, held in the syringe-like manual sampling device (Supelco, Bellefonte, PA). To obtain an SPME sample from a culture flask, the fiber was placed ~4 cm inside the Teflon outlet tube and along its central axis. After 30 min, the sample was analyzed by gas chromatography (GC) or GC/mass spectrometry (GC/MS), as described below.

GC, MS, and Chemical Identifications. GC was conducted using a Hewlett-Packard 5890 instrument, equipped with a cool on-column inlet and flame ionization detector and controlled by a Hewlett-Packard ChemStation data system. Two types of columns were used successfully. The first was a 30-m DB-1 with 0.32 mm i.d. and 5-μm film thickness, and the second was a 30-m DB-5MS with 0.25 mm i.d. and 1-μm film thickness (both from J&W Scientific, Folsom, CA). In either case, a 10-cm retention gap of 0.53 mm i.d. deactivated fused silica tubing was placed between the column and the inlet. A press-fit connector was used to attach the retention gap to the column. The retention gap had a large enough internal diameter to allow SPME injections. The usual GC program was 50 °C for 1 min, increasing at 10 °C/min to 250 °C, and then holding at the maximum for 3 min. Inlet temperature was 200 °C, and detector temperature was 250 °C. Carrier gas was helium. GC injections of SPME samples were of 30-s duration. The SPME fiber was then conditioned for at least 2 min in a second inlet at 200 °C before reuse.

Mass spectrometry (GC/MS) was done using a Hewlett-Packard 5973 mass selective detector, which was interfaced to a Hewlett-Packard 6890 GC. Columns, inlets, and temperatures were as above. Tentative identifications of compounds **1**−**9**, **11**, **12**, and **14**−**16** (Figures 2 and 4 and text below) were based on mass spectral library searches; these identifications were verified by demonstrating that authentic standards had identical GC retentions and mass spectra.

Quantitation Calculations. The properties of the SPME fibers allow absolute quantitation to be conducted simply (Bartelt, 1997; Bartelt and Zilkowski, 1999). Two key characteristics of SPME fibers are (1) that analytes equilibrate between the fiber coating and the gas phase during sampling and (2) that the material accumulated by the fiber is transferred essentially completely onto the GC column during injection. Once sampling equilibrium is established, the mass of analyte in the fiber (in nanograms, as measured by GC) is directly proportional to the concentration in the air (nanograms per milliliter). The proportionality constant is the "calibration factor" and has units of milliliters. Calibration factors do differ among analytes and also vary with temperature, but they can be readily calculated for nearly any specific situation once the compounds have been identified (Bartelt, 1997). Fortunately, all fibers of a given type afford nearly the same results because of very uniform manufacture, and the fibers change very little over hundreds of injections; thus, the characteristics of individual fibers do not need to be measured unless extreme accuracy is required. The final values needed for quantitation are the detector response factors (nanograms per area unit), for converting detector peak areas to masses of analyte (a response factor is a characteristic of the GC instrument, not of SPME). Using the measured response factors (determined by injecting quantitative standard solutions) and the calculated calibration factors, the GC peak areas can be converted directly to absolute concentrations of analyte in the air (in nanograms per milliliter), without further standardization.

The above principles apply regardless of whether the sampled air is moving or static, but concentration in an air stream (nanograms per milliliter) is less meaningful biologically than is analyte flux (nanograms per second). Therefore, concentration was converted to flux by multiplying the concentration by the volumetric air flow rate (milliliters per second), and only the flux values (or "delivery rates") are presented.

Beetle Culture and Wind-Tunnel Bioassays. The culture of *C. humeralis* used for the bioassay experiments was originally obtained from Oasis, CA, and was maintained on artificial diet as described previously (Zilkowski et al., 1999). The bioassay experiments were conducted in a wind tunnel (Bartelt et al., 1990), using the procedures of Zilkowski et al. (1999). Briefly, the wind tunnel contained 500−1000 beetles of mixed sex and up to 1 week of age. Starvation for several hours induced the beetles to take flight so that as many as 100 would be airborne at any instant. For preliminary bioassays, culture flasks were hung directly in the wind tunnel, as described below, and the numbers of beetles landing on them were recorded.

