Determination of Allelochemicals in the Environment Surrounding Ceratiola Ericoides.

Elizabeth Douglas Jordan

Louisiana State University and Agricultural & Mechanical College

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Determination of allelochemicals in the environment surrounding
*Ceratiola ericoides*

Jordan, Elizabeth Douglas, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1990
DETERMINATION OF ALLELOCHEMICALS IN THE ENVIRONMENT SURROUNDING
CERATIOLA ERICOIDES

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in
The Department of Chemistry

by

Elizabeth Douglas Jordan
B.A., Mississippi University for Women, 1985

May, 1990
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FOREWORD

This dissertation contains an introduction and three chapters that describe investigations of the release of allelochemicals by Ceratiola ericoides. Each chapter will be submitted to Journal of Chemical Ecology as a separate paper. An appendix is included that describes experimental details that, for reasons of journal space economy, preclude their inclusion in the chapters.
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ABSTRACT

As part of an investigation into the allelopathic interactions of members of the Florida scrub community of plants, the release of allelochemicals by the Florida wild rosemary, Ceratiola ericoides (Empetraceae) was monitored.

Aqueous extracts of whole leaves, water-rinses of leaves, aqueous litter extracts, and aqueous soil extracts were monitored monthly, or twice monthly in the summer months, for the dihydrochalcone ceratiolin, its decomposition product hydrocinnamic acid, the microbial degradation product of hydrocinnamic acid, acetophenone, and trans-cinnamic acid. High performance liquid chromatography was used to quantify the four compounds. The amounts of ceratiolin, hydrocinnamic acid, acetophenone, and trans-cinnamic acid in the leaf extracts varied seasonally; the largest amounts were observed in the months of September and October. These months are in the midst of the rainy season in the Florida scrub. Observed amounts of the four analytes in the leaf mists were much lower, and there was no seasonal variation. The soil and litter extracts also contained hydrocinnamic acid in varying amounts; the maximum concentrations again occurred in the late summer.

The efficiency of extraction of exogenously applied hydrocinnamic acid to scrub soil was determined to be dependent on the level at which the compound was applied, and ranged from 18% at a level of 1 μg/g soil to 79% at a level of
64 μg/g soil in non-sterile soil. In sterile soil, the recovery was 91%, 78%, and 80% for levels of 2 μg/g, 4 μg/g, and 8 μg/g soil respectively.

Allelochemicals that were released into the environment as volatile compounds were collected by dynamic headspace sampling and trapping on Tenax TA adsorbent. Thermal desorption and cryogenic focusing were utilized to introduce the compounds into a capillary column for analysis by gas chromatography-mass spectrometry. The volatile compound composition of whole leaves varied seasonally. Among the more abundant compounds were ethyl acetate, 3-methyl-1-butanol, and 1-hexanol. Of the volatile compounds collected from the litter and soil, 1-octene, 3-methyl-1-butanol, and 3-octanol were the most abundant.
CHAPTER I

Introduction
ALLELOPATHY

Interactions between plants have been observed and recorded throughout history. Theophrastus, in 300 B.C., commented that chick pea exhausted soil and inhibited weed growth (Hort, 1916). The concept that this interaction between plants was chemical in nature was introduced by Plenck in 1795. Molisch (1937) then defined the term allelopathy- the inhibitory or stimulatory biochemical interactions between all types of plants, including microorganisms.

One of the first examples of allelopathic activity to be characterized was that of the black walnut tree, Juglans nigra. The inhibitory nature of the canopy drip from the black walnut was observed by Stickney and Hoy (1881), and the chemical constituent responsible for this activity was identified later as juglone [Figure 1.1] (Davis, 1928).

Many types of secondary metabolites have been implicated in allelopathic interactions. These include compounds of such diverse structural types as monoterpenes (Muller, 1965; Halligan, 1975; reviewed by Harborne, 1982; Rice, 1984), lactones (Veldstra and Havinga, 1943-45; reviewed by Moreland, 1966; Fischer, 1986), and alkaloids (Knypl and Janas, 1977; Kusumi, 1981; Wink, 1983). In general, allelopathic compounds have been divided into two categories: 1) water-soluble compounds, and 2) volatile compounds (Muller and Chou, 1972).
FIG 1.1 Structure of juglone

FIG 1.2 Structures of benzoic acid derivatives
WATER-SOLUBLE ALLELOCHEMICALS

Perhaps the most highly scrutinized group of water-soluble allelochemicals is the organic acids, and particularly the aromatic acids. Many organic acids are intermediates of metabolism of plants and microbes (Flaig, 1971).

Many phenolic acids [see Figures 1.2 and 1.3] are distributed throughout the angiosperms; gymnosperms and ferns also contain phenolic acids, although fewer in number (Hartley and Whitehead, 1985). Early references to the occurrence of phenolic acids in plant tissues include Hillis and Isoi (1965), who reported vanillic, gallic, ferulic, caffeic, chlorogenic, and p-coumaric acids in *Eucalyptus camaldulensis* leaves, and Bende (1956), who reported the occurrence of gallic acid in litter of *Acer platanoides*.

Ubiquitous in higher plants are the cinnamic acids and their derivatives. These acids are found primarily in the *trans* forms (Hartley and Whitehead, 1985). The phenolic acids often occur in their bound form, with an attached sugar as a glycoside, or as an ester, such as chlorogenic acid, the quinic acid ester of caffeic acid (Harborne, 1980). Cinnamic acids have been identified as free acids including, for example, p-coumaric and ferulic acids in water extracts of sub-tropical grasses (Chou and Young, 1975). In addition, coumarin [Figure 1.3], the lactone of o-hydrocinnamic acid, and substituted coumarins occur widely in the higher plants (Brown, 1979).
FIG 1.3 Structures of cinnamic acid derivatives
The primary precursor of the benzoic and cinnamic acids in the synthesis by plants is thought to be the aromatic amino acid L-phenylalanine (Gross, 1979). Phenolic acids have been detected in the cell walls of some higher plants and are attached by ester links involving the carboxyl group of the acids and hydroxyl groups of the sugar units (Hartley, 1973).

Aromatic acids have also been detected in soils. Whitehead (1964) identified p-hydroxybenzoic, vanillic, p-coumaric, and ferulic acids in four different soils. Other acids, such as syringic, protocatechuic, gallic, and genistic, have also been detected as soil constituents (Evans, 1977). Decomposing plant materials (surface litter or root tissues) have been shown to contain the following aromatic acids: benzoic, caffeic, cinnamic, o-coumaric, o-hydroxyphenylacetic, phenylacetic, 4-phenylbutyric, salicylic (Chou and Patrick, 1976), gallic and chlorogenic (Chou and Muller, 1972), and p-hydroxyphenylpropionic and 3,4-dihydroxyphenylpropionic (Lynch, et al., 1980). The water-soluble aromatic acids can be washed into the soil in leachates of decomposing plant or root material, or in leachates of live plant material, as in the case of canopy drip.

Many early investigations of the phenolic acid content of plant tissues on soils involved the extraction of the compounds with harsh reagents, such as strong alkali (Whitehead, 1964; Wang, et al., 1967; McPherson, et al., 1971; Hartley and Jones, 1977). Extraction of plant tissues with water often releases
phenolic acids in their glycosidic or esterified form (Harborne, 1973), but reports of free acids obtained through aqueous extraction are also numerous (Chou and Young, 1975; Horsley, 1977; Chou and Yang, 1982). Methanol has also been used to extract phenolic acids from plant tissue and soil (Carballiera, 1980).

Strong alkaline solutions, either aqueous or ethanolic, were originally employed to extract "free" organic acids, including those reversibly bound to soil clay particles or organic matter. It was later realized that the use of these reagents most likely also released acids that had been incorporated into soil humus, polymerized organic matter. These acids are not "free" under normal soil conditions (Hartley and Whitehead, 1985). The use of chelating agents, such as Na$_2$EDTA (sodium ethylenediamine-tetraacetic acid) was proposed by Kaminsky and Muller (1977) to avoid the denaturing properties of alkaline conditions, and the authors recommended the use of Na$_2$EDTA in studies involving allelopathic interactions (Kaminsky and Muller, 1978). Whitehead, et al. (1981), have investigated the effect of extractant pH on the release of phenolic compounds from four types of soils and have reported that the amounts of phenolic compounds extracted by water alone were equivalent to concentrations in the soil solution (the soil solution comprises the natural moisture in the soil and any substances dissolved in this moisture). More recently, a study comparing many different extraction procedures (Dalton, et al., 1987) reported significant differences in recovery of exogenously applied ferulic
acid from four soils with water and EDTA. No significant differences in recovery were reported for soils extracted with water and methanol, and extraction times of more than three hours did not result in an increase in the recovery.

It is evident that the concentrations of phenolic compounds determined in soils is dependent upon the method of extraction. Whitehead (1964) reported concentrations of p-hydroxybenzoic acid in the soil solution of four types of soils to be from 0.08 to 0.39 μM, while ferulic acid concentrations for the same sample types ranged from 0.007 to 0.32 μM. These extractions were with strong alkali. Further investigations of acids in soils report concentrations of 1.4 μM and less than 10 nM for p-hydroxybenzoic and ferulic acids, respectively, when extracted with water (Whitehead, et al., 1981).

The aromatic acids identified in plant tissues and soils exhibit biological activities of varying degrees. Early reports by Bende (1956) and Grümmer (1961) showed that gallic acid and several cinnamic acids were toxic to germination or seedling growth. Wang, et al. (1967) reported the toxic effects of p-hydroxybenzoic, ferulic, vanillic, syringic, and p-coumaric acids on the growth of sugar cane at concentrations of 50 ppm in hydroponic bioassays. Benzoic, p-coumaric, p-hydroxybenzoic, hydrocinnamic (3-phenylpropionic), salicylic, syringic, and vanillic acids all were tested for inhibition of root growth of barley seed grown in sand and watered with acid in solution at 5 moles/m³ (Lynch, 1980).
p-Coumaric and 3-phenylpropionic acids were the most inhibitory and had approximately the same activity. Reynolds (1989) tested trans-cinnamic and hydrocinnamic acids and coumarin for germination inhibition of lettuce seed (*Lactuca sativa* L. cv. Great Lakes). trans-Cinnamic and hydrocinnamic acid exhibited equal activity, and coumarin was about twenty-fold more active.

The most commonly employed techniques for the analysis of phenolic acids and related flavonoid compounds are chromatographic techniques. Thin-layer chromatography (TLC) has replaced paper chromatography for much of the qualitative analyses. TLC, using a mobile phase of toluene and formic acid (4%) has been used to separate *cis* and *trans* isomers of substituted cinnamic acids (Hartley and Jones, 1975). High performance TLC (HPTLC) has been used to analyze a number of phenolic compounds including flavonoids (aglycones), phenolic acids, and related compounds (Vanhaelen and Vanhaelen-Fastré, 1979). Reversed-phase C₈ (RP-8) bonded stationary phase with ethanol/water (55:45) as the mobile phase afforded improved resolution over RP-2, RP-18, or polymide stationary phases. Gas liquid chromatography (GLC) has also been employed for the quantitative determination of phenolic compounds; however, prior to analysis, the compounds had been converted to their trimethyl silyl derivatives to impart volatility (Hartley and Jones, 1976; Vanhaelen and Vanhaelen-Fastré, 1979).
High performance liquid chromatography (HPLC) has become the primary technique of analysis of phenolic compounds. The use of ultra-violet adsorption in the detection of phenolic acids takes advantage of the strong and characteristic band of adsorption of the aromatic portion of these compounds; therefore, detection in the aromatic region of the spectrum reduces interference by non-aromatic species. Reversed-phase C\textsubscript{18} stationary phases and methanol/water or ethanol/water mobile phases containing 0.1% to 5.0% acetic acid have been employed with success (Wulf and Nagel, 1976; Vanhaelen and Vanhaelen-Fastré, 1979).