Subsequently, to permit quantitation and better control of culture volatiles, the culture flasks were placed outside the wind tunnel and air streams were passed through them to entrain the emitted volatiles. This air was then directed through a piece of Teflon tubing into the upwind end of the wind tunnel (Bartelt and Zilkowski, 1998). The outlet was in

Table 1. Strains of *F. verticillioides* Investigated and Summary of Maximum Attraction and Maximum Production of Two Selected Volatiles

		max compd delivery rate (ng/s)	
strain	max index of attraction (day)	ethanol (day)	ethylguaiacol (day)
NRRL 13586	49 (2)	13 (2)	0.075 (5)
NRRL 13993	71 (2)	14 (1)	0.017 (8)
NRRL 20956	106 (2)	13 (1)	0.0032 (5)
NRRL 20960	104 (2)	18 (2)	0.0067 (2)
NRRL 20984	144 (4)	36 (2)	0.10 (5)
NRRL 22055	120 (3)	18 (3)	0.012 (5)
NRRL 22172	110 (2)	21 (2)	0.0046 (4)
NRRL 25102	77 (2)	19 (2)	0.021 (5)
NRRL 25457	*a*	45 (5)	0.096 (8)

[a] NRRL 25457 was bioassayed in earlier experiment; chemical data are included here for comparison.

the center of a circular disk of white cardboard, which was 8 cm in diameter and was mounted vertically, perpendicular to the direction of air movement through the wind tunnel. This disk served as a landing target for flying beetles responding to the scent. The bioassay was quantified by counting the number of beetles alighting on the cardboard disk during the test period. The sample air streams were under the control of a splitter system, as described by Bartelt and Zilkowski (1998). When the air streams were monitored by SPME, absolute rates of volatile delivery from the outlet into the wind tunnel (in nanograms per second) could be readily calculated. (In contrast, when the culture flasks were simply hung in the wind tunnel, there was no way to know what levels of compounds the bioassay beetles actually experienced.)

Initial Bioassays with *F. verticillioides* NRRL 25457. Autoclaved corn was inoculated with strain NRRL 25457 of *F. verticillioides* at intervals so that cultures of ages 0, 1, 2, 3, 4, and 7 days after inoculation could be compared for wind-tunnel activity all at one time. Uninoculated, control corn was the seventh experimental treatment. The flasks were covered with fine cloth for the bioassays, to prevent beetle entry. The experiment was conducted using a balanced incomplete block design, so that each possible pair of treatments was tested once (a total of six replications per treatment). For each comparison, the two test flasks were suspended in the upwind end of the wind tunnel, separated by 30 cm, and the numbers of beetles alighting on them during a 3-min test were recorded. Statistical analysis was done according to the method of Yates (1940), after transformation to $\log(X + 1)$ to stabilize variance. The results prompted the following, more systematic study of chemistry and beetle attraction.

Chemical and Behavioral Comparison of *F. verticillioides* Strains. Flasks with mature corn kernels were prepared as above and inoculated with one of the *F. verticillioides* strains (Table 1) or left uninoculated as controls. The cultures were set up with air flow at 5 mL/min. Each treatment flask was sampled by SPME and evaluated by bioassay 1, 2, 3, 4, 5, and 8 days after inoculation; additional SPME samples were taken at later times for some cultures. It was impossible to design the experiment so that all bioassays would be conducted on one day because the required number of tests was too large, and, furthermore, it was desirable to monitor the chemical and attractive properties of the individual culture flasks over time. Therefore, the experiment was conducted over a series of bioassay days, but an attractive standard treatment was included each day so that any day-to-day variability in beetle responsiveness could be compensated for. The attractive standard was 290 mg of ethanol and 300 mg of 3-hydroxy-2-butanone dissolved in 100 mL of water, a mixture found to be highly attractive in earlier research (Nout and Bartelt, 1998). With an air flow rate of 5 mL/min over this solution, the flux of ethanol was 50 ng/s and the flux of of 3-hydroxy-2-butanone was 10 ng/s. The bioassays were conducted in the following order: The first test was the attractive standard, followed by the eight culture treatments and uninoculated control. There were two such replications, followed by a final test of the attractive standard. The activity of each treatment on each day was expressed as an index of activity (I), which was calculated from the means as

$$I = \frac{\text{treatment} - \text{control}}{\text{attractive standard} - \text{control}} \times 100$$

The index of activity expresses the attractiveness of each treatment as a percentage of the attractive standard, after correction for the control response.

Dose−Response Study and Culture Simulations. A highly attractive autoclaved corn culture of *F. verticillioides* was connected to a splitter system (Bartelt and Zilkowski, 1998) so that the responses to reduced volatile levels could be evaluated and information obtained about the response threshold. The effluent from the attractive culture was quantitated at the 100% level by SPME. The bioassay activity was then evaluated at 100, 50, 25, 13, 6, and 3% of the maximum level. The bioassays were then repeated in reverse order.

After the dose−response relationship had been established, three cultures were selected that were clearly attractive but did not elicit the maximum response. The culture air streams were analyzed by SPME, and within several hours aqueous solutions of synthetic compounds were prepared that would simulate the cultures both qualitatively and quantitatively when air was passed over them [see Bartelt and Zilkowski (1998)]. These synthetic mixtures were analyzed by SPME and compared to the corresponding natural samples for bioassay activity (see Results and Discussion for details).

Manipulations of Laboratory Conditions. Inoculated autoclaved corn (NRRL 25457) was incubated at 20 and 30 °C in addition to 25 °C, and amounts of inoculum were varied (0.1, 0.5, and 1.5 mL instead of the standard 1.0 mL). Amounts of corn (10 or 50 g) and flask sizes (50 or 500 mL) were also varied, all to evaluate whether the volatile emission patterns were robust over a range of conditions. Finally, an 8-day-old culture was freeze-dried and, after 2 days, was rewetted with 10 mL of sterile distilled water and incubated again (for 5 days at 25 °C). This procedure was intended to approximate, quickly, the desiccating effects of winter, followed by the subsequent warming and moistening due to spring weather.

Field Study. Field corn (Pioneer 3394) planted at the University of Illinois, Illinois Valley Sand Farm Experiment Station, Kilbourne, IL, was evaluated for volatile production from inoculated kernels and attraction of nitidulid beetles. Ears were wounded on July 23, 1998 (milk stage), by making a knife cut along the axis of the ear (∼5 cm long and deep enough to cut into the kernels). *F. verticillioides* inoculum (NRRL 20960 or NRRL 25457) was applied as a conidial suspension using a sterile pipe cleaner (8 cm) inserted into the wound, and the pipe cleaner was left to mark the wound site at harvest. Alternatively, the pipe cleaner was dipped in sterile water as a control. There were five ears in each of 10 blocks with each treatment. One ear from each treatment and block was collected on the day of inoculation, and four subsequent samples were taken at weekly intervals.

Three ears from each day and treatment were analyzed for volatile production. This was done by removing the husk and then carefully removing the damaged kernels and a row of adjacent, undamaged kernels on either side. Removal was by cutting the kernels at their attachment to the cob. The kernels were placed in a 50-mL flask and weighed, and an inlet/outlet adapter was fitted to the flask to allow metered air flow through the headspace (20 mL/min). Within 1−5 h of the flasks being set up, volatile emission rates (flux) were measured by SPME. (In the sequence of analyses, each group of three samples contained one from each treatment. Order did not seem to affect results, however, because a sample analyzed at the beginning of the day and again at the end gave virtually identical results.) All 10 ears from each day were examined for the presence of nitidulid beetles or larvae.

RESULTS AND DISCUSSION

Initial Bioassay of Strain NRRL 25457. The preliminary experiment indicated that *F. verticillioides*

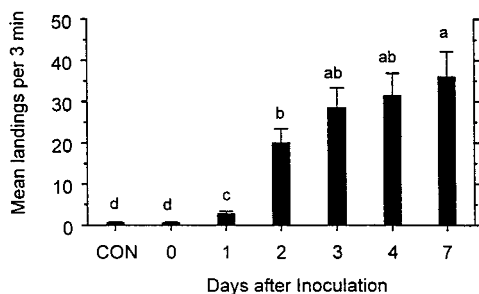

Figure 1. Mean wind-tunnel attractiveness of corn samples inoculated with *F. verticillioides* NRRL 25457 to *C. humeralis* ($N = 6$). Analysis was in $\log(X + 1)$ scale, but means and standard errors were untransformed for presentation. Bars accompanied by the same letter are not significantly different (LSD, $P = 0.05$).

NRRL 25457, when grown on autoclaved corn, was attractive in the wind tunnel bioassay to *C. humeralis* (Figure 1). The inoculum itself was not attractive, but activity was detectable after 1 day of incubation. Attraction was very clear after 2 days and remained high until the test was terminated after 7 days.