**VOLATILE ALLELOCHEMICALS**

Volatile monoterpenes are important constituents of the essential oils of many plant species. Muller (1965) suggested that monoterpenes volatilized from *Salvia leucophylla* and *S. mellifera* might contribute to the inhibitory effect the shrubs exerted upon neighboring grasses. The monoterpenes α-pinene, β-pinene, camphene, cineole, and camphor [Figure 1.4] were detected in macerated *Salvia* leaves, and camphor and cineole were detected in the air surrounding *Salvia* shrubs (Muller, 1966). Camphor and cineole were the most inhibitory of the *Salvia* monoterpenes (Muller, 1965). Muller and del Moral (1966) demonstrated that soil, when exposed to *Salvia* monoterpenes, adsorbed terpenes from the atmosphere, thus reducing the concentrations of terpenes in the atmosphere and inducing toxicity in the soil. In addition, monoterpenes were detected in the soil.
FIG. 1.4 Monoterpenes from *Salvia leucophylla*
collected near *Salvia* shrubs (Muller, 1966). This observation laid the foundation for the hypothesis that terpenes volatilized from the surfaces of leaves could be adsorbed by soils, where they might then act to inhibit seed germination (Muller and del Moral, 1966). Monoterpenes were also detected in *E. camaldulensis* (del Moral and Muller, 1970) and *Artemisia californica* (Halligan, 1975).

More recently, volatile compounds associated with *Amaranthus palmeri* have been identified as toxic allelochemicals (Bradow and Connick, 1987). These volatiles consisted of C$_4$-C$_{11}$ methyl ketones and secondary alcohols, 3-methyl-1-butanol, hexanal, and two furans (Connick, *et al.*, 1987). 2-Heptanol and 2-heptanone were major components and were found to be active on several seed in germination assays. Further bioassays of vapors of volatile compounds from *A. palmeri* for germination inhibition of onion, carrot, *A. palmeri*, and tomato seed have indicated that the inhibitory activities of the compounds follow the order: 2-nonanone, 2-heptanol, 2-octanone > 2-undecanone > 3-methyl-1-butanol, 1-hexanol > 2-heptanone > 2-hexanone > 3-methyl-2-butanol, hexanal, 2-pentanol, 3-pentanone, 2-pentanone, acetaldehyde, 3-hydroxy-2-butanone > ethanol, 2-butanol, 2-methyl-1-propanol (Bradow and Connick, 1988).

Reports of toxicity of volatile compounds have often been based on inhibitory effects displayed by these compounds in the vapor phase. The monoterpenes have long been considered, as a group, to be non-water soluble.
However, bioassays of compounds in solution (Reynolds, 1987) indicate that several monoterpenes, such as camphor, exhibit activity in aqueous solution, though the monoterpene hydrocarbons did not exhibit high activity. It has been suggested (Fischer, et al., 1988) that natural tensides that co-occur on the surface of leaves with monoterpenes may increase their water solubility through the formation of micelles. Additionally, many volatile alcohols and ketones, such as those identified from A. palmeri, are quite water-soluble and have been shown to be germination inhibitors in solution (Reynolds, 1987). Therefore, the activity of these volatile allelochemicals could be manifested in the vapor phase or in solutions.

Analysis of plant volatiles is normally by gas chromatography, and it is the preparation of samples and the collection of volatiles that has seen many changes. Muller (1965) collected volatiles in the atmosphere near live Salvia shrubs in the field using 2ml-5ml syringes. More often, plant tissues have been macerated, and the essential oil distilled (Halligan, 1975; Bestmann, et al., 1988). Another common method of collecting volatile compounds involves passing air or helium over the plant material and trapping the volatiles in a cooled solvent, such as pentane or dichloromethane (Muller, 1965; Tanrisever, 1986). Recently, various adsorbents, such as charcoal, Tenax (Lewis, et al., 1988; Patt, et al., 1988; Hernandez, et al., 1989; Lwande, et al., 1989), and Poropak Q (Tollsten and Bergstrom, 1988), have been used to strip the volatiles from air helium passed
over the plant material. Subsequent removal of the trapped volatiles has been by solvent elution (Tollsten and Bergström, 1988) or by thermal desorption, in which no solvents are employed (Lewis, et al., 1988; Hernandez, et al., 1989).

EFFECTS OF SOIL CHEMISTRY AND MICROBIOLOGY ON ALLELOCHEMICALS

The soil environment into which allelochemicals are released is quite complex. The physical characteristics of the soil, as well as the microbial populations, can have a marked effect on any substance released into the soil environment.

The inorganic fraction of soils can be separated into classes based on the size of the particulate matter: gravel (>2.0 mm); sand (2.0-0.05 mm); silt (0.05-0.002 mm); and clay (0.002 mm). These classes all consist primarily of silicates, though the form differs for the various classes (Tan, 1986). The organic fraction of soils can be separated into classes on the basis of its solubility; free organic compounds are soluble in water, and humus is an amorphous material that is insoluble in water. Humus comprises humic acid (soluble in alkali, but not in acid), fulvic acid (soluble in both alkali and acid), and humin (insoluble in alkali) (Gray and Williams, 1971). The characteristics of a particular soil are governed by its texture and organic matter content. These physical characteristics can have a marked effect on organic compounds in soil. For example, monoterpenes have been shown to be adsorbed by soils with
a high clay content (del Moral and Muller, 1970), while sorption of ferulic acid was shown to be related to the amount of organic matter present (Dalton, et al., 1989).

An important component of the soil environment is the soil microbiota. The effects of soil microorganisms have largely been overlooked in investigations of allelopathy (Blum and Shafer, 1988). Soil organic matter is metabolized by microflora, and although not all soil organic matter is available to the microbes, almost any organic substance can be attacked by one or more organisms under the correct environmental conditions. Aromatic compounds, however, are relatively resistant to attack (Gray and Williams, 1971).

Soil microbes colonize plant materials in the soil. The result of this colonization is the breakdown of complex organic polymers, such as lignin, and the release of phenolic acids, such as p-hydroxybenzoic, vanillic, and syringic, into the soil (Stevenson, 1967). In addition to the decomposition of plant tissues, microbes will attack the phenolic acids in the soil. Through investigations using $^{14}$C labeled acids, Haider and Martin (1975) have determined the pathway by which phenolic acids are decomposed by soil microorganisms [Figure 1.5]. The side chain of p-coumaric acid is cleaved by $\beta$-oxidation, thus releasing acetic acid. The formation of a dihydroxy-acid with hydroxy groups attached to adjacent carbon atoms is achieved and is necessary for the enzymatic opening of the aromatic ring.
FIG 1.5 Microbial metabolism of p-hydroxycinnamic and caffeic acids (Haider & Martin, 1975)
The time required for this process is dependent upon many factors such as temperature, moisture, soil pH, nutrient availability, and soil texture (Martin and Haider, 1986). Microbes require high water activity for growth, and except for short periods following rain or other addition of water to the soil, many soils do not contain sufficient water to fully support microbial growth. This is especially true for sandy soils, as sand particles retain little water against gravitational pull (Stotsky, 1986). Additionally, low pH and low nitrogen availability reduce microbial activity (Kowalenko, et al., 1978).

ALLELOPATHIC INTERACTIONS IN THE FLORIDA SCRUB COMMUNITY

The Florida scrub community is the lesser of two communities that occupy the well-drained sands of the Florida and the southeastern coastal plain. The more prevalent community, the sandhill, shares the same sub-tropical climate as the scrub, with dry winters and heavy rains during the summer rainy season. The flora of the two communities differ greatly, however.

The scrub is characterized by a canopy of sand pine, *Pinus clausa*, a branched tree that reaches a height of less than 30 feet (Nash, 1895), and an understory of scrub oaks, (*Quercus myrtifolia, Q. chapmanni*). There is no herbaceous ground cover in the scrub, but the scrub does contain quite a few shrubs, such as *Ceratiola ericoides, Calamintha ashei*, and *Conradina canescens*. These shrubs tend to colonize disturbed sites (Williamson, unpublished).
The sandhill flora is characterized by longleaf pine (*P. palustris*), a tall pine that is branched only near the top (Nash, 1895). Other trees include the turkey oak (*Q. laevis*) and sand live oak (*Q. geminata*). Shrubs are uncommon, but there is a complete ground cover of grasses, such as wiregrass (*Aristida stricta*) and beard grasses (*Andropogon gyrans* and *Schizachyrium scoparium*).

There exists a sharp boundary between the two communities (Nash, 1895), and the two do not intermix [Table 1.1]. Thus, a variety of explanations have been put forth for the observed phenomena. Exhaustive soil analyses have shown the soil to be over 90% sand with low amounts of organic matter (less than 1%) and available nutrients. However, no evidence for differences in pH, soil texture, moisture, or cationic nutrients between scrub and sandhill soil has been provided (Webber, 1935; Laessle, 1958, 1968; Kalisz, 1982; Kalisz and Stone, 1984).

Another striking difference between the two communities is the frequency of fires. The sandhill community burns quite frequently, once every 3-5 years. The community is able to recover from these fires, as plant reproduction and development are stimulated by post-fire conditions. The scrub burns only every 40-60 years, and the shrub species are completely consumed by fire. If left undisturbed, however, the scrub species will colonize sandhill areas (Burns, 1968; Britt, 1972; Veno, 1976; Richardson, 1977).
Table 1.1. Contrast in scrub and sandhill vegetation types (Fischer, et al., 1988)

<table>
<thead>
<tr>
<th>Property</th>
<th>Scrub</th>
<th>Sandhill</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shrub layer:</td>
<td>Very dense</td>
<td>Open</td>
</tr>
<tr>
<td>Herbaceous layer:</td>
<td>Nearly None</td>
<td>Complete</td>
</tr>
<tr>
<td>Foliage phenology:</td>
<td>Evergreen</td>
<td>Deciduous</td>
</tr>
<tr>
<td>Surface litter:</td>
<td>Light</td>
<td>Heavy</td>
</tr>
<tr>
<td>Fire frequency:</td>
<td>20-50 years</td>
<td>3-8 years</td>
</tr>
<tr>
<td>Plant relative growth rates:</td>
<td>Slow</td>
<td>Fast</td>
</tr>
<tr>
<td>Age of plants at first reproduction</td>
<td>Old</td>
<td>Young</td>
</tr>
</tbody>
</table>

Investigations were therefore initiated to test the hypothesis that allelopathic activity by scrub species deters the encroachment of sandhill grasses (fuel for fire) into the proximity of scrub species, while frequent surface fires in the sandhill subdue the colonization of sandhill areas by scrub species (Richardson and Williamson, 1988). Initial investigations of the possible allelopathic activity included bioassays of leaf leachates of several scrub species and reciprocal transplant experiments. The reciprocal transplant experiments have indicated that allelopathic effects, rather than effects of nutrient competition, are dominant. The bioassay experiments involved collecting monthly samples of leaf and litter leachates from several scrub species, including *C. ericoides*. These leachates were tested for inhibition of germination and
radicle growth of *S. scoparium*, a native sandhill grass. Results [Table 1.2] have shown that *C. ericoides* litter leachates are more inhibitory than leaf leachates and that there is a seasonal variability of toxicity, with the highest activity occurring in the summer months (Fischer, *et al.*, 1988).