Identities of Volatiles and Patterns over Time. The volatiles emitted from corn kernel cultures of NRRL 25457 and entrained in the air stream through the flask were easily detected by SPME. Example gas chromatograms are given in Figure 2 and show the changes in volatile emission pattern over time. (Note that because SPME is more sensitive to heavier compounds, the peaks to the right in the GC traces are considerably larger than would occur if all compounds were sampled with equal sensitivity.) From MS analysis, the first compounds to be detected after inoculation were among those commonly associated with fermentation: acetaldehyde (**1**), ethanol (**2**), 1-propanol (**3**), ethyl acetate (**4**), and 2-methyl-1-propanol (**5**) (Figure 2, upper panel). These compounds became more abundant over the next few days, and the larger alcohols, 3-methyl-1-butanol (**6**) and 2-methyl-1-butanol (**7**), became evident. In addition, a number of higher molecular weight compounds also became prominent (Figure 2, middle panel); these were mostly aromatic compounds, with the most abundant being ethylguaiacol (4-ethyl-2-methoxyphenol, **11**). Others were 4-ethylanisole (1-ethyl-4-methoxybenzene, **8**), 4-ethylphenol (**9**), and 4-ethyl-1,2-dimethoxybenzene (**12**). Compound **10** may be a 10-carbon ketone with two additional double-bond/ring equivalents, based on MS features and comparisons with library spectra [m/z (% of base peak): 152 (20%, M^+), 137 (3%), 123 (0.3%), 121 (0.3%), 109 (100%), 91 (1%), 81 (60%), 79 (36%), 66 (3%), 65 (4%), 53 (16%), 43 (8%), 41 (19%)].

After 8 days of incubation (Figure 2, lower panel), the "fermentation" volatiles essentially disappeared, but the aromatic compounds and apparent ketone became even more abundant. After additional time (several days for most flasks), emission of all volatiles subsided, even though the fungal culture remained vigorous and healthy in appearance. A group of nine apparent sesquiterpenes (molecular weight = 204) occurred at low levels in cultures of all ages (the most abundant of these is labeled 13 in the GC traces in Figure 2), but these were undetectable from some strains and remain unidentified. Rigorous identification based on matching GC retention times and mass and/or NMR spectra with authentic standards was beyond the scope of the current project. The above patterns over time were consistent from flask to flask within a given strain, although there was some variability in how quickly each "stage" (exemplified by the panels of Figure 2) was reached.

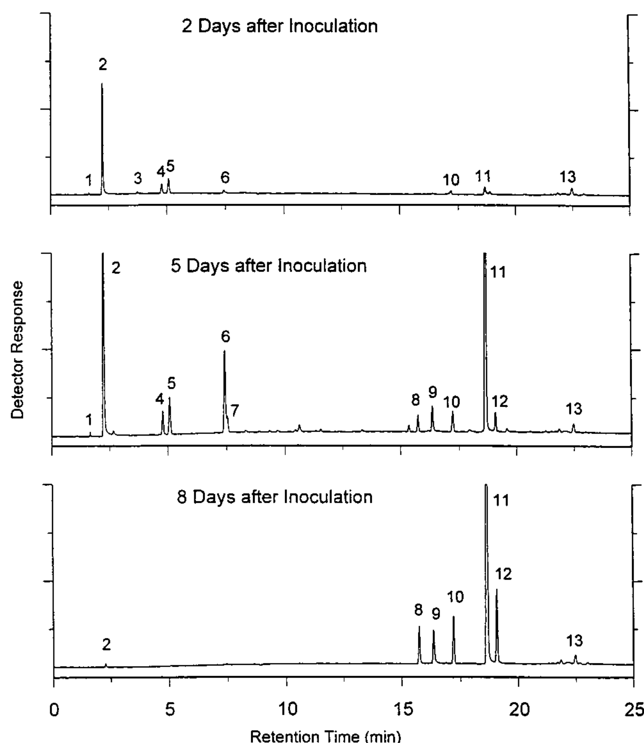

Figure 2. SPME gas chromatograms of volatiles from *F. verticillioides* NRRL 25457 grown on corn for 2, 5, and 8 days after inoculation. Incubation temperature was 25 °C, and air flow rate through flask was 5 mL/min. Peaks: 1 = acetaldehyde; 2 = ethanol; 3 = 1-propanol. 4 = ethyl acetate; 5 = 2-methyl-1-propanol; 6 = 3-methyl-1-butanol; 7 = 2-methyl-1-butanol; 8 = 1-ethyl-4-methoxybenzene; 9 = 4-ethylphenol; 10 = unknown; 11 = ethylguaiacol; 12 = 4-ethyl-1,2-dimethoxybenzene; 13 = unknown sesquiterpene.