**Table 1.2.** Effect of water washes of *Ceratiola ericoides* on *Schizachyrium scoparium* germination (G) and radicle length (RL)\(^1\) (Fischer, *et al.*, 1988)

<table>
<thead>
<tr>
<th></th>
<th><em>Ceratiola ericoides</em></th>
<th></th>
<th><em>Ceratiola ericoides</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fresh plant</td>
<td>litter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G    RL</td>
<td>G    RL</td>
<td></td>
</tr>
<tr>
<td>March</td>
<td>80   114</td>
<td>51*  28</td>
<td></td>
</tr>
<tr>
<td>April</td>
<td>103* 91</td>
<td>133 84*</td>
<td></td>
</tr>
<tr>
<td>May</td>
<td>83   99</td>
<td>81   81</td>
<td></td>
</tr>
<tr>
<td>June</td>
<td>85   83</td>
<td>9*    79</td>
<td></td>
</tr>
<tr>
<td>July</td>
<td>55*  80</td>
<td>48*  93</td>
<td></td>
</tr>
<tr>
<td>August</td>
<td>84   102</td>
<td>100 108</td>
<td></td>
</tr>
<tr>
<td>September</td>
<td>93   129*</td>
<td>85*  101*</td>
<td></td>
</tr>
<tr>
<td>October</td>
<td>74   108</td>
<td>70   95</td>
<td></td>
</tr>
<tr>
<td>November</td>
<td>147  84</td>
<td>95   91</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Numbers are germination as a per cent of the control and radicle lengths as per cent of control. An asterisk indicates a significant difference (p<0.05).

Chemical investigation of *C. ericoides* resulted in the identification of the dihydrochalcone ceratiolin [Figure 1.6] (Tanrisever, *et al.*, 1987). In bioassays, ceratiolin has shown no effect on germination or radicle growth of lettuce seed (*Lactuca sativa*) or *S. scoparium*; however, ceratiolin decomposes either neat or
FIG 1.6 Structures of allelochemicals from *Ceratiola ericoides*
in solution to dihydrocinnamic acid (HCA) [Figure 1.6], which has been shown to be inhibitory of both lettuce seed and *S. scoparium* [Table 1.3] (Fischer, *et al.*, 1988).

**Table 1.3.** Effects of hydrocinnamic acid (HCA) and ceratiolin on germination and radicle growth of *Lactuca sativa* and *Schizachyrium scoparium*¹ (Fischer, *et al.*, 1987)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (ppm)</th>
<th><em>L. sativa</em></th>
<th></th>
<th><em>Schizachyrium scoparium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>G</em></td>
<td><em>RL</em></td>
<td><em>G</em></td>
</tr>
<tr>
<td>HCA</td>
<td>1000</td>
<td>0*</td>
<td>-</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0*</td>
<td>-</td>
<td>2*</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>4*</td>
<td>9*</td>
<td>50*</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>95</td>
<td>40*</td>
<td>68*</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>109</td>
<td>56*</td>
<td>74*</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>100</td>
<td>72*</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>104</td>
<td>76*</td>
<td>109</td>
</tr>
<tr>
<td>Ceratiolin</td>
<td>125</td>
<td>102</td>
<td>83</td>
<td>102</td>
</tr>
</tbody>
</table>

¹Germinations (G) and radicle lengths (RL) are in per cent of the controls. An asterisk indicates significant difference from the control (p<0.05).

The objective of the current research is to monitor the release of allelochemicals by *C. ericoides*, and thus to determine if phytotoxins are present at sufficient concentrations in the environment surrounding the shrub to cause or contribute to the observed inhibition of neighboring species.
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release of phenolic compounds from soils, plant roots and leaf litter. *Soil

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CHAPTER II

Recovery and Decomposition of Hydrocinnamic Acid

in Florida Scrub Soil
INTRODUCTION

Phytotoxic aromatic acids have been found in various soils (for reviews see Stevenson, 1967; Patrick, 1971; and Hartley and Whitehead, 1985), and many of these acids have been implicated as allelopathic compounds. Bioassays have shown the cinnamic acid derivatives p-coumaric, ferulic, and hydrocinnamic acids to be seed germination and seedling growth inhibitors (Lynch, 1980; Blum, et al., 1984; Fischer, et al., 1988; Blum, et al., 1989); however, these acids are readily metabolized or decomposed in the soil by microbes (Haider and Martin, 1967, 1975; Martin and Haider, 1971; Turner and Rice, 1975; and Blum and Shafer, 1988). In addition, extraction procedures and soil characteristics can have a marked effect on the amounts of aromatic organic acids recovered from the soil (Dalton, et al., 1987); therefore, in studies addressing the allelopathic role of aromatic acids, such factors as the stability of the compound in the environment and the ease of extraction should be determined if ecologically relevant concentrations of these compounds are to be reported.

Hydrocinnamic acid (HCA) is a decomposition product of the dihydrochalcone ceratiolin. Ceratiolin has been isolated from Ceratiola ericoides, a shrub endemic to the Florida scrub community (Tanrisever, et al., 1987). A bare zone surrounds mature C. ericoides shrubs, and previous investigations have indicated that allelochemicals contribute to this phenomenon (Richardson, 1988). HCA has been shown to inhibit germination and radicle growth of grasses
common to the neighboring Florida sandhill community (Fischer, et al., 1988).
The objective of this work was to determine the fate and recovery of HCA
applied exogenously to scrub soil.

METHODS AND MATERIALS

General. Soil was collected in November of 1989 near a stand of C. ericoides
shrubs in a scrub area near Sun Ray, Florida, and was then cooled and shipped
overnight to Baton Rouge, Louisiana. To ensure a representative sample, the
soil was mixed well and was used immediately. Large pieces of organic matter
were removed by hand. For each sample, 25 g soil in a 125 ml Erlenmeyer
flask were used, and for each addition of HCA, 2.5 ml of an aqueous solution of
HCA were applied. After addition of HCA in solution, samples were allowed to
equilbrate for 2 hours (Graham-Bryce and Briggs, 1970). Samples were
maintained at approximately 24°C in darkness until extraction. The number of
replicates was three.

Samples were extracted in 50 ml distilled water. The flasks were sealed
with parafilm and were shaken for 2 hours at 24°C. The samples were then
filtered through Whatman No. 2 filter paper. Prior to analysis, samples were
filtered through 0.2 μm Nylon 66 membrane filters.

HCA and acetophenone were quantified by HPLC using a Hewlett
Packard 1090 Liquid Chromatograph equipped with a diode array detector and
auto-injector (250 μl syringe). Detection was at 257 nm (wavelength of
maximum absorbance for HCA) with a band width of 8 nm. Chromatograms were integrated using a Hewlett Packard HPLC ChemStation (Series 300 computer).

A C₁₈ reversed phase column, 10 μm Carbosphere, (250 mm x 4.6 mm I.D., Phenomenex, Palo Alto, California) was used for separation of the compounds. Two solvents were used: (A) HPLC grade methanol and acetic acid (99.5:0.5); and (B) doubly distilled deionized water and acetic acid (99.5:0.5). The gradient was from 50% A to 100% A in 17 minutes. Identification of HCA and acetophenone was confirmed by comparison of retention times and UV spectra with those of standards. HCA was quantified by comparison of peak areas with those of standards (least-squares method was used). Acetophenone was quantified by calculating a response factor for acetophenone relative to HCA using prepared standards. One set of soil samples to which no HCA was added was extracted. No HCA was recovered from these samples.

Recovery of HCA. Seven levels of application were used to test the efficiency of recovery of HCA from non-sterile soil. The amounts of HCA added to the soil were 1 μg/g, 2 μg/g, 4 μg/g, 8 μg/g, 16 μg/g, 32 μg/g, and 64 μg/g. For the sterile soil sets, soil was sterilized by 12 hour exposure to ethylene oxide in a closed polyethylene bag. Flasks were autoclaved. Solutions of HCA and extractant were filter sterilized using a 0.2 μm triacetate Membrane (Gelman Sciences) membrane filter. Three levels of application were used for sterile soil: 2 μg/g, 4
μg/g, and 8 μg/g. After addition of HCA, each sample was allowed to equilibrate for 2 hours before extraction. All samples at an application rate of 16 μg/g or less were concentrated twenty-fold prior to analysis by lyophilizing 20 ml solution and taking up the residue in 1 ml of 50/50 water/methanol. This solution was then filtered through 0.2 μ Nylon 66 membrane filters.

Persistence of HCA. In this study, all flasks and equipment were sterilized by autoclaving. HCA solution and extractant were filter sterilized. Non-sterile soil was used. To each sample, 3.75 mg HCA (2.5 ml HCA solution, 1.5 g/l, pH adjusted to 5.0 with NaOH) were aseptically transferred. The flasks were then plugged with sterile cotton. Sets of samples (3 reps each) were then extracted and analyzed at 12, 30, 60, and 144 hours.

One ml of filtered sterilized deionized water was added to each flask daily to maintain the soil moisture content. After three days the soil was quite wet, and these additions were terminated. As a control, 3.75 mg was added to one sample of sterilized soil. This sample was extracted after 60 hours.

Accumulation of HCA. All flasks and equipment were sterilized by autoclaving, and HCA solution and extractant were filter sterilized. Two and one half ml HCA solution (1.5 g/l) were added to the soil every 48 hours. Sets of samples were then extracted 48 hours later. A total of six applications of HCA were carried out in order to determine if HCA would accumulate in the soil if supplied on a continuous basis (Blum and Shafer, 1988).
Data Analysis. Least squares regression and determination of concentrations were performed using a LOTUS 1-2-3 spreadsheet (Lotus Development Corporation, 1985). Data were further analyzed using analysis of variance, one way (SAS Institute, Inc., 1985). Least significant differences were determined at a level of 0.05.

RESULTS AND DISCUSSION

Recovery of HCA from Soil. The per cent recovery of exogenously applied HCA from non-sterile soil varied significantly with the level of application [Figure 2.1]. At a level of 1 μg/g soil, 18% of the HCA was recovered, while at 64 μg/g soil, 79% of the HCA was recovered. The recovery of HCA from sterile soil also varied with level of application and was significantly higher than the recovery from non-sterile soil at the same level of application. For applications of 2 μg/g, 4 μg/g, and 8 μg/g, the recovery from non-sterile soil was 26%, 28%, and 34%, while from sterile soil the recovery was 91%, 78%, and 80%, respectively.

Sorption of phenolic acids tends to increase with organic matter content of soils (Dalton, et al., 1989). The scrub soil is primarily sand (>93%), with only small amounts of clay (3%) and organic matter (0.5%) (Weidenhamer and Romeo, 1989). Thus, the effect of microbial breakdown of HCA during the 2 hour equilibration time and the 2 hour extraction time was much more important than the sorption of HCA in the reduction of per cent recovery.
Persistence of HCA. Amounts of water-extractable HCA declined with time [Figure 2.2]. At 30 hours, only 36% of the HCA added to the soil was extracted, and no HCA was detected after 60 hours. After 12 hours, an additional peak appeared in the chromatogram. This peak was identified as acetophenone (ACP). The amount of ACP in non-sterile soil samples increased to a maximum at 60 hours, and decreased to near zero at 144 hours; however, in sterile soil extracted 60 hours after the application of HCA, ACP was not detected. Thus, the ACP is from microbial, rather than air-oxidative decomposition of HCA. In addition, HCA in aqueous solution left at room temperature for approximately 10 days decomposed to ACP.

Accumulation of HCA. When applied to soil on alternate days and extracted 48 hours later, HCA accumulated in the soil [Figure 2.3]. After 5 additions, the amount of HCA extracted reached a level 75% higher than the rate at which HCA was applied. ACP was detected in the soil extracts, but the level of ACP did not vary significantly throughout the duration of the experiment. Thus, the rate of increase in the amount of HCA applied to the soil in this study, 150 μg/g per 48 hours (1 μ mole/g per 48 hours), was greater than the rate of formation of ACP; however, the rate of formation of ACP from HCA is approximately equal to the rate of decomposition of ACP.

The phenolic acids ferulic, p-coumaric, p-hydroxy benzoic, and vanillic have been shown to accumulate in soils in a similar manner. In addition, the
phenolic acids were shown to stimulate bacterial populations in the soil (Blum and Shafer, 1988).

Organic acids released into the soil by plants can be utilized as a carbon source by many heterotrophic bacteria and fungi (Wang, 1967; Flaig, 1971; Haider and Martin, 1975; Stevenson, 1982). However, the rate of this decomposition is dependent on many factors such as temperature, soil texture, and nutrient and moisture content (Haider and Martin, 1975; Kowalenko, et al., 1978; Kassim, 1982; Blum and Shafer, 1988). The sandy soil of the Florida scrub is nutrient poor (Kalisz and Stone, 1984; Weidenhamer and Romeo, 1989). In addition, high soil temperatures in the summer and low moisture content in the dry season and during summer dry periods (Richardson, 1985) are factors that can affect microbial populations in the field. In this study, temperatures were moderate and soil moisture was maintained at a high level. Therefore, the rate of decomposition of HCA under laboratory and field conditions could be quite different. It should be noted that the effect of the decomposition of HCA during extraction procedures could affect concentrations of HCA determined in field samples.
ACKNOWLEDGMENTS

The authors wish to thank D.K. Granger for technical assistance, Dr. J.D. Weidenhamer for his assistance in experimental design, and Dr. Don Richardson for collection of soil samples. This material is based upon work supported by the Cooperative State Research Service, U.S. Department of Agriculture under Agreement No. 88-33520-4077 of the Competitive Research Grants for Forest and Rangeland Renewable Resources.
FIG 2.1 Recovery of exogenously applied hydrocinnamic acid (HCA) to sterile and non-sterile soil at different levels of application (1, 2, 4, 8, 16, 32, and 64 µg/g)
FIG 2.2 Time-dependent depletion of hydrocinnamic acid (HCA) applied to the soil (1 μ mole/g); and recovery of the decomposition product acetophenone (ACP) Values represent means (n=3).
FIG 2.3 Accumulation of hydrocinnamic acid (HCA) applied to soil every 2 days (150 ug/g, indicated on plot by dotted line) and extracted 48 hours later; and recovery of the decomposition product acetophenone (ACP)
REFERENCES


CHAPTER III

Determination of Water Soluble Allelochemicals

Released by Ceratiola ericoides
INTRODUCTION

The sand pine scrub community exists on the well-drained sandy ridges of relict shorelines in Florida. The scrub is characterized by groups of sand pine [Pinus clausa (Chapm. ex Engelm) Vasey ex Sarg.] and oaks forming a dense canopy with no herbaceous ground cover (Laessle, 1958). Disturbed sites are colonized by shrubs such as Ceratiola ericoides Michx., and Conradina canescens (Torr & Gray) A. Gray. These shrubs are surrounded by open areas in which there is little or no herbaceous growth. Grasses that are members of the neighboring sandhill community, such as Schizachyrium scoparium (Michx.) Nash, Andropogon gyrans Ashe, and Leptochloa dubia (H.B.K.) Nees, are also excluded from these bare zones.

The possibility of chemical inhibition of grasses and herbs by scrub species has been investigated (Tanrisever, et al., 1987; Fischer, et al., 1988; Richardson and Williamson, 1988; Weidenhamer and Romeo, 1989; Williamson, et al., 1989). Bioassays, testing the effect of aqueous extracts of C. ericoides leaves and litter on germination and radicle growth of S. scoparium, have shown litter extracts to be inhibitory. In addition, this activity was seasonal in degree, and the highest activities were exhibited in extracts taken during the summer months (Richardson, 1985).

Chemical investigation of C. ericoides has resulted in the identification of the dihydrochalcone ceratiolin [Figure 3.1] in aqueous extracts of whole leaves
FIG 3.1 Water soluble allelochemicals released by *Ceratiola ericoides* and their degradation products
(Tanrisever, et al., 1987). Bioassays have shown ceratiolin to be noninhibitory of germination and radicle growth of *S. scoparium*; however, ceratiolin decomposes readily in aqueous solution to hydrocinnamic acid (HCA), which has shown considerable inhibitory activity (Fischer, et al., 1988). In addition, hydrocinnamic acid is decomposed in non-sterile soil or in aqueous solution to acetophenone (Jordan, previous chapter).

It was the objective of the current study to monitor the release of ceratiolin and hydrocinnamic acid into the environment surrounding *C. ericoides* shrubs. In addition, trans-cinnamic acid (TCA) and acetophenone (ACP) were determined.

**METHODS AND MATERIALS**

**Collection and Extraction of Samples.** Three healthy, mature, individual *C. ericoides* shrubs in a scrub area near Sun Ray, Florida, were chosen for this study. Four types of samples—leaf soak, leaf mist, litter, and soil—were taken monthly from December, 1986 to April, 1987; twice monthly from March, 1987 to October, 1987; and monthly from October, 1987 to December, 1988. Samples were packed with ice packs and sent to Baton Rouge, LA overnight. All samples were processed immediately. For the leaf soaks, 50 g whole leaves were soaked in 100 ml distilled water for 24 hours (during shipping) at 10-20°C. The samples were then filtered and the filtrate stored at -40°C. Leaf mists consisted of 100 ml distilled water that was misted over the leaves of each of
the three shrubs and collected using a funnel. The resulting extract was filtered and the filtrate stored at -40°C.

Surface litter was collected from beneath each of the three shrubs, and 50 g of this litter was shaken with 150 ml water for 2 hours at approximately 24°C. The samples were then filtered and the filtrate stored.

A soil plug, 8.5 cm diameter x 8.5 cm depth, was taken from beneath each of the three shrubs. After mixing the soil thoroughly, 25 g was extracted by shaking with 50 ml distilled water for 2 hours at approximately 24°C. The samples were then filtered and the filtrate stored. All extracts were stored at -40°C.

Prior to analysis, soil and litter extracts were concentrated by lyophilizing an aliquot (20 ml for soil, 10 ml for litter) of the extract. The residue was taken up in 4 x 1 ml portions of methanol, which was evaporated to 1 ml in a 1 ml volumetric flask. This procedure, when applied to HCA and TCA standards, showed no loss of HCA or TCA. Leaf soaks and leaf mists were analyzed without concentration. All samples were filtered through Nylon 66 membrane filters before HPLC analysis.

**HPLC Analysis of Extracts.** Compounds were quantified using a Hewlett Packard 1090 Liquid Chromatograph equipped with a photodiode array detector and an auto-injector (250 μl syringe). The detection channel was set at 257 nm with a band width of 8 nm. A Hewlett Packard HPLC ChemStation (Series 300
computer) was used to record and integrate chromatograms. The column was a 10 μm Carbosphere C18 column (250 mm x 4.6 mm I.D.). Two solvents were used: A) HPLC grade methanol and acetic acid, and B) double distilled, deionized water and acetic acid (99.5:0.5). The gradient was from 50% A to 100% A in 17 minutes. Injections of 250 μl were made of leaf soak and leaf mist extracts. For the soil and litter extracts, an injection program was used to mix 125 μl sample and 125 μl water prior to injection. This procedure yielded improved peak shapes over injections of pure methanolic samples.

Identification of HCA, TCA, ACP, and ceratiolin was confirmed by comparison of retention times and UV spectra with those of standard compounds. HCA was quantified by applying least squares regression using HCA standards. Ceratiolin, ACP, and TCA were quantified by calculating a response factor to HCA using standard compounds.

Data Analysis. Regression analysis and other calculations were performed using a Lotus 123 spreadsheet (Lotus Development Corp., 1985).

RESULTS AND DISCUSSION

Leaf Soaks. HCA, TCA, ACP, and ceratiolin were all detected consistently in the leaf soaks from the three shrubs (Figures 3.2-3.5); however, quantities of the compounds differed significantly, and μ molar amounts present in the soaks followed the order ceratiolin > HCA > ACP > TCA. Although the rate of decomposition of ceratiolin to HCA is not known, the relative amounts of
ceratiolin, HCA, and ACP in leaf soaks suggest that the rate of release of ceratiolin into solution was greater than the rate of decomposition into HCA. This decomposition is spontaneous and not microbial (Tanrisever, et al., 1987). The rate of formation of HCA was in turn greater than the rate of formation of ACP. It is important to note, however, that microbial activity is affected by factors such as substrate concentration and temperature (Gray and Williams, 1971); therefore, some differences in concentrations seen in samples from different months may have resulted from slightly different conditions present during shipping of the samples.

There were differences in the concentrations of compounds found in the leaf soaks from the individual shrubs. The concentrations of ceratiolin was generally higher in Plant 1 than in Plant 2 or 3. The concentrations of HCA and ACP varied in the soak from the three shrubs, but no individual plant produced consistently higher concentrations of these compounds. It might be expected that the leaf soaks that contained the highest concentrations of ceratiolin would also contain high concentrations of HCA or ACP, as ceratiolin is the precursor for these compounds (Tanrisever, et al., 1987; Jordan, unpublished). This was not always the case, however. The early July leaf soak for Plant 1 contained a significantly higher amount of ceratiolin than the leaf soak for the sampling of Plants 2 and 3. The concentrations of HCA in the
early July soaks for Plants 1 and 2 were similar, and the concentration of ACP was highest for Plant 3.

The concentrations of the quantified compounds varied seasonally in the soaks from the three shrubs. Over the two year sampling period, all three shrubs exhibited maximum concentrations of ceratiolin in soaks obtained in July, August, or October of 1987 and August or September of 1988 (Figure 3.2). Minimum concentrations of ceratiolin were detected during the period from March to May of both years. The concentration of ceratiolin reached a local minimum in September of 1987 in soaks from all three individuals. The concentrations of HCA also varied seasonally (Figure 3.3), although for 1987, the concentration of HCA in Plant 1 did not reach a sharp maximum. The concentrations of ACP varied also, with high concentrations detected in July, August, and October of 1987; however, near-maximum concentrations were also detected in January and February of the same year.

**Leaf Mists.** Ceratiolin, HCA, and ACP were present much less consistently in the leaf mists than in the soaks (Figures 3.6-3.9). Also, quantities of ceratiolin were 100 to 1000 times less in the mists than the soaks, and HCA quantities were about ten-fold less. In contrast to the leaf soaks, in which a source of ceratiolin may have been present for 24 hours during shipping, the leaf mists contained a finite amount of dissolved ceratiolin. The breakdown of this ceratiolin without a source of renewal may have resulted in smaller quantities of
ceratiolin relative to HCA in the leaf mists. Quantities of ACP were also about
100 to 1000 times smaller in the rinses than in the soaks. TCA was also present
in much smaller quantities, but was again ubiquitous.

The leaf rinses were designed to mimic the action of natural rainfall, and
thus to indicate the quantities of allelochemicals released under field conditions.
However, the action of misting water over the leaves may have been somewhat
more gentle than the action of natural rain. Therefore, the concentrations
determined in the rinses may have been either higher or lower than those that
occur under field conditions.

Because the occurrence of the compounds in the mists was intermittent, a
seasonal trend was not manifested; however, ceratiolin was not detected in mists
collected in the winter and early spring months of January, February, and March
of 1987 or 1988 (Figure 3.6).