Generally for all cultures incubated at 25 °C, fungal growth was not visible to the naked eye 1 day after inoculation but was clearly visible as a fine whitish pubescence on the kernel surfaces after 2 days. The white fungal mat grew and thickened until by day 5 the kernels were fully engulfed and could not be seen from above through the solid mat. There was no obvious change between 5 and 8 days.

Comparison of Fungal Strains. Figure 3 shows the bioassay activity and chemical emission rates for two representative *F. verticillioides* strains. Strain NRRL 20984 (solid bars) was attractive to the beetles for at least 5 days, and it produced both the smaller "fermentation" compounds and the larger aromatic compounds quite abundantly. Volatile production was relatively sustained. In contrast, the attractiveness of NRRL 22172 (Figure 3, partially shaded bars) waned rapidly after 2 days, as did the production of many compounds (**1**−**7**). The aromatic compounds were always nearly absent with NRRL 22172.

NRRL 20894 was very similar to NRRL 25457 (Figures 1 and 2) with respect to the types and amounts of compounds detected and the duration of attractiveness. However, the onset and termination of volatile production occurred slightly earlier with NRRL 20894. The pattern for NRRL 22172 was more typical of the *F. verticillioides* strains we examined in that bioassay activity and copious volatile production did not last for more than a day or two.

Maximum bioassay activity, maximum rate of ethanol production, and maximum rate of ethylguaiacol produc-

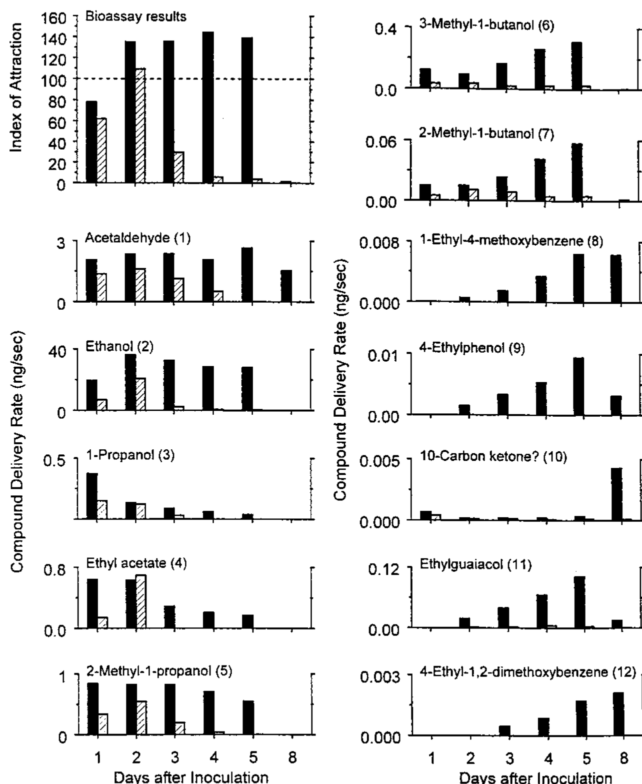

Figure 3. Bioassay activity (upper left) and delivery rates of 12 compounds from cultures of *F. verticillioides* of various ages. Solid bars represent NRRL 20984, and partially shaded bars represent NRRL 22172. Overall mean beetle counts for the 3-hydroxy-2-butanone/ethanol standard (index of attraction = 100) and the sterile-corn control (index of attraction = 0) were 205.4 and 2.6, respectively (2-min counts); see text for further explanation.

tion for all strains are given in Table 1. Also shown is the time after inoculation when these levels were achieved. All of the strains were attractive in the wind tunnel, and all of them produced ethanol, ethylguaiacol, and most of the other volatiles shown in Figure 2. In no case was there a prominent GC peak in these strains that was not among the numbered ones in Figure 2.