Litter Extracts. Results of the analyses of aqueous litter extracts are given in
Figures 3.10 and 3.11. The method of extraction of the litter and soil was
designed to extract ecologically significant amounts of compounds. Amounts of
phenolic compounds extracted with water without adjusting the pH have been
shown to be equivalent to the natural soil solution (Whitehead, et al., 1984).
Dalton, et al. (1987) have shown that there is not a significant increase in
amounts of phenolic acids extracted in water when extraction times were greater
than 2-3 hours. Only HCA and TCA were detected in the litter samples.
Lyophilization precluded the detection of the semi-volatile ACP. The concentrations of HCA, expressed in μ moles/kg, were significantly higher in litter collected from Plant 2, ranging from 2 to 107 μ moles/kg in samples containing HCA. Amounts of HCA determined in Plants 1 and 3 were from 1 to 35 μ moles/kg. In addition, amounts of HCA were much higher in extracts of litter sampled in 1987 than in those of 1988. Samples were taken twice monthly in the summer of 1987 and only once monthly in 1988.

The amounts of HCA extracted from litter samples also followed a pattern of seasonal variation, most notably in extracts of litter from Plant 3. This effect was not as marked as the effect observed in the leaf soaks. The release of compounds from litter is a product of microbial activity and water leaching, and the process can require months. The litter is colonized by microbes, and much of the colonization is confined to internal portions of the leaf. As incorporation of the organic matter into the soil occurs, external colonization takes place (Gray and Williams, 1971). Ceratiolin has been detected as a major component of C. ericoides leaves, but several other flavonoids have been isolated from ground leaves. These flavonoids, like ceratiolin, lack B-ring oxidation (Tanrisever, et al., 1987); therefore, the release of HCA from litter could be a product of microbial degradation of internal leaf flavonoids, and this process may be much slower than the degradation of the external compound ceratiolin.
Soil Extracts. Results of the analyses of aqueous soil extracts are shown in Figures 3.12-3.14 (soil data from July-December, 1988 are not available). Ceratiolin was detected only sporadically and at levels comparable to those reported in leaf mists. HCA was also detected in soil extracts, and the levels in $\mu$ moles/kg are shown in Figure 3.13. Soil taken from beneath Plant 1 contained detectable levels of HCA most often, and the levels were significantly higher than those in the soil collected from beneath Plants 2 and 3. The levels of HCA in the soil associated with Plant 1 showed a pattern of seasonal dependence, and like the leaf soaks, there was a large drop in the levels detected in mid-summer of 1987.

The effects of soil associated with sand pine scrub species (Weidenhamer and Romeo, 1989), as well as the effect of leaf soaks and litter leachates upon germination and growth of sandhill grasses, have been shown to follow a pattern of seasonal dependence (Richardson and Williamson, 1988). In particular, leachates of litter associated with *C. ericoides* were shown to be inhibitory, and the inhibition was concentrated in the late spring and summer. Leaf soaks were not inhibitory (Richardson and Williamson, 1988). Therefore, the concentrations of toxic allelochemicals released by *C. ericoides* were expected to vary during the year. However, the interactions that result in the presence of a toxic substance in the soil at sufficiently high concentrations to be toxic to target seeds are quite complex. In this case, ceratiolin, released by leaves, decomposes to
HCA, which is toxic. There is evidence (Tanrisever, 1986) that the decomposition of ceratiolin may be promoted by high temperatures. HCA is, in turn, microbially transformed into ACP, and microbial decomposition of litter may also release additional allelochemicals (Takijima, 1964; Gray and Williams, 1971; Lynch, 1983). Microbial activity can be affected by a number of factors. High soil moisture and temperature promote activity. Soils with high clay content have higher activity, and the type of substrate and its concentration can affect activity (Gray and Williams, 1971; Martin and Haider, 1979; Kassim, et al., 1982; Stotsky, 1986; Blum and Shafer, 1988). The onset of the summer rainy season in the scrub occurs in May, and the precipitation is highest from June through September. In addition, temperatures are highest in June through September (NOAA, 1983). These factors may influence the available concentrations of toxins in the soil.

HCA has been shown to significantly inhibit the sandhill grass *Schizachyrium scoparium* germination and radicle growth at 63 ppm (Fischer, et al., 1988), and chronic applications of 15-25 ppm have been shown to affect root growth (Williamson, unpublished). In this study, the maximum concentration of HCA in the leaf soaks was 24 ppm. In addition, the HCA detected in the soil, if in solution in the moisture present in the soil at approximately 2% moisture by weight, ranged from less than 1 ppm to greater than 270 ppm. The actual effect of HCA on target species in the field may be different from that shown in
bioassays. Plants in the scrub experience adverse conditions, such as high soil temperatures, moisture stress during the dry season (Richardson, 1985), and low availability of soil nutrients (Kalisz and Stone, 1984; Weidenhamer and Romeo, 1989). These factors can intensify the effects of toxins (Einhellig and Eckrich, 1984). In addition, allelochemicals can induce water-stress, interfere with photosynthetic mechanisms (Einhellig, et al., 1970; Lodhi and Nickell, 1973), and interfere with nutrient uptake (Glass, 1974a, 1974b).

Conclusion. In summary, the results presented here provide evidence that a compound previously isolated from C. ericoides and its toxic decomposition product are released into the environment through leaching from leaves or litter. Further investigation of the toxicity of ACP and possible synergistic or additive effects of the compounds reported here is needed to more fully understand the observed allelopathic activity of C. ericoides.

ACKNOWLEDGMENTS

The authors would like to thank D.K. Granger for technical assistance. This material is based upon work supported by the Cooperative State Research Service, U.S. Department of Agriculture under Agreement No. 88-33520-4077 of the Competitive Research Grants Program for Forest and Rangeland Renewable Resources.
FIG 3.2 Concentration of ceratiolin in *Ceratiola ericoides* leaf soaks
FIG 3.3 Concentration of hydrocinnamic acid in *Ceratiola ericoides* leaf soaks
FIG 3.4 Concentration of acetophenone in *Ceratiola ericoides* leaf soaks
FIG 3.5 Concentration of trans-cinnamic acid in *Ceratiola ericoides* leaf soaks
FIG 3.6 Concentration of ceratiolin in *Ceratiola ericoides* leaf mists
FIG 3.7 Concentration of hydrocinnamic acid in *Ceratiola ericoides* leaf mists
FIG 3.8 Concentration of acetophenone in *Ceratiola ericoides* leaf mists
FIG 3.9 Concentration of trans-cinnamic acid in Ceratiola ericoide leaf mists
FIG 3.10  Concentration of hydrocinnamic acid in *Ceratiola ericoide* litter
FIG 3.11 Concentration of trans-cinammic acid in *Ceratiola ericoides* litter
FIG 3.12 Concentration of ceratiolin in *Ceratiola ericoides* soil
FIG 3.13 Concentration of hydrocinnamic acid in *Ceratiola ericoides* soil.
FIG 3.14 Concentration of trans-cinnamic acid in *Ceratiola ericoides* soil
REFERENCES


CHAPTER IV

Volatile Compounds from \textit{Ceratiola ericoides}
PART I

Whole Leaves
INTRODUCTION

*Ceratiola ericoides* is an evergreen shrub endemic to the Florida scrub community, which occurs on the upland, well-drained sandy soils along Florida’s central ridge. The herbaceous vegetation surrounding mature *C. ericoides* shrubs is sparse. Field and laboratory studies (Richardson and Williamson, 1988) have provided strong evidence of the allelopathic activity of the shrub, although the chemical nature of this activity has not been well understood. An early study (Richardson and Williamson, 1988) of *C. ericoides* aqueous leaf extracts has indicated a mild inhibitory effect on germination and radicle growth of grasses native to the neighboring Florida sandhill community. This activity followed a pattern of seasonal dependence, with the highest activity occurring in the summer months.

In a previous chemical investigation of *C. ericoides*, several flavonoids, including the dihydrochalcone ceratiolin, were identified. Although ceratiolin exhibited little phytotoxicity, it decomposed in water to dihydrocinnamic acid, a considerably more phytotoxic compound (Tanrisever, et al., 1987). In addition, several monoterpenes and sesquiterpenes were detected in the steam distillate of a hexane extract of ground *C. ericoides* leaves (Tanrisever, 1986); however, there has been no reported investigation of volatiles from whole *C. ericoides* leaves. In the present study, we investigated headspace volatiles released by whole *C. ericoides* leaves collected in the spring, summer, and fall. The volatiles were
collected by dynamic headspace sampling and trapped on Tenax TA. Subsequently, they were desorbed thermally with partial-column cryofocusing, separated, and identified by GC-MS. This technique avoids the introduction of solvent impurities and other artifacts formed during sample preparation.

METHODS AND MATERIALS

Plant material. Whole leaves and stems of *Ceratiola ericoides* shrubs were collected near Sun Ray, Florida, in the fall of 1988 and the spring and summer of 1989 and placed in glass jars with Teflon-lined caps. The jars were then packed with ice and shipped overnight to Baton Rouge, Louisiana. The jars of plant material were stored at 0-5°C, and the plant material was analyzed within ten days.

Collection of volatiles. Whole leaves and stems (40 g) were placed in a 0.5-l glass purge vessel fitted with an inlet and an exit port. High purity He (99.999%), further purified by passage through an O₂ scrubber and a hydrocarbon trap, was forced by positive pressure into the glass vessel through the inlet port. Connected to the exit port was a sorbent trap, consisting of a stainless steel tube fitted with brass nuts and ferrules and containing 0.30 g of Tenax TA (2,6-diphenyl-p-phenylene oxide polymer, 60-80 mesh, Chrompack, Raritan, NJ), held in place by silanized glass wool plugs. The Tenax TA trap was initially conditioned at 340°C for 2 hrs with a He flow at 30 ml/min. Immediately before sample purging and trapping, the Tenax TA trap was baked
at 225°C for 10 min to ensure no carry-over compounds from previous analysis. The He contacted copper tubing, glass, and teflon only; metal tubing to glass tubing connections were made using brass fittings and teflon ferrules. The glass purge vessel had a ground glass closure that was sealed from the outside with teflon tape and held in place with springs. The flow rate of the He (40 ml/min) was measured at the exit end of the Tenax TA trap using a bubble meter. The leaves were purged for 16 hrs at 20-24°C. Control blank runs using the glass purge vessel with no plant material were run in the same manner. After sample, the trap was connected directly to the purified He and purged for 40 minutes to remove any accumulated moisture according to the procedure of Williams, et al. (1988).

Desorption and GC-MS. The volatiles were thermally desorbed (185°C, 15 min) from the Tenax trap installed in a Tekmar model 4000 Dynamic Headspace Concentrator and flushed through a heated transfer line (120°C) into a 60 m length x 0.25 mm i.d. x0.25 μm film thickness Supelcowax 10 column (Supelco Inc., Bellafonte, PA). Prior to desorption, a ca. 10 cm loop of the column at the injector end was lowered into a liquid N₂ bath, where it remained during desorption in order to cryogenically focus the desorbed compounds in the GC column. During desorption, the carrier gas head pressure was 30 psi, and the septum purge and the split vent were closed. After the desorption period, the liquid N₂ bath was removed and the oven temperature programming was begun.
Chromatograms were obtained with a Hewlett Packard 5792 gas chromatograph interfaced to a model 5970 mass selective detector under the following conditions: carrier gas head pressure, 15 psi; carrier gas linear velocity, 25 cm/sec; carrier gas flow rate, 0.74 ml/min; electron ionization at 70 eV; and electron multiplier voltage, 1800 V. The GC oven temperature was programmed from an initial temperature of 30°C, held for 10 min, to 175°C at 2°/min. The final temperature was held for 15 min.

The column was baked at 195°C for 15 min. before reuse. Per cent peak area was calculated by electronic integration with an HP59970C MS ChemStation program with a threshold of 16 and a peak width of 0.15.

Calculation of Retention Indices. Standard normal alkanes (C₈-C₂₂) were used as retention references, and retention indices were calculated according to van den Dool and Kratz (1963). The summer collection of leaves was spiked with the alkanes by adding 3 µl of n-alkanes in solution in hexane (1000 ng/µl) to the side of the glass purge vessel. Collection of volatiles, desorption, and GC-MS was carried out as above, except for a 7 min. solvent delay in the GC-MS procedure.

GC-MS of Standard Compounds. One µl of a standard mixture containing 1000 ng/µl of standard and 1000 ng/µl of each n-alkane (C₈-C₂₂) was injected at a 30:1 split ratio. The solvent delay was 7 min., and all other GC and MS conditions were the same as above. Retention indices were calculated for the standards,
and the RI’s and the mass spectra were used to identify the leaf volatiles. Where authentic standards were not available, tentative identification of leaf volatiles was made based on computer matching of the unknown spectra with reference mass spectra of the NBS/NIH/EPA/MSDC Data Base (1986) installed on the HP MSD ChemStation.

RESULTS AND DISCUSSION

Gas chromatograms of volatile compounds from spring, summer, and fall C. ericoides leaves are shown in Figures 4.I.1-4.I.3. Airborne volatiles identified from whole C. ericoides leaves are listed in Table 4.I.1. A total of 103 volatile compounds were detected, of which 91 were identified, while 12 were partially characterized. In addition to these compounds, several unidentified peaks, detected from each sample, were in low abundance and are not listed in Table 4.I.1. Compounds were identified by comparing their retention indices (RI) and mass spectra with authentic reference compounds. Those without standard RI’s were tentatively identified by computer matching of their mass spectra with library mass spectra.

The composition of volatile compounds in C. ericoides differs in leaves collected in spring, summer, and fall. Among the compounds identified, 30 occur in 2 of the 3 samplings, and 19 occur in all three.

Aromatics. Alkyl benzenes were detected in all samples of C. ericoides leaves in low abundance, as indicated by %peak area in Table 4.I.1. The spring collection
of leaves contained the widest variety of alkyl benzenes, including the ortho-, meta-, and para-isomers of dimethyl- and ethyl toluenes, styrene, and 1,3,5-trimethyl benzene. Ethyl benzene was identified in all of the samples. Oxygenated aromatics appeared more consistently, with acetophenone and ethyl benzoate occurring at moderate to low abundance in all 3 samples. Other aromatics identified were benzylalcohol, benzaldehyde, and methoxybenzene, all of which appeared in low abundance. Aromatic compounds accounted for only about 3%, 1%, and 1% of the spring, summer, and fall leaf collections, respectively.

**Esters.** The esters make up a more substantial portion of the volatiles collected, contributing about 21%, 5%, and 38% of the total volatiles from leaves collected in spring, summer, and fall, respectively. Esters were the major volatile compounds of leaves collected in the fall, and ethyl acetate and ethyl butanoate were the most abundant compounds, each constituting about 10% of the total volatiles of this sample. Ethyl acetate was also the major compound found in spring leaves at about 11% of the total peak area. Ethyl 2-methylbutanoate was also present in the fall leaves at about 10% of the total fall leaf volatiles. Other esters include various combinations of ethyl-, propyl-, butyl-, and hexyl- esters of acetic, propanoic, butyric, isovaleric, and hexanoic acids. In addition, several esters that have unsaturation either in the ester or the acid moiety were
detected. One cyclic ester, γ-butyrolactone, was detected in the volatiles collected from leaves sampled in the spring, summer, and fall.

Esters have been shown to have germination inhibitory activity on lettuce seeds (*Lactuca sativa* L. cv. Great Lakes) (Reynolds, 1989). The inhibitory properties of the ester are influenced by the length of the ester chain, the length of the acid moiety, and the degree of unsaturation in the molecule. For example, the inhibitory activity of ethyl acrylate is about 10 times that of ethyl propionate, which is about twice as active as propyl acetate. Propyl acetate is in turn about twice as active as ethyl acetate. In general, activity of these compounds increases with lipophilicity; however, linear esters are usually less active than their precursor acid and alcohol (Reynolds, 1989). There were no acids detected in the volatiles collected from any of the *C. ericoides* leaf samples. Lactonization can cause an increase in inhibitory activity over the parent acid, but in the case of γ-butyrolactone, the converse is true. However, γ-butyrolactone is mildly inhibitory against lettuce seed germination (Reynolds, 1989). Therefore, the esters present in *C. ericoides* volatiles may exhibit a wide range of inhibitory activity.

**Alcohols, Aldehydes, and Ketones.** The alcohols, aldehydes, and ketones make up a large part of the volatiles collected from *C. ericoides* leaves. This is the major group of compounds detected in the summer foliage, supplying 75% of the total peak area. Carboxyl compounds make up only 5-8% of the total peak
area for all samples, and 2,3-butanedione is the most abundant of these (4.2%, 5.2%, and 5.0% of all volatiles from spring, summer, and fall leaves). The most abundant constituent of the volatiles collected from the summer foliage, and unique to this collection of leaves, was 1-octen-3-ol, making up 34% of the total peak area. Other prominent compounds were 1-hexanol, 3-methyl-1-butanol, and 2-hexen-1-ol.

Two substituted furans were detected in low abundance as volatiles from leaves of *C. ericoides*. From leaves sampled in the fall, 2,3-dihydro-4-methylfuran was identified, and from leaves sampled in the summer, 3-(4-methyl-3-pentenyl)furan was identified. Both compounds were present in an abundance of <1%. Many alcohols, aldehydes, and ketones exhibit biological activity. The ketones 2-butanone and 3-pentanone, the alcohols 1-hexanol and 3-methyl-1-butanol, and the aldehyde 1-hexanal have been detected in plant residues (Connick, *et al.*, 1987) and are germination inhibitors for a variety of test seed (Bradow and Connick, 1988a; Bradow and Connick, 1988b). 1-Butanol and 6-methyl-5-hepten-2-one also have been shown to be germination inhibitors of lettuce seed (Reynolds, 1987; Reynolds, 1989). Reynolds (1989) and Bradow and Connick (1988a, 1988b) have found that, among similar compounds, increasing lipophilicity increased inhibitory activity. Methyl ketones have exhibited a stimulatory effect on some weed species (French, 1984; French, *et al.*, 1986), and it is thought that these compounds interact with seed membranes.
during the early stages of germination (Bradow and Connick, 1988a).

**Hydrocarbons.** A large number of hydrocarbons was detected from foliage, and these compounds accounted for 42%, 5%, and 8% of the volatiles collected from leaves sampled in spring, summer, and fall, respectively. These compounds included straight-chain and branched hydrocarbons, some of which contain 1 to 2 double bonds. These compounds were not positively identified because of difficulty in obtaining pure isomeric reference standards.

**Monoterpenes.** Eight monoterpenes were identified in *C. ericoides* leaf volatiles, seven of which are monoterpane hydrocarbons and one of which is an oxygenated monoterpane. Six of these compounds were positively identified, and the remaining 2 were partially characterized or tentatively identified. The monoterpenes occurred in very low abundance, however, with the most abundant, linalool (1% of total peak area), appearing in volatiles collected from the fall leaf sample.

Farnesol, geraniol propanoate, β-ionone, γ-ionone, camphor, and α-pinene have been detected previously in the steam distillate of a hexane extract of ground *C. ericoides* leaves (Tanrisever, 1986). α-Pinene was detected in this study in spring leaf volatiles. Monoterpenes that were found in *C. ericoides* have been detected in other shrubs endemic to the sand pine scrub community. p-Cymene has been identified in the steam distillate of whole leaves of *Conradina*
canescens (Williamson, et al., 1989), and α-pinene and limonene have been detected in the atmosphere surrounding Calamintha ashei (Tanrisever, 1986).

The monoterpene hydrocarbons myrcene, limonene, α-terpinene, and p-cymene exhibit moderate inhibitory action against lettuce seed germination, while the monoterpene alcohol linalool exhibits considerably higher activity (Reynolds, 1987).

Sesquiterpenes. A large number of sesquiterpene hydrocarbons (C_{15}H_{24}) were detected in C. ericoides leaf volatiles; however, none were positively identified. Six sesquiterpenes were tentatively identified by matching their mass spectra with those stored in a spectral library, while the remaining 10 were partially characterized. The sesquiterpenes occurred in low abundance, and α-murolene and 6-cadinene were the most abundant, each representing 1.9% of the total peak area for spring leaves.

Caryophyllene, humulene, and γ-amorphene have been reported previously from C. ericoides (Tanrisever, 1986). Caryophyllene, detected in spring, summer, and fall leaf samples, has been identified as a constituent of the steam distillate of Conradina canescens (Tanrisever, 1986).

Comparison of seasonal leaf samples. There was a marked difference in the number and abundance of different types of compounds detected in C. ericoides leaf volatiles from leaves sampled in the spring, summer, and fall. In spring leaves, alcohols, ketones, and aldehydes made up 10.9% of the total peak area,
esters accounted for 22.7%, and hydrocarbons were the most abundant at a total of 41.5%. Interestingly, the spring leaf sample contained the largest number of sesquiterpenes (16; 10.4% total peak area) and monoterpenes (7, accounting for only 1.3% peak area); however, the oxygenated monoterpe linalool was not detected in spring leaves.

Of the volatiles found in the summer leaf sample, alcohols, aldehydes, and ketones were by far the most abundant class of compounds, accounting for 75.5% of the total peak area. Two compounds, 1-octene-3-ol and 1-hexanol, together accounted for 50% of the total peak area.

Esters (38.2%) and alcohols, aldehydes, and ketones (18.9%) were the most abundant types of compounds detected in the fall leaf sample. This sample contained only 3 sesquiterpenes.

**Conclusion.** Dynamic headspace sample (DHS) of volatiles followed by thermal desorption has previously been used to study plant volatiles (Connick, *et al.*, 1987; Hernandez, *et al.*, 1989; Lwande, *et al.*, 1989) and DHS with on-column cryofocusing is used in food flavor analysis (Vejaphan, *et al.*, 1988; Hsieh, *et al.*, 1989). Although it has been reported (Tanrisever, 1986) that fresh *C. ericoides* leaves are odorless, DHS of leaves showed a large yield of volatile compounds. Many of these compounds have anti-germination activities (Reynolds, 1987; Bradow and Connick, 1988a; Bradow and Connick, 1988b; Reynolds, 1989); however, the role of these compounds as allelopathic agents cannot be assumed.
Volatile compounds have strong activities as insect attractants or repellents (Visser, 1983; Williams, et al., 1988). Therefore, an investigation of the volatiles present in the litter and soil surrounding *C. ericoides* is part of our on-going research.

**ACKNOWLEDGMENT**

The authors would like to thank J. Matiella and U. Tanchotikul for their technical assistance and Dr. D. Richardson for collection of plant samples. This material is based upon work supported by the Cooperative State Research Service, U.S. Department of Agriculture under Agreement No. 88-33520-4077 of the Competitive Research Grants Program for Forest and Rangeland Renewable Resources.
### Table 4.1.1. Volatile compounds in the dynamic headspace of *Ceratiola ericoides* leaves collected in the spring, summer, and fall.