Example Dose−Response. As shown in Table 2, the behavioral response to attractive fungal volatiles exhibited the expected "sigmoid" dose−response relationship. A surprising finding was that the fairly low volatile production level of this culture (the most abundant component being ethanol, at 18 ng/s) was nevertheless considerably higher than the threshold for attraction. Even at 13% of the maximum level, which corresponded to only 2.8 ng/s of ethanol and proportionately reduced levels of the other compounds, the fungal volatiles were still clearly (>10 times) more attractive than the lowest level tested.

Relationships among Volatile Abundance and Bioassay Activity. The correlations involving volatile levels and bioassay indices of activity are given in Table 3 for the *F. verticillioides* strains and the controls for the six durations of incubation (ranging from 1 to 8 days). Bioassay activity was most closely correlated with the ethanol level ($r = 0.91$), but ethanol level was itself correlated closely with the levels of several other volatiles, especially 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and acetaldehyde. Because of these relationships, the results do not allow the bioassay activity data to be attributed specifically to any

Table 2. Chemical Analysis of Emission from *F. verticillioides* Culture 1 Day after Inoculation (A) and Bioassay Activity for a Range of Doses (B)

A. Chemical Analysis (for 100% Level)

compd	emission rate (ng/s)
acetaldehyde	2.9
ethanol	18.
2-methyl-1-propanol	0.25
3-methyl-1-butanol	0.16
2-methyl-1-butanol	0.027
1-ethyl-4-methoxybenzene	0.017
4-ethylphenol	0.0098
ethylguaiacol	0.072
4-ethyl-1,2-dimethoxybenzene	0.0038

B. Dose−Response Bioassay

treatment (% of max)	corresponding emission rate for ethanol (ng/s)	mean landings ($n = 2$)
100	18.	363.0
50	9.0	327.0
25	4.5	232.5
13	2.8	86.0
6	1.4	8.5
3	0.7	6.0

one compound, but the key components are likely to be from among ethanol, the other alcohols, and acetaldehyde.

Mixtures of synthetic compounds that simulated culture emissions were clearly attractive to *C. humeralis* (Table 4). Both mixtures that included ethanol, acetaldehyde, 1-propanol, 2-methyl-1-propanol, and 3-methyl-1-butanol were particularly effective. The cultures and synthetic mixtures in Table 4 represent volatile levels that would be in the "steep" portion of the dose−response curve (see Table 2); therefore, even slight differences in attractiveness should be reflected by relatively large differences in beetle counts, and the comparisons between cultures and simulating mixtures should be quite sensitive. In fact, the synthetic blends were not always as attractive as the corresponding cultures, but neither did they quite match the cultures chemically (Table 4). Despite conscientious efforts to mimic the cultures, the levels of compounds in the synthetic mixtures were usually lower than in the cultures; hence, imperfect agreement in level of activity was not surprising. The exact contribution of each of the fungal volatiles to attraction has not yet been defined, but these data demonstrate several attractive combinations. It is likely that some day-to-day variability in thresholds exists and that an absolute standard does not exist.

Earlier results showed that *C. humeralis* was not strongly attracted to either ethanol or acetaldehyde by itself but that the compounds were synergistic with each other (Bartelt and Zilkowski, 1998; Nout and Bartelt, 1998). The specific effects of the three higher alcohols on this species have not yet been described, but they were shown to be active in other nitidulids (Lin and Phelan, 1992; Phelan and Lin, 1991). Essentially the same volatiles have been identified from bread dough fermented by *Saccharomyces cervisiae* (Phelan and Lin, 1991; Bartelt and Zilkowski, 1998). The thresholds in this study were lower than those reported by Bartelt and Zilkowski (1998) for bread dough. Although it is possible that this effect was due to compounds that were not detected but that were synergistic with those listed above, we feel it is more likely that the overall lower levels used in these bioassays resulted in less habituation and therefore lower thresholds.

Table 3. Correlation Coefficients among Emission Rates for Eight Fungal Volatiles and Measured Bioassay Activity[a]

compd or activity	compd							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**11**
acetaldehyde (**1**)	1.00[b]							
ethanol (**2**)	0.76***	1.00						
1-propanol (**3**)	0.49***	0.60***	1.00					
ethyl acetate (**4**)	0.53***	0.61***	0.75***	1.00				
2-methyl-1-propanol (**5**)	0.75***	0.94***	0.75***	0.68***	1.00			
3-methyl-1-butanol (**6**)	0.69***	0.78***	0.31*	0.27*	0.71***	1.00		
2-methyl-1-butanol (**7**)	0.72***	0.77***	0.30*	0.30*	0.70***	0.97***	1.00	
ethylguaiacol (**11**)	0.23	0.30*	−0.16	−0.09	0.15	0.54***	0.52***	1.00
bioassay activity	0.78***	0.91***	0.48***	0.51***	0.86***	0.79***	0.81***	0.29***