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### HYDROCARBONS

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HYDROCARBONS (continued)

| 19  | a-pinene\(^b\)            | 1004   | 0.4    | --     | --     | --     | --     | 1003   |
| 31  | \(\beta\)-myrcene         | 1155   | 0.1    | 1158   | 0.2    | 1154   | 0.2    | 1166   |
| 32  | \(\alpha\)-terpinene      | 1162   | 0.1    | --     | --     | --     | --     | 1166   |
| 36  | 1-limonene                | 1180   | 0.4    | 1180   | 0.6    | 1180   | 0.2    | 1186   |
| 45  | cis-ocimene               | 1243   | 0.1    | 1235   | 0.1    | 1256   | 0.1    | --     |
| 48  | \(\beta\)-cymene          | 1258   | 0.1    | --     | --     | --     | --     | 1265   |
| 49  | monoterpene               | 1266   | 0.1    | --     | --     | 1285   | 0.1    | --     |
| 80  | linalool                  | --     | --     | 1533   | 0.1    | 1534   | 1.0    | 1549   |

MONOTERPENES

| 78  | sesquiterpene             | --     | 0.3    | 1497   | 0.1    | 1495   | 0.2    | --     |
| 82  | caryophyllene\(^b\)       | 1576   | 0.1    | 1571   | 0.1    | 1570   | 0.2    | --     |
| 87  | sesquiterpene             | --     | 0.1    | --     | 0.1    | --     | --     | --     |
| 90  | sesquiterpene             | --     | 0.1    | --     | --     | --     | --     | --     |
| 91  | sesquiterpene             | --     | 0.7    | --     | 0.1    | --     | --     | --     |
| 92  | sesquiterpene             | --     | 1.1    | --     | 0.1    | --     | 0.1    | --     |
| 93  | sesquiterpene             | --     | 0.3    | --     | --     | --     | --     | --     |
| 94  | \(\alpha\)-muurolene      | --     | 1.9    | --     | 0.1    | --     | --     | --     |
| 95  | \(\delta\)-cadinene       | --     | 1.9    | --     | --     | --     | --     | --     |
| 96  | sesquiterpene             | --     | 0.5    | --     | --     | --     | --     | --     |
| 97  | sesquiterpene             | --     | 0.4    | --     | --     | --     | --     | --     |
| 98  | sesquiterpene             | --     | 0.4    | --     | 0.1    | --     | --     | --     |
| 99  | \(\beta\)-cedrene         | --     | 0.2    | --     | --     | --     | --     | --     |
| 100 | 4-isopropyl-1,6-dimethyltetralin | -- | 1.6 | -- | 0.1 | -- | -- | -- |
| 102 | sesquiterpene             | --     | 0.5    | --     | 0.1    | --     | --     | --     |
| 103 | 8-isopropyl-2,5-dimethyltetralin | -- | 0.3 | -- | -- | -- | -- | -- |

SESQUITERPENES

\(\ast\) RI = Retention Index
\(\ast\) Compounds previously identified in *C. ericoides* volatiles (Tanrisever, 1986)
Figure 4.1.1 Total ion chromatogram of volatile compounds from *Ceratiola ericoides* leaves collected in the spring.
Figure 4.1.2 Total ion chromatogram of volatile compounds from *Ceratiola ericoides* leaves collected in the summer.
Figure 4.1.3 Total ion chromatogram of volatile compounds from *Ceratiola ericoides* leaves collected in the fall
REFERENCES


*Phytochemistry* 26: 175.


PART II

Associated Litter and Soil
INTRODUCTION

During the course of investigation of allelopathic interactions between members of the Florida scrub community and those of the neighboring sandhill community, laboratory and field studies have provided evidence of the allelopathic activity of Ceratiola ericoides (Florida wild rosemary), a shrub endemic to the scrub community. Mature shrubs or groups of shrubs are surrounded by a bare zone containing little or no herbaceous growth. A visible layer of litter collects directly beneath C. ericoides shrubs, and water leachates of litter have been shown to have an inhibitory effect on germination and radicle growth of grasses native to the sandhill (Richardson and Williamson, 1988).

Volatile compounds that inhibit germination or plant growth have been detected in soil and plant residues (del Moral and Muller, 1970; Halligan, 1975; Bradow and Connick, 1987).

Recently, the volatile compound composition of whole C. ericoides leaves has been reported. Identified from whole leaves were volatile esters, alcohols, and hydrocarbons in high abundance, and mono- and sesquiterpenes in low abundance (Jordan, Part I). The objective of the current study was to identify the major volatile compounds in soil and litter taken from beneath C. ericoides in the field.
METHODS AND MATERIALS

Plant material. Soil and litter were collected from beneath Ceratiola ericoides shrubs in a scrub area near Sun Ray, Florida, in the summer of 1989 and placed in separate glass jars with Teflon-lined caps. The jars were then packed with ice and shipped overnight to Baton Rouge, Louisiana. The jars of litter and soil were stored at -40°C.

Collection of volatiles. Headspace volatiles of soil (200 g) and litter (100 g) were collected according to the procedure described in Part 1 of Chapter IV. Subsequent analysis was as in Part 1.

RESULTS AND DISCUSSION

Volatile compounds identified in the headspace of soil and litter associated with C. ericoides are given in Table 4.II.1. The volatile compound compositions of the soil and litter were qualitatively and quantitatively different, although several of the major compounds were the same for both soil and litter. The compounds were quite well resolved (Figures 4.II.1 and 4.II.2) by this system, and 64 compounds were identified while 4 more were partially characterized.

The most abundant compounds detected (based on per cent peak area of each compound) in litter volatiles were 1-octene, 3-octanol, and 1-pentanol, together accounting for more than 45% of the total volatiles in the litter. Detected in highest abundance in the soil volatiles were 3-methyl-1-butanol, 3-
octanol, and 3-octanone. The soil volatiles contained significantly fewer and less abundant hydrocarbons than the litter. Both the litter and the soil volatiles contained a number of benzenoids; however, the abundance of these compounds was low. The same was true for the monoterpenes detected in soil and litter volatiles. The major class of compounds for both samples comprised alcohols, aldehydes, and ketones.

A number of the compounds detected in the litter or soil volatiles have been previously found in volatiles of whole C. ericoides leaves by dynamic headspace sampling (Jordan, et al., Part I). Hexane was a major component of spring leaf volatiles, while 1-hexanol and 1-octen-3-ol were prominent in the summer leaf volatile profile. As a class, esters accounted for a large part of C. ericoides leaf volatiles, but these compounds were not found in soil and litter volatiles with the exception of butyrolactone.

Many of the compounds listed in Table 1 have been shown to have phytotoxic activity. Bradow and Connick (1988a, 1988b) have tested vapors of several volatile alcohols, aldehydes, and ketones for germination inhibition of various test seed. In these assays, C7-C9 ketones were shown to be the most inhibitory of those tested, which included 3-methyl-2-butanone, 2-octanone, and 2-pentanone. Hexanal and 3-methyl-2-butanone exhibited similar activity to 2-pentanone. For the alcohols, there was no relationship between carbon chain length and activity, and 3-methyl-1-butanol showed consistently high activity.
In bioassays for germination inhibition of lettuce seed (*Lactuca sativa* c.v. Great Lakes) by compounds in solution, 1-butanol, butyrolactone, and 1-pentanol were all shown to be moderately active, and benzene and other alkyl benzenes were shown to be no more inhibitory than their straight chain analogues (Reynolds, 1989). In addition, linear esters were shown to be less inhibitory than the parent acid and alcohol; therefore, the alcohols detected in the litter and soil volatiles, such as 1-butanol, 1-hexanol, and 3-hexen-1-ol would be more active than the butyl-, hexyl-, and 3-hexenyl-esters detected in the leaf volatiles. The presence of a double bond in conjugation with a carbonyl or hydroxy-moiety, as in 2-methyl-1-peten-3-ol has been shown to increase inhibitory activity by a factor of 2 (Reynolds, 1987).

Early investigations of volatile toxins in soil and litter involved monoterpenes (Muller, 1965; Muller and del Moral, 1966; del Moral and Muller, 1970; Halligan, 1975). In investigations of the *Eucalyptus camaldulensis*, del Moral and Muller (1970) determined α-pinene and cineole to be highly toxic soil volatiles. Reynolds has shown camphor to be 100 times as inhibitory as cineole. Other monoterpenes shown to be active are β-myrcene, limonene, and borneol. The activities of these compounds were limited by their water solubility (Reynolds, 1987).

There are many factors that can affect the appearance, stability, availability, and activity of allelochemicals in the complex soil environment.
Microbial decomposition of compounds from leaves washed into the soil by rain or decomposition of litter on the soil surface can change the volatile profile of soil. Many of the volatile aldehydes and alcohols are products of the oxidative decomposition of leaf lipids (Frankel, 1982; Visser, 1983), a process soil microorganisms carry out readily (Moucawi, et al., 1981). In addition, soil microbes can transform many terpenes and benzenoids in the soil (Dagley, 1971; Muller and Chou, 1972); however, the microbial activity of soils is greatly affected by factors such as soil moisture, pH, temperature, and texture (Kowalenko, 1978; Allison, 1973; Moucawi, 1981; Kassim, et al., 1982). The scrub soil is over 90% sand, and the per cent organic matter is low (Weidenhamer and Romeo, 1989). In addition, the scrub experiences periods of moisture stress in the dry winter season and during dry periods in the summer (Richardson, 1988). The effect of these factors on the activity of soil volatiles in the scrub has not been characterized; therefore, further study of this phenomenon is in order.

ACKNOWLEDGMENT

The authors would like to thank J. Matiella and U. Tanchotikul for their technical assistance. This material is based upon work supported by the Cooperative State Research Service, U.S. Department of Agriculture under Agreement No. 88-33520-4077 of the Competitive Research Grants Program for Forest and Rangeland Renewable Resources.
Table 4.II.1. Volatile compounds identified in the headspace of litter and soil associated with *Ceratiola ericoides*.