[a] Based on chemical emission rates (ng/s) and behavioral analyses (index of activity) for 8 different *F. verticillioides* cultures on autoclaved corn, over 6 different culture ages, and the respective corn controls (54 total samples). Compound numbers are as in Figure 2 and text. Correlation coefficients that are significantly different from zero at the 0.05 and 0.001 levels are indicated by * and ***, respectively. [b] Equals 1.00 by definition.

Table 4. Chemical and Bioassay Comparisons among Three Moderately Attractive *F. verticillioides* Strains and Their Synthetic Imitations

description of line	NRRL 20984 (day 1)[a]		NRRL 25457 (day 3)[a]		NRRL 13586 (day 3)[a]	
	culture	synthetic	culture	synthetic[b]	culture	synthetic
chemical analysis (emission rate in ng/s)						
acetaldehyde	1.8	1.5	2.2	1.8	1.4	1.0
ethanol	4.9	3.0	28	19.	5.4	4.2
1-propanol	0.15	0.092	0.073	0.083	ND[c]	0
ethyl acetate	ND	0	0.84	0.92	ND	0
2-methyl-1-propanol	0.26	0.21	0.58	0.53	0.12	0.086
3-methyl-1-butanol	0.038	0.030	0.058	0.056	ND	0
2-methyl-1-butanol	ND	0	0.020	0	ND	0
bioassay activity (mean landings per 2-min test, $n \geq 3$)[d]	95.0	58.8	105.7	100.2	16.3	8.3

[a] Age of culture when corresponding synthetic mixture prepared and bioassay conducted. [b] Synthetic mixture for comparison 2 prepared by adding to 100 mL of water: 0.91 mg of acetaldehyde, 190 mg of ethanol, 0.24 mg of 1-propanol, 0.19 mg of ethyl acetate, 1.10 mg of 2-methyl-1-propanol, and 0.10 mg of 3-methyl-1-butanol; then air passed through headspace of flask at 5 mL/min. Amounts for other mixtures adjusted proportionally. [c] ND, not detected. [d] Means for controls were 3.8 ($n = 5$) on day 1 and 1.3 ($n = 16$) on day 3.

Table 5. Volatile Emission Rates from Milk-Stage Field Corn That Had Been Wounded and Inoculated with Strains of *F. verticillioides* or Treated with Sterile Water 1 Week Previously[a]

compd	volatile emission rate (ng/s, ±SD)		
	NRRL 25457	NRRL 20960	wounded control
acetaldehyde	7.6 ± 4.0	7.4 ± 3.4	1.3 ± 1.2
ethanol	77 ± 55	76 ± 44	8.6 ± 6.5
1-propanol	0.070 ± 0.12	0.047 ± 0.081	ND[b]
ethyl acetate	0.47 ± 0.53	0.42 ± 0.40	ND
2-methyl-1-propanol	0.37 ± 0.39	0.40 ± 0.23	0.022 ± 0.039
3-hydroxy-2-butanone	0.063 ± 0.11	0.12 ± 0.13	0.080 ± 0.072
3-methyl-1-butanol	0.32 ± 0.33	0.29 ± 0.20	0.019 ± 0.016
2-methyl-1-butanol	0.068 ± 0.076	0.066 ± 0.058	ND
ethylguaiacol	0.00017 ± 0.00029	0.0019 ± 0.0009	ND

[a] Samples included about eight wounded kernels and adjacent, unwounded kernels so that the total wet weight was ~5 g. There were three samples per treatment. [b] ND, not detected.

Additional attractants for *C. humeralis* from *F. verticillioides* include ethyl acetate (Bartelt and Zilkowski, 1998) and ethylguaiacol (Zilkowski et al., 1999). The levels detected during this study were high enough to contribute to attraction, but the interactions among these components are undoubtedly complex and will require additional research to fully resolve. Zilkowski et al. (1999) tested 4-ethylphenol, 1-ethyl-4-methoxybenzene, and 4-ethyl-1,2-dimethoxybenzene for bioassay activity but none was detected.

Emission Patterns under Various Laboratory Conditions. The patterns of volatile emission (Figure 2) were generally robust to changes in culture conditions. Although a lower incubation temperature (20 °C) retarded the progression in pattern and a higher temperature (30 °C) accelerated it, the patterns were qualitatively similar. Inoculation with a smaller amount of fungal material (0.1 or 0.5 mL of conidial suspension) slowed the initial appearance of volatiles but did not affect the eventual evolution of patterns. Similarly, inoculation with 1.5 mL instead of 1.0 mL had little effect. On the other hand, freeze-drying did dramatically change the pattern (Figure 4). Two days after the addition of water to the freeze-dried culture and incubation at 25 °C, ethyl acetate (peak 4) was by far the most abundant volatile, and the additional esters, propyl acetate (peak 14), 2-methylpropyl acetate (peak 15), and 3-methylbutyl acetate (peak 16), were evident for the first time. Thus, the patterns of emitted volatiles have some degree of plasticity, and this needs to be considered when one is making conclusions regarding ecological relationships involving fungal volatiles. These results may be ecologically relevant if there is a similar release of volatiles from *Fusarium*-rotted and overwintered corn ears following spring thaw.

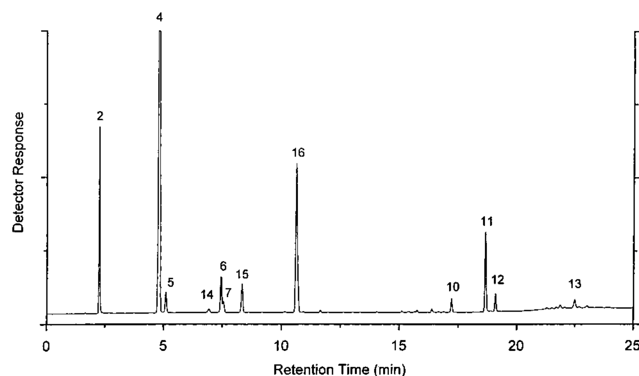

Figure 4. SPME gas chromatogram of volatiles from *F. verticillioides* NRRL 25457, after freeze-drying and then rehydrating and incubating for 2 days. Peaks: 14 = propyl acetate; 15 = 2-methylpropyl acetate; 16 = 3-methylbutyl acetate; see Figure 2 or text for the others.

Inoculated Field Corn. Field corn in the early dough stage that had been wounded and inoculated with *F. verticillioides* 1 week prior to sampling produced volatiles that were similar in types and quantities to cultures on autoclaved corn (Table 5). Even though sterile conditions were not possible and various other microorganisms eventually invaded the wounds, the cut but uninoculated controls produced far less of the beetle-attracting compounds. After kernels from the samples were placed onto agar plates, *F. verticillioides* was present on the inoculated corn samples, but only one of the three control samples had this species, presumably a native strain, and this was the only sample in which 2-methyl-1-propanol and 3-methyl-1-butanol were detected (Table 5). Controls were found to be infected by *Alternaria alternata*, *Mucor* sp., *Cladosporium cladosporioides*, and an unidentified yeast, and *A. alternata* was also present in one of the inoculated samples. 3-Hydroxy-2-butanone was identified from most of the field samples. Although *F. verticillioides* was never observed to produce 3-hydroxy-2-butanone, live corn tissue or other associated microbes can (Nout and Bartelt, 1998; unpublished observations). From a comparison of Tables 2, 4, and 5, it is suggested that *F. verticillioides*, growing naturally on small numbers of wounded kernels in field corn (as few as eight), could easily produce volatiles at a rate that is significantly attractive to nitidulid beetles. In fact, 83% of the wounded ears in the experimental plot (85% of inoculated ears and 80% of controls) were infested by *C. lugubris* beetles on the day the sample ears were collected. *F. verticillioides* may have contributed to this attraction but was clearly not totally responsible for it. The *F. verticillioides* volatiles were not evident as the corn proceeded to dry down 2−4 weeks after wounding and inoculation.

F. verticillioides, like a number of other fungal and yeast species, produces a bouquet of small alcohols, esters, aldehydes, and other compounds that is quite attractive to nitidulid beetles. This situation would lead to more than just incidental contact between this fungus and these beetles in the field and increases the likelihood that the beetles can be significant vectors. However, it is also clear that transmission of this fungus can involve a number of mechanisms, and much additional research will be required to establish their relative contributions to the distribution of *F. verticillioides*.

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