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**AROMATICs**

**HYDROCARBONS**

- hexane\(^b\) 0.6 600 - -- 600
- 2-methyl-2-pentene 0.2 -- - -- --
- heptane\(^b\) 1.1 -- - -- --
1 2-heptene 0.7 -- - -- --
- octane\(^b\) 2.1 800 - -- 800
- 1-octene\(^b\) 17.4 -- 2.0 -- 837
- nonane 0.6 900 0.3 -- 900
- 1-methyl-2-pentylocyclopropane 0.6 -- - -- --
- 1,3-octadiene\(^b\) 1.2 -- - -- --
2 2,2,3,4-tetramethylpentane - -- 0.2 -- --
- 2,2,4,6,6-pentamethylheptane 0.9 1010 - -- --
- 5-(2-methylpropyl)nonane 0.6 -- - -- --
- 2,6-dimethyl undecane 0.4 -- - -- --
- undecane 8.6 1100 - -- 1100
Table 4.11.1.  Continued

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</table>

**HYDROCARBONS (continued)**

| 12  | branched alkane                  | 8.4    | 1144|      |     |     |     |     |
| 18  | 2,2,3,3-tetramethylhexane        |        |     | 1246 |     |     |     |     |
| 19  | branched alkane                  | 1.6    |     |      |     |     |     |     |
|     | branched alkane                  | 8.4    | 1144|      |     |     |     |     |
| 18  | 2,2,3,3-tetramethylhexane        | 0.1    | 1246|      |     |     |     |     |
|     | 4,7-dimethylundecane             | 0.2    | 1144|      |     |     |     |     |
|     | tridecane                        | 1.0    | 1300|      |     |     | 1300|     |

**MONOTERPENES**

| 19  | α-pineneb                        | 3.1    |     |      |     |     | 1003|     |
| 35  | camphor                          | 0.2    |     | 1705 |     |     |     |     |
|     | 3-methyl-2-butanone              | 0.7    |     |      |     |     |     |     |
| 4  | 2,3-butanedione                 | 1.5    |     | 928  |     |     |     |     |
|     | 2-methyl-2-bornene               | 1.5    |     |      |     |     |     |     |
| 11  | β-myrceneb                       | 0.1    | 1105|      |     |     |     |     |
| 14  | 1-limoneneb                      | 0.3    | 1166|      |     |     |     |     |
| 35  | camphor                          | 0.2    | 1517|      |     |     |     |     |
| 39  | endo-borneol                     | 0.3    | 1705|      |     |     |     |     |

**ALCOHOLS, ALDEHYDES, KETONES**

| 3   | 3-methyl-2-butanolone            | 0.7    |     |      |     |     |     |     |
| 4   | 2,3-butanedione                 | 1.5    |     | 928  |     |     |     |     |
|     | 2-methyl-3-pentane              | 0.6    |     |      |     |     |     |     |
|     | 3,4,4-trimethyl-2-cyclopenten-1-one | 0.2 |     |      |     |     |     |     |
| 7   | 2-hexenal                        | 0.1    | 1052|      |     |     |     |     |
| 8   | hexanalb                        | 0.5    | 1068|      |     |     | 1063|     |
|     | 3-heptanone                      | 0.1    |     |      |     |     |     |     |
| 15  | 1-butanolb                      | 1.5    | 1174| 1134 |     |     |     |     |
| 16  | 3-methyl-1-butanolb             | 6.5    | 1193| 1206 |     |     |     |     |
| 17  | 2-pentylfuran                    | 0.2    | 1196| 1237 |     |     |     |     |
| 19  | 1-pentanol                      | 13.4   | 1218|      |     |     |     |     |
| 20  | 3-octanoneb                     | 4.4    | 1258| 1247 |     |     |     |     |
| 23  | 2-methyl-1-penten-3-ol           | 0.7    | 1249|      |     |     |     |     |
Table 4.II.1.  Continued

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<td>RI</td>
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<td>0.1</td>
<td>1639</td>
<td>1638</td>
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**ALCOHOLS, ALDEHYDES, KETONES (continued)**

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<td>RI</td>
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<td>-</td>
<td>sesquiterpene</td>
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<td>1583</td>
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<td>1569</td>
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<sup>a</sup> No. corresponds to peak number on chromatogram.

<sup>b</sup> Compounds previously detected in *C. ericoides* whole leaf volatiles.

<sup>a</sup> RI = Retention Index (Van den Dool and Kratz, 1963). Compounds with no RI were tentatively identified from mass spectral data.
Figure 4.11.1 Total ion chromatogram of volatile compounds from litter associated with *Ceratiola ericoides*
Figure 4.II.2   Total ion chromatogram of volatile compounds from soil associated with Ceratola ericoides


In: P.A. Hedin, ed. Plant Resistance to Insects. ACS Symposium Series
No. 208, American Chemical Society, Washington, D.C.

Weidenhamer, J. and Romeo, J.T. 1989. Allelopathic properties of Polygonella

Abstracts of the 39th Pittsburgh Conference and Exposition on Analytical
FIG A.1 Calibration of Hydrocinamic acid (HCA) by HPLC

<table>
<thead>
<tr>
<th>CONC</th>
<th>RT</th>
<th>AREA A</th>
<th>AREA B</th>
<th>AVG AREA</th>
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<tbody>
<tr>
<td>20.0000</td>
<td>10.3600</td>
<td>125.4700</td>
<td>123.9900</td>
<td>124.7300</td>
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<tr>
<td>40.0000</td>
<td>8.40-8.46</td>
<td>242.4200</td>
<td>242.9000</td>
<td>242.6600</td>
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<tr>
<td>80.0000</td>
<td>8.40-8.45</td>
<td>482.0800</td>
<td>484.7900</td>
<td>483.4350</td>
</tr>
<tr>
<td>100.0000</td>
<td>8.40-8.45</td>
<td>602.3200</td>
<td>601.8700</td>
<td>602.0950</td>
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</tbody>
</table>

**HCA STANDARDS**

**Regression Output:**

- **Constant:** 4.5785
- **Std Err of Y Est:** 0.9707
- **R Squared:** 1.0000
- **No. of Observations:** 4.0000
- **Degrees of Freedom:** 2.0000

**X Coefficient(s):** 5.9775

**Std Err of Coef.:** 0.0153

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<th>X</th>
<th>Y</th>
<th>D = 16000.0000</th>
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<tbody>
<tr>
<td>20.0000</td>
<td>124.1290</td>
<td>SIGB = 1.0410</td>
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<tr>
<td>40.0000</td>
<td>243.6795</td>
<td>ERRAS = 1.4234</td>
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<td>80.0000</td>
<td>482.7805</td>
<td>%ERRM = 0.2568</td>
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<tr>
<td>100.0000</td>
<td>602.3310</td>
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Figure A.2 Chromatogram of extracted hydrocinnamic acid at 0 hours after application

MOBILE PHASE:
A = MEOH/HOAC (99.5/0.5)
B = H2O/HOAC (99.5/0.5)

GRADIENT:
50% A TO 100% A IN 17 MIN

WAVELENGTH = 257 nm
Figure A.3 Chromatogram of extracted hydrocinnamic acid and acetophenone 12 hours after hydrocinnamic acid application
Figure A.4 Chromatogram of extracted hydrocinnamic acid and acetophenone 30 hours after hydrocinnamic acid application.
Figure A.5 Chromatogram of extracted hydrocinnamic acid and acetophenone 60 hours after hydrocinnamic acid application

MOBILE PHASE:
A = MEOH/HOAC (99.5/0.5)
B = H2O/HOAC (99.5/0.5)

GRADIENT:
50% A TO 100% A IN 17 MIN

WAVELENGTH = 257 nm
Figure A.5 UV spectra of hydrocinnamic acid and acetophenone
<table>
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<tr>
<th>NAME</th>
<th>RT</th>
<th>CONC (ppm)</th>
<th>AREA A</th>
<th>AREA B</th>
<th>AREA C</th>
<th>AVE</th>
<th>AHT Cug</th>
<th>RF</th>
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<tr>
<td>Acetophenone</td>
<td>7.51-7.57</td>
<td>20.0000</td>
<td>8934.9200</td>
<td>8911.4600</td>
<td>8914.6100</td>
<td>8920.3300</td>
<td>3.9570</td>
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<td>7.52-7.55</td>
<td>40.0000</td>
<td>16866.0000</td>
<td>16951.0000</td>
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<td>16896.6667</td>
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<td>7.46-7.52</td>
<td>80.0000</td>
<td>30163.0000</td>
<td>30035.0000</td>
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<td>30126.0000</td>
<td>15.9200</td>
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<td>7.51-7.54</td>
<td>100.0000</td>
<td>33561.0000</td>
<td>33627.0000</td>
<td>33498.0000</td>
<td>33565.3333</td>
<td>19.7850</td>
<td>43.1304</td>
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</tbody>
</table>

| TCA   | 8.73-8.75 | 20.0000 | 12482.0000 | 12498.0000 | 12499.0000 | 12489.0000 | 3.9570 | 49.9246 |
|       | 8.69-8.74 | 40.0000 | 26584.0000 | 26581.0000 | 26565.3333 | 26556.3333 | 7.9140 | 77.4624 |
|       | 8.68-8.75 | 80.0000 | 43211.0000 | 43499.0000 | 47469.3333 | 47469.3333 | 15.0200 | 85.3139 |

|       | 8.18-8.21 | 40.0000 | 311.4000 | 311.8400 | 311.3683 | 7.9140 |
|       | 8.18-8.20 | 80.0000 | 641.7500 | 640.4200 | 640.8567 | 15.0200 |
|       | 8.22-8.23 | 100.0000 | 778.2400 | 778.2000 | 778.2500 | 19.7850 |

<table>
<thead>
<tr>
<th>Y MOLES</th>
<th>MOLAR RF</th>
<th>MOLAR A HCA</th>
<th>RF = average of areas R, B, &amp; C (3 reps at X ppm)</th>
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<tr>
<td>Acetophenone</td>
<td>0.0329</td>
<td>44.7816</td>
<td>394.5123</td>
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<tr>
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<td>0.0658</td>
<td>42.8039</td>
<td>40.0474 = MRF Avg.</td>
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<tr>
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<td>0.1317</td>
<td>38.3700</td>
<td>4.7123 = STD DEV</td>
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<td>0.1646</td>
<td>34.2343</td>
<td>380.4605</td>
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</tbody>
</table>

| TCA | 0.0267 | 76.9239 |
|     | 0.0534 | 82.8017 | 78.0514 = MRF Avg. |
|     | 0.1058 | 74.4208 | 4.2988 = STD DEV |
|     | 0.0263 | 76.9239 |
|     | 0.0527 | 82.8017 |
|     | 0.1317 | 78.0514 |

| HCA | 0.1054 |

AVE = average of areas R, B, & C (3 reps at X ppm)
MRF (ug) = concentration x 19785 = no. of ug's
RF = average area for that amount / average area for same amount HCA

121
LEAF SOAK FROM AUGUST OF 1987

MOBILE PHASE:
A = MeOH/HOAc (99.5/0.5)
B = H2O/HOAc (99.5/0.5)

GRADIENT:
50% A TO 10% A IN 17 MIN
WAVELENGTH = 257 NM

Figure A.7 Chromatogram of leaf soak
LEAF MIST FROM AUGUST OF 1987

MOBILE PHASE:
A = MeOH / HoRC (99.5/0.5)
B = H2O / HoRC (99.5/0.5)

GRADIENT:
50% A TO 100% A IN 17 MIN.

WAVELENGTH = 257 NM

Figure A.8 Chromatogram of leaf mist
Figure A.9 Chromatogram of litter extract
Figure A.10 Chromatogram of soil extract

MOBILE PHASE = 49.75% MEOH-49.75% H2O- 0.5% HOC
ELUTION: ISOCRATIC
WAVELENGTH = 257 nm
Figure A.11 UV spectra of allelochemicals released by *Ceratiola ericoides*
FIG A.12 Monoterpenes from *Ceratiola ericoides*
FIG A.13 Sesquiterpenes from *Ceratiola ericoides* leaves
A = (UHP) Ultra High Purity Helium
B = Metering Valve
C = Shut-Off Valve
D = Hydrocarbon Trap
E = Oxygen Trap
F = Kovar Metal-Glass Connection
G = Glass Sample Holder
H = Stainless Steel Column with Tenax TA
I = Metal Springs

FIG A.14 Dynamic headspace sampling apparatus
VITA

Elizabeth D. Jordan was born on April 4, 1963 in Athens, Georgia. She attended Washington School in Greenville, Mississippi, where she received her high school diploma in 1981. She received a B.A. with honors in mathematics and chemistry from Mississippi University for Women in Columbus, Mississippi in 1985. Since the fall of 1985, she has been working under Dr. N.H. Fischer in the Chemistry Department at Louisiana State University. She is a member of the American Chemical Society and Phi Kappa Phi National Honorary Society. She is currently a candidate for the Ph.D. degree in the Department of Chemistry.
Candidate: Elizabeth Douglas Jordan

Major Field: Chemistry (Analytical)

Title of Dissertation: Determination of Allelochemicals in the Environment Surrounding Ceratiola Ericoides

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: