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IN SEARCH OF ALLELOPATHY IN THE FLORIDA SCRUB: THE ROLE OF TERPENOIDS[1]

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Abstract—The hypothesis was tested that allelopathic agents released from fire-sensitive plants of the Florida scrub community deter the invasion of fire-prone sandhill grasses. The structures of the constituents of four endemic scrub species, *Conradina canescens*, *Calamintha ashei*, *Chrysoma pauciflosculosa*, and *Ceratiola ericiodes*, were established and their phytotoxic activity against two grasses of the sandhill was examined. Effects of the secondary metabolites from the above scrub species and their degradation products upon the germination and radicle growth of little bluestem (*Schizachyrium scoparium*) and green sprangletop (*Leptochloa dubia*), two native grasses of the Florida sandhill community, were determined. The studies included determination of the water solubility and release mechanism of terpenes and other allelopathic agents from the source plants and their aqueous transport to the target species. Some of the natural products were nontoxic until activated by light and/or oxidation after release from the source plant into the environment.

Key Words—*Calamintha ashei* (Labiatae), *Conradina canescens* (Labiatae), *Chrysoma pauciflosculosa* (Asteraceae), *Ceratiola ericiodes* (Empetraceae), terpenoids, allelopathy, mechanisms of release, water transport, activation.

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[1] Dedicated to one of the pioneers in allelopathy, Cornelius H. Muller.

INTRODUCTION

In the ongoing debate about the existence or nonexistence of allelopathy, this poorly understood area of chemical ecology is still considered an "illegitimate child" by opponents and a "stepchild" by proponents. Much of the criticism expressed in the past stems from the wealth of anecdotal information in the ecological and agricultural literature of alleged allelopathic actions and the paucity of experimental verification. The term "allelopathic action" in connection with the description of phytotoxic effects of a source plant upon ecologically unrelated target species has caused further consternation among ecologists. This has led to critical statements that "almost all species can, by appropriate digestion, extraction and concentration, be persuaded to yield a product that is toxic to one species or another" (J.L. Harper, 1975, 1977). Another cause for the extensive criticism of allelopathy resides in the fact that "chemistry [or the lack of it] has been the Achilles heel of allelopathy" (Putnam and Tang, 1986b), because most studies have been performed with little or no knowledge of the active principles, their chemical nature, their transport mechanism, quantities of released agent(s) in aqueous leachates, and concentrations in the soil. In recent years, in a collaborative effort between chemists and ecologists, we have studied the chemical interaction of higher plants in two adjacent plant communities in Florida, the scrub and the sandhill, in an attempt to provide a better understanding of the complex interactions among plant species. The goals of this project were to shed light on the multiple biological interactions in the two plant communities, to test the "influence of one plant upon another" (Molisch, 1937) and to determine the role of a biotic component in determining plant community composition.

The Florida scrub, in particular its early seral stage, represents an ecosystem with numerous advantages for the study of possible plant–plant interactions:

1. The sandy soil of the scrub contains little organic matter (Kalisz and Stone, 1984), thereby facilitating detection and quantification of allelotoxins in the soil below and near the source plant.
2. The quartz sand reduces uncontrolled absorptions of allelochemicals by clay soil particles (Muller, 1965a).
3. The source and target species are clearly defined. Early bioassays (Richardson and Williamson, 1988) provided evidence for allelopathic activity of several fire-sensitive scrub perennials against fire-prone sandhill species.
4. Last but not least, the Florida scrub hosts about 400 plant species including some 40 endemics, many of which are threatened or endangered (Menges and Salzman, 1992). This little studied community chemically represents a valuable resource for undiscovered natural products with potential biological activities (Eisner et al., 1990).

Following several years of ecological studies, four endemic shrubs common in immature white sand scrub communities were initially chosen for our combined chemical and biological studies. In this review, data will be presented from studies of two mints, *Calamintha ashei* (Weatherby) Shinners (Labiatae) and *Conradina canescens* (Torr. and Gray) (Labiatae), the Florida rosemary [*Ceratiola ericoides* (A. Gray)] of the family Empetraceae, and the woody goldenrod [*Chrysoma pauciflosculosa* (Michx.) Greene] of the Asteraceae family. Besides the new chemical data, which had to be established for all four species, their effects on the putative target species of the sandhill community were determined.

Our discussion will also address questions related to the production and storage of potentially allelopathic agents from the above scrub source plants. Furthermore, we will present evidence for possible mechanisms of release of potential phytotoxins and/or pretoxins and their transport from the leaf surface and/or roots into the soil and their inhibition of germination and growth of graminoid target plants of the sandhill community, which are used as test species to estimate the magnitude of allelopathic effects. Examples from *Chrysoma* and *Ceratiola* will be provided that demonstrate that after release into the environment photochemical and/or air-oxidative modifications can alter plant products to form derivatives of increased activity. Results of our studies on the qualitative and quantitative aspects of terpenoids and other plant products will hopefully contribute to a better understanding of the complex nature of the ecological roles of plant products in plant–plant interactions.

The allelopathic and phytotoxic properties of plant terpenoids have received attention in numerous publications and reviews. Sigmund (1924) was the first to demonstrate that essential oils as well as pure monoterpenes strongly inhibit seed germination and plant growth. Earlier treatments of phytotoxic and possible allelopathic actions of secondary plant metabolites including terpenoids were presented in books by Rice (1984), Thompson (1985), Putnam and Tang (1986a), Waller (1987), and Harborne (1988). Phytotoxic terpenoids and their possible involvement in allelopathy were covered in reviews on mono- and sesquiterpenes (Evanari, 1949; Fischer, 1986, 1991) and biological activities of sesquiterpene lactones were reviewed by Picman (1986) and Stevens and Merrill (1985). A summary of terpenoid allelopathy in desert ecosystems was presented by Friedman (1987), and a recent general review on allelopathic plant terpenoids was provided by Fischer (1991).

FLORIDA SCRUB AND SANDHILL COMMUNITIES

In the southeastern coastal plains of the United States, two plant communities, the sandhill and the scrub, are found on the sandy ridges of relict shorelines and coastal dunes. The two communities share a subtropical climate with frequent heavy rains, particularly during the summer months of June to October. Although both plant communities are dominated by pines and oaks, their species composition is distinctly different. The sandhill is an open woodland with pines and oaks and a dense ground cover of grasses, mainly wiregrass (*Aristrida stricta* Michx.) and beard grasses (*Schizachyrium* and *Andropogon* spp.). Common fire-adapted pine species are slash pine (*Pinus elliottii* Engelm.) and longleaf pine (*P. palustris* Mill.). Oak species in the sandhill include sand live oak (*Quercus germinata* Small), turkey oak (*Q. laevis* Walt.) and bluejack oak (*Q. incana* Bartr.). With the exception of patches of saw palmetto [*Serenoa repens* (Bartr.) Small] shrubs are not common in the sandhill.

Throughout the sandhill vegetation are strands and islands of the much less common sand pine scrub community. The young scrub is mainly composed of endemic shrubs. A few such as *Ceratiola* are universally present, but most, such as *Chrysoma* and the mints, are restricted to local sites. In contrast, mature scrub has a closed canopy of sand pine (*P. clausa* Vasey ex Sarg.) and a dense understory of scrub oaks (*Q. chapmanii* Sarg., *Q. germinata*, and *Q. myrtifolia* Willd.), but like young scrub, there is almost no herbaceous ground cover. On sunny days, the well-drained white sandy soil has surface temperatures greater than 50°C (Richardson, 1985), and during the dry season as well as between summer rains moisture stress is significant in the upper horizons. In addition, the scrub and sandhill soils are considered nutrient poor with low levels of potassium and nitrogen (Christensen, 1988; Kalisz and Stone, 1984; Richardson, 1985), perhaps adding to the stress of scrub species.

Nearly 100 years ago, Nash (1895) noted that "The scrub flora is entirely different from that of the high pine land [sandhill], hardly a single plant being common to both; in fact these two floras are natural enemies and appear to be constantly fighting each other . . . . Later, Webber (1935) called the Florida scrub "a fire-fighting machine." Fire susceptibility and fire frequency of the two communities differ drastically. Grasses and pine needles are the fuel for surface fires that sweep through the sandhills every three to eight years (Williamson and Black, 1981). Surface fires move through the sandhills until they reach the ecotone, separating the sandhill from patches or strands of scrub. In most instances the fires are extinguished due to the lack of fuel caused by dramatic reduction of the surface vegetation in the ecotone. Postfire conditions in the sandhill stimulate plant reproduction and development without overall change of species composition.

Every 20–50 years crown fires devastate the slow-growing, fire-sensitive evergreen scrub stands. However, the community regenerates successfully by resprouting of oak species and fire-initiated release of sand pine seeds from serotinous cones (R.M. Harper, 1915; Richardson, 1977; Williamson et al., 1992b). The characteristic differences distinguishing the two plant communities are summarized in Table 1 (Williamson et al., 1992b).

TABLE 1. COMPARISON OF FLORIDA'S SCRUB AND SANDHILL COMMUNITIES AND CALIFORNIA'S CHAPARRAL AND GRASSLAND COMMUNITIES (Williamson et al., 1992b)

Community		
Florida	scrub	sandhill
California	chaparral	grassland
Physiognomy		
Ground cover	little	complete
Shrub cover	very dense	very sparse
Fuel traits		
Surface litter quantity	low	high
Surface litter quality	compressed	loose, aerated
Crown litter quantity	high	none
Foliage phenology	evergreen	deciduous
Fire traits		
Frequency	20-50 years	3-8 years
Type	crown	surface
Evidence of allelopathy	yes	no

Fire appears to be the major factor in preventing invasion of scrub plants into the sandhill. In fire-free intervals in the sandhill, scrub species will colonize and grow well, often faster than in the scrub, but then they are killed by the next natural surface fire sweeping through the graminoid ground cover (Laessle, 1958; Veno, 1976; Hebb, 1982). In mature scrub the closed canopy seems to prevent colonization of sandhill (or scrub) species, which generally require full sunlight. However, this does not explain the lack of sandhill species colonizing young scrub communities, which are open. Numerous studies of soil profiles of the two communities have produced meager differences in physical and chemical soil characteristics (R.M. Harper, 1914; Webber, 1935; Laessle, 1958, 1968). The most recent study concludes that any differences are attributable to the different plant communities and their fire regimes (Kalisz and Stone, 1984). The lack of significant differences in soil nutrients (Kalisz and Stone 1984; Richardson, 1985) and the fact that the addition of fertilizers in scrub plots did not increase germination or seedling growth relative to unfertilized control plots (Richardson, 1985) led to our studies in search for allelopathic actions in the scrub.

As summarized in Table 1, differences in the Florida scrub and the adjacent sandhill parallel the differences between California's chaparral and adjacent grasslands (Williamson, 1990). In addition, the sometimes sharp ecotone between scrub and sandhill resembles the bare zone between chaparral and grasslands. These similarities cannot be explained on the basis of soils, mainly clays in California versus sands in Florida, or on the basis of climate, dry in California with precipitation concentrated in the winter versus wet in Florida with precipitation concentrated in the summer. However, parallels exist in fire regimes and in evidence of allelopathic suppression of grasses and herbs in both the California chaparral and the Florida scrub (Williamson, 1990).

ROLE OF MONOTERPENES IN FLORIDA SCRUB

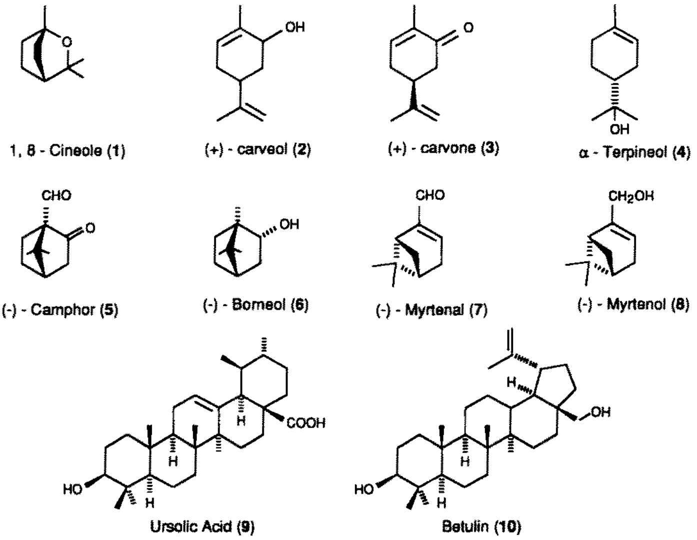
We have tested the hypothesis (Richardson and Williamson, 1988) that allelopathy has evolved as a mechanism to prevent the invasion of fire-prone grasses and pines into the scrub, in particular, in immature or disturbed scrub communities (Williamson, 1990; Williamson et al., 1992b). Since graminoids provide fuel for frequent surface fires, the slow-growing, fire-sensitive scrub colonizers would be adversely affected by these fires. It was, therefore, proposed that allelopathic agents released from scrub species inhibit the growth of the fire-fueling grasses and create fuel breaks around individual plants and ecotones along dense scrub communities (Richardson and Williamson, 1988; Williamson et al., 1992b).

Our recent studies have provided evidence that biotic instead of abiotic factors are mainly responsible for the patterns of the scrub and sandhill communities (Richardson, 1977, 1988; Richardson and Williamson, 1988; Williamson and Black, 1981; Fischer et al., 1988; Fischer, 1991; Williamson et al., 1992b; Weidenhamer and Romeo, 1989). Earlier studies involved monthly collections of fresh leaves of *Calamintha ashei*, *Conradina canescens*, and *Ceratiola ericoides* from central Florida for bioassays. The aqueous soakings of fresh foliage of each of the three species, as well as decaying litter in the case of *Ceratiola*, were tested against three native sandhill grasses, *Schizachyrium scoparium* (Michx.) Nash, *Andropogon gyrans* Ashe, and *Leptochloa dubia* (HBK) Nees, for germination and radicle growth inhibitions (Richardson and Williamson, 1988). The effects of the leaf washes of all three scrub species upon the germination of target sandhill grasses was significant, with an overall average of inhibition of 20%. *Calamintha ashei*, *Conradina canescens*, and *Ceratiola ericoides* leaf washes gave 44%, 34%, and 20% inhibition of *Leptochloa*, respectively (Williamson et al., 1992b). Effects of the leaf washes on *Schizachyrium* were lower but decaying *Ceratiola* litter showed a 26% reduction of germination.

Monoterpenes of Conradina canescens *and their Biological Activities*

This member of the mint family is an evergreen of the sand pine scrub. It exists in two major disjunct populations, one in the Florida Central Ridge in Highland and Polk counties and the other in the Gulf Coastal region from Horn Island, Mississippi, to near Tallahassee, Florida. Within its range, it is relatively common in areas where full sunlight occurs. Under the shady canopy of sand pine and the subcanopy of oaks of the mature scrub, it is less abundant. Recurved, undamaged leaves of *Conradina* emit no odor; however, even mild mechanical disturbance results in the emission of a strong terpene odor (Williamson et al., 1989). Scanning electron micrographs of the leaf surfaces of *Conradina* showed glandular trichomes encircled by the filamentous ones (Williamson et al., 1989). Cross-sections revealed secretory cells under a layer of the cuticle that harbored the secretion. Similar glandular and nonglandular trichomes are found in some, but not all, mint species (Metcalfe and Chalk, 1965). Dipping the leaves into organic solvents dissolved the fold of cuticle and the underlying secretions, whereas the filamentous trichomes remained intact. The resulting extract contained a mixture of monoterpenes and triterpenes.

Aqueous leaf soaks of fresh *Conradina* leaves were strongly inhibitory to sandhill grasses, and their chemical analysis indicated the presence of monoterpenes as well as the triterpenes, ursolic acid **(9)** and betulin **(10)**, which appear to be present in copious amounts in the cutical wax (Figure 1). Detailed chemical analysis of *Conradina* foliage provided a mixture of monoterpenes, typical of the mint family and also the active components of other allelopathic mints (Muller et al., 1964; Muller, 1965a,b; Muller and del Moral, 1966; Fischer, 1986, 1991). Besides several minor monoterpenes, the known entities, 1,8-cineole **(1)**, camphor **(5)**, borneol **(6)**, myrtenal **(7)**, myrtenol **(8)**, α-terpineol



1, 8 - Cineole (1)

(+) - carveol (2)

(+) - carvone (3)

α - Terpineol (4)

(-) - Camphor (5)

(-) - Borneol (6)

(-) - Myrtenal (7)

(-) - Myrtenol (8)

Ursolic Acid (9)

Betulin (10)

FIG. 1. Monoterpenes and triterpenes found in *Conradina canescens*.

(4), carveol **(2)**, and carvone **(3)** were the major constituents (Figure 1). Several of these monoterpenes (e.g., borneol, camphor, and 1,8-cineole) have been previously described as potent plant germination and growth inhibitors (Asplund, 1968, 1969; Muller, 1965a; Fischer, 1986, 1991).

Biological activities of saturated aqueous solutions of the major monoterpenes of *Conradina* on *Schizachyrium* and *Leptochloa* as well as lettuce were determined. Petri dish bioassays of the monoterpenes, applied to *Leptochloa* and *Schizachyrium*, revealed complete inhibition of germination by five compounds and statistically significant inhibition of germination in each case with the exception of 1,8-cineole, which had no effects on *Leptochloa* (Table 2). Thus, saturated aqueous solutions of most of the monoterpenes tested provided strong phytotoxicity. This result was highly unexpected since this group of natural products was assumed to have negligible water solubility due to the nonpolar character of the monoterpenes relative to other more polar plant metabolites such as organic acids and phenolics (Harborne, 1988). The above bioassay data strongly suggested that the monoterpenes in *Conradina* are not only potent phytotoxins but appear to have sufficiently high water solubilities to cause strong inhibitory effects in aqueous solution. In contrast, previous allelopathic effects of monoterpenes in the California chapparal had involved volatility as a mechanism whereby the volatile monoterpene reach the soil to be adsorbed on lipophilic clay particles and seed/seedling membranes (Muller and del Moral, 1966).

Storage, Water Solubility and Aqueous Transport of Monoterpenes

Secondary plant products are generally localized in specialized cells, tissues, organs, and organelles. Storage sites include glandular trichomes, resin ducts, secretory cavities, surface wax, vacuoles, and cell walls (Gershenzon, 1993). Defensive natural products could in principle have multiple functions, that is, be active against herbivores and also act as allelopathic agents (Langenheim, 1994). For instance, leaf surface waxes and resins (Gershenzon, 1993; Fischer et al., 1988) as well as constituents of glandular trichomes (Duke and Paul, 1993; Spring, 1991; Kelsey et al., 1984) may be active against herbivorous insects and also inhibit the germination and growth of competing plant species (Duke, 1991). Furthermore, lipophilic constituents in resin ducts of plant roots can be released into the surrounding soil at concentration levels sufficient to act as potent allelopathic agents (Tang, 1986; Kobayashi et al., 1980; Menelaou et al., 1992). Lipophilicity and the site of storage of secondary plant products appear to correlate since lipophilic plant metabolites, including most terpenoids, accumulate in secretory structures or in plant surface wax, whereas hydrophilic compounds are present in cell walls and/or vacuoles (Gershenzon, 1993).

The function of plant metabolites in allelopathic actions differs distinctly

Table 2. Effects of Saturated Aqueous Solutions of Monoterpenes Detected in Leaf Soaks of *Conradina canescens* on Germination (G) and Radicle Lengths (R) of *Leptochloa dubia* and *Schizachyrium scoparium*, as Percent of Water Controls[a]

	Leptochloa		Schizachyrium	
Compound	G	R	G	R
1,8-Cineole	100	97	20**	26**
(−)-Camphor	12**	34**	16**	61
(−)-Borneol	0**		8**	47*
(+)-α-Terpineol	0**		0**	
Myrtenal	2**	5**	8**	13**
Myrtenol	0**		0**	
(−)-Carvenol	0**		0**	
(+)-Carvone	0**		0**	

[a] Treatment was significantly different from water controls at $*P < 0.05$; and $** < 0.01$.

from those of antiherbivore defense compounds in that the defense actions seem to be exclusively beneficial to the living plant tissue. In contrast, allelopathic activity may also be caused by phytotoxins released from dead tissue, such as plant leaf litter, or plant residues. Litter of evergreens in natural ecosystems, e.g., *Ceratiola ericoides*, as well as decomposing plant residues in agricultural monocultures are often highly phytotoxic (Rice, 1984; Williamson et al., 1992a). Sometimes, significantly higher allelopathic effects have been observed when decomposing plant material is involved (Rice, 1984; Fischer et al., 1988).

In general, aqueous transport may be essential to effective allelopathy where the aqueous runoff during frequent rains is transported to the soil solution and the target species. Alternative transport as volatiles was proposed in the California chaparral (Muller, 1965a,b; Muller and del Moral, 1966) and still may require aqueous dissolution in dew or soil solution (Muller et al., 1964). The "volatility" mechanism seemed less likely to operate within the immature scrub of the Florida coastal regions where frequent winds and high soil surface temperature would prevent condensation of monoterpenes on soils in the proximity of the plant.

Due to the relatively nonpolar character of monoterpenes, the group has been classified as volatile and assumed to have negligible water solubility in comparison to other more polar, organic natural products. For instance, Harborne (1984) states that "terpenoids are generally lipid-soluble" whereas "phenolic substances tend to be water-soluble." In spite of reports of significant solubilities (Rhode, 1922; Seidell, 1940–41; Smyrl and LeMaguer, 1980), citations in standard chemical references continued to represent monoterpenes as insoluble in water (Budavari, 1989; Weast, 1976, 1989).

Based on our preliminary data, allelopathic effects of the two scrub mints, *Calamintha ashei* and *Conradina canescens*, seemed to exist and are most probably due to monoterpenes as active constituents (Richardson and Williamson, 1988; Tanrisever et al., 1987, 1988; Macias et al., 1989; Williamson et al., 1989). Bioassays of saturated aqueous solutions of monoterpenes suggested that the most likely mechanism of allelochemical release was aqueous leaching of foliage and litter in apparent incongruity with the assumed aqueous insolubility of monoterpenes. Therefore, as part of our ongoing investigations of allelopathic mechanisms, we determined the water solubility of a structurally diverse group of monoterpenes, eight hydrocarbons and 23 oxygen-bearing monoterpenes (Weidenhamer et al., 1993). Their solubilities were also compared to known phenolic phytotoxins, juglone, ferulic acid, and hydrocinnamic acid (Davis, 1928; Blum and Dalton, 1985; Tanrisever et al., 1987; Harborne, 1988), which are presumed to be water soluble.

Solubility among the monoterpenes varied considerably, ranging from < 10 ppm to 6990 ppm (Table 3). The hydrocarbon monoterpenes had low solubilities, all under 35 ppm, but oxygenated monoterpenes such as aldehyde, ketones, alcohols, and ethers had solubilities 10–100 times greater than the hydrocarbons with a similar skeleton. Alcohols were somewhat more soluble than related ketones in the monocyclic skeletons. In bridged bicyclic monoterpenes, ring strain favors formation of the geminal diols from the ketones, making them more soluble than comparable alcohols, for instance, camphor (550 ppm) versus borneol (274 ppm) and verbenone (6990) versus myrtenol (1010 ppm). The monoterpenes exhibited a broad range of aqueous solubilities unexpectedly comparable to the common allelopathic phenolics and organic acids, juglone (52 ppm), ferulic acid (174 ppm), and hydrocinnamic acid (3490 ppm).

These results demonstrate that generalization about the biological activities and ecological functions of monoterpenes based on putative differences in aqueous solubilities need to be reevaluated. For example, Tukey (1969) in his classic foliar leaching studies concluded that "carbon dioxide, ethylene and terpenes" are released as volatiles, while rain and dew leach "mineral nutrients, carbohydrates, amino and organic acids, and growth regulators." Describing the role of secondary metabolites in litter decomposition, Horner et al. (1988) stated that "Leaching losses of fairly water soluble components (e.g., most simple phenolics, phenylpropanoids, flavonoids, and tannins) should exceed those of components that are only slightly or negligibly soluble in water (e.g., terpenes and lignin, respectively)." In regard to allelopathy, we and other authors have in the past differentiated the "volatile terpenes" from the "water-soluble" phenolics and aromatic acids (Whittaker, 1971; Harborne, 1988; Fischer et al., 1989; Williamson et al., 1992b). Apparently, the pioneering research of C.H.

TABLE 3. SOLUBILITIES OF MONOTERPENES IN WATER (from Weidenhamer et al., 1993)

Name of Compound	Water solubility (ppm)
[(1*S*)-*endo*]-(−)-Borneol	274
Bornyl acetate	23
Calaminthone	972
Calaminthone, desacetyl	1005
Camphene	23
(1*S*)-(−)-Camphor	550
(1*R*)-(+)-Camphor	531
(−)-Carveol	1115
(*S*)-(+)-Carvone	596
1,8-Cineole	332
p-Cymene	15
(*S*)-(+)-Dihydrocarveol	727
(*S*)-(+)-Dihydrocarvone	461
(+)-Evodone	409
Geraniol	404
Geranyl acetate	18
Limonene	13
Linolyl acetate	<10
Menthol	183
Menthone	155
Myrcene	<10
(1*R*)-(−)-Myrtenal	305
(1*R*)-(−)-Myrtenol	1010
Nerol	332
α-Pinene	22
β-Pinene	32
(1*R*)-(+)-Pulegone	385
(+)-Sabinene	<10
α-Terpinene	14
4-Terpineol	1360
(1*S*)-(−)-Verbenone	6990

Muller and coworkers (Muller et al., 1964; Muller, 1965a,b, 1966, 1969; McPherson et al., 1971; Muller and Chou, 1972) might have unintentionally contributed to this perception, as they found several monoterpenes emitted as volatiles from *Salvia leucophylla* (Labiatae) and *Artemisia california* (Asteraceae), whereas phenols and phenolic acids were washed from the leaves of *Adenostoma fasciculatum* (Roaseceae) and *Arctostaphylos glandulosa* (Ericaceae).

Phytotoxic effects of monoterpenes vary as much as their water solubilities, although in many cases compounds are active well below their aqueous solubility levels (Fischer, 1991). A comparison of bioactivity of the two monoterpenes borneol and (+)-camphor with the phenol juglone, the active constituent of *Juglans nigra* (Davis, 1928; Harborne, 1988), gave unexpected results (Figures 2 and 3). Although there is variation among these compounds and variation between target species, the biological activity of the two monoterpenes matched or exceeded in several cases that of juglone, the well known allelopathic agent of black walnut. Furthermore, the solubility of borneol and camphor exceeded that of juglone by 5–10×. Thus, the potential activity of the monoterpenes is much greater than juglone where toxicity is mediated through aqueous soil solutions. Reaching active concentrations requires solubilities and solubilization rates sufficiently high for activity. Some presumed insoluble compounds may dissolve

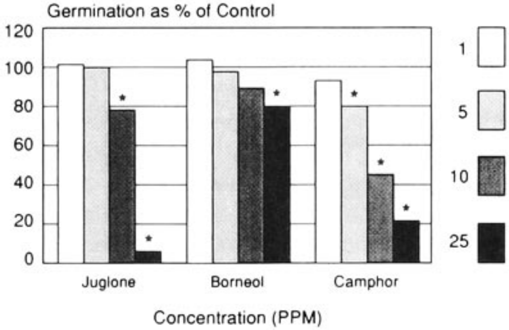


FIG. 2. Germination of *Lactuca sativa* in aqueous solutions of juglone, borneol, and camphor. Asterisk indicates significant difference from the control at $P \leq 0.05$.

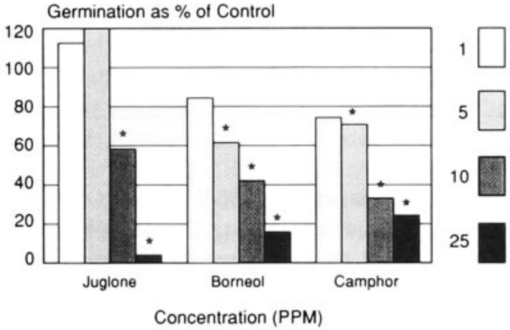


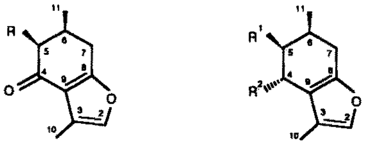
FIG. 3. Germination of *Rudbeckia hirta* in aqueous solutions of juglone, borneol, and camphor. Asterisk indicates significant difference from the control at $P \leq 0.05$.

through formation of micelles with other natural products (Fischer et al., 1988; Fischer, 1991; Williamson et al., 1992a). The natural surfactant, ursolic acid **(9)**, is found in copious amounts on the leaf surface of all three scrub species, *Ceratiola, Conradina*, and *Calamintha*. It forms micelles in leaf washes, but does not seem to increase aqueous solubility of monoterpenes (Fischer et al., 1988; Weidenhamer et al., 1993). Even if solubilities were improved by micellization, activities may decrease if the compounds remain bound by the surfactant and thus, unable to penetrate target plant membranes. Therefore, the major ecological role of cuticular waxes in allelopathic processes and other biological functions may be due to their fixative property, which enhances long-term retention of active volatiles that would otherwise be lost to volatilization.

Monoterpenes of Calamintha ashei *and their Biological Activities*

Calamintha ashei, commonly referred to as Ashe's savory, is endemic to the central ridge of Florida and is locally common in hickory and sand pine scrub (Menges and Salzman, 1992; Van der Kloet, 1986). Extensive chemical analysis of this mint provided copious amounts of ursolic acid **(9)**, small amounts of the known sesquiterpene, caryophyllene epoxide, and a series of new menthofuran-type monoterpenes (Figure 4). In addition, six known highly methoxylated flavones were obtained (Tanrisever et al., 1988; Macias et al., 1989; Hernandez and Fischer, 1988; Menelaou, 1990).

When fresh aerial parts of *C. ashei* were soaked in water at ambient temperature followed by reextraction of the water soaks with dichloromethane, the organic extracts contained (+)-evodone **(11)**, (−)-calaminthone **(12)**, and desacetylcalaminthone **(13)** (Menelaou, 1990). Besides these three major monoterpenes, six highly unstable minor menthofurans **(15–20)** were isolated from the



		R
11	(+) - Evodone	H
12	Calaminthone	OAc
13	Desacetyl - calaminthone	OH
14	Menthofuran	H, 4-desoxy

	R¹	R²
15	OAc	OAc
16	OAc	H
17	H	OAc
18	OH	OH
19	OAc	OH
20	OH	OAc

FIG. 4. Monoterpenes isolated from fresh leaves of *Calamintha ashei*.

water and dichloromethane extracts but could not be tested for biological activity due to lack of sufficient quantities.

The proposed mechanism for the release of allelochemicals from *Calamintha* and other scrub perennials is the leaching of monoterpenes from leaves and decaying litter by rainfall (Tanrisever et al., 1987, 1988; Fischer et al., 1988). In order to ascertain the role of menthofuran monoterpenes in the allelopathic potential of *Calamintha ashei*, we evaluated the phytotoxicity of the menthofuran monoterpenes in bioassays.

Earlier studies (Tanrisever et al., 1988) had shown that an aqueous solution of unknown concentration of a chromatographic fraction containing calaminthone **(12)**, (+)-evodone **(11)**, and caryophyllene oxide completely suppressed germination of *Schizachyrium* but had no effect on lettuce germination. Significant inhibitory effects were observed when (+)-evodone was administered as a volatile or in a saturated aqueous solution of ursolic acid **(9)**, the latter showing no significant effects when tested alone. Two facts from the earlier bioassay are of interest: (1) the highly specific activity of a mixture of calaminthone **(12)**, (+)-evodone **(11)**, and caryophyllene oxide on little bluestem contrasts with insignificant inhibition of lettuce; and (2) the strong inhibitory effect exhibited by the terpene mixture toward *Schizachyrium*. Dramatic synergistic phytotoxic effects by mixtures of camphor, pulegone, and borneol were previously observed by Asplund (1968, 1969), although we have failed to find such synergisms in other tests (see below).

More recent bioassays of (+)-evodone **(11)** at 0.05–1 mM concentration revealed strong inhibitory activity against four test species, *Schizachyrium*, *Leptochloa*, the sandhill herb *Rudbeckia hirta*, and *Lactuca* (Weidenhamer et al., 1994). In all cases, germination was inhibited more than radicle growth (Figure 5). All four target species were affected, with germination of *Rudbeckia* and *Lactuca* significantly inhibited even at 0.05 mM. In contrast, germination of the two grasses, *Schizachyrium* and *Leptochloa*, was inhibited only at the higher concentrations of 1.0 and 0.5 mM, respectively. Radical elongation was reduced in three of the four target species, but generally to a lesser degree than germination. Activity of the second major constituent of *Calamintha*, desacetylcalaminthone **(13)**, was limited to inhibition of germination and radicle elongation of *Leptochloa* at 1.0 mM, and germination of *Rudbeckia* was inhibited at concentrations as low as 0.25 mM.

The equimolar mixtures of (+)-evodone and desacetylcalaminthone showed inhibitory effects that were additive rather than synergistic or antagonistic. *Schizachyrium* germination was 53%, 67%, and 59% for 0.05 mM solutions of desacetylcalaminthone, (+)-evodone, and the equimolar mixture, respectively. *Lactuca* germination was 100%, 33%, and 66% for 0.25 mM solutions of desacetylcalaminthone, (+)-evodone, and their equimolar mixture, respectively. *Rudbeckia* germination was only slightly reduced (20%) by 0.25 mM desace-

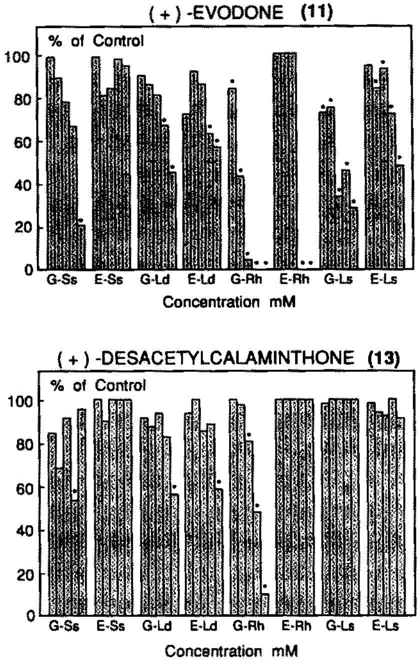


FIG. 5. Germination (G) and radicle elongation (E) of *Schizachyrium scoparium* (Ss), *Leptochloa dubia* (Ld), *Rudbeckia hirta* (Rh), and *Lactuca sativa* (Ls) at five concentrations: right to left 1.0, 0.5, 0.25, 0.10, and 0.05 mM for (+)-evodone and (+)-desacetylcalaminthone.

tylcalaminthone, but was drastically inhibited by 0.25 mM (+)-evodone (3% germination) and the 0.25 mM equimolar mixture (6% germination). Effects of the other three monoterpenes were considerably lower. Calaminthone exhibited strong inhibition of *Rudbeckia* at 0.8 mM but showed few effects at lower concentrations or on the other three species. Menthofuran stimulated 30–100% increases in radical elongation of *Leptochloa* at all five concentrations.

Water Solubility of Calamintha *Monoterpenes*

Determination of the aqueous solubility of the *Calamintha* monoterpenes was necessary to evaluate their potential phytotoxicity. Aqueous solubilities of the three major *Calamintha* monoterpenes were determined by gas chromatography, with values of 2.49 mM (409 ppm) for (+)-evodone **(11)**, 5.58 mM (1005 ppm) for desacetylcalaminthone **(13)**, and 4.36 mM (972 ppm) for calaminthone **(12)** (Weidenhamer et al., 1994). Menthofuran **(14)** slowly degraded, giving an unreliable value of 1.69 mM (254 ppm). The aqueous solubilities of desacetylcalaminthone **(13)** and (+)-evodone **(11)**, the two major menthofuran constituents of *Calamintha ashei*, exceeded concentration levels necessary for inhibition of germination and radicle growth of the sandhill species tested. Calaminthone **(12)** and menthofuran **(14)**, which had low activity in the bioassays, also had relatively high solubilities (< 1 mM).

Prior to determination of the aqueous solubilities (Fischer et al., 1988; Tanrisever et al., 1988), we proposed that the allelopathic activity of *Calamintha* must be mediated by the solubilization of the *Calamintha* menthofurans with naturally occurring surfactants such as the triterpene ursolic acid **(9)**, which occurs in large quantities in *Calamintha* foliage (Fischer et al., 1988; Tanrisever et al., 1988). We had previously demonstrated that formation of micelles in leaf soaks of these plants occurs (Tanrisever et al., 1988), but no measurements of water solubilities or solubilization rates of pure compounds were made. The solubility data presented above clearly showed that the *Calamintha* menthofurans were soluble in excess of 100 ppm, sufficient for these compounds to be potent phytotoxins. The new quantitative data showed clearly that oxygenated monoterpenes are sufficiently water soluble to exert biological activity, and ursolic acid does not increase monoterpene solubility (Weidenhamer et al., 1993). Therefore, our previous hypothesis of the importance of micelle formation by biological detergents such as ursolic acid needs revision.

The ecological significance of the formation of micelles in leaf soaks of *Calamintha* and other allelopathic shrubs of the Florida scrub may have been overestimated based on the presumed insolubilities of the menthofuran monoterpenes. However, triterpenes and other lipids may play a significant role in the fixation of leaf volatiles, in general, and volatile monoterpenes, in particular, to prevent their otherwise rapid release into the atmosphere. Furthermore, effects of triterpenes and other micelle-forming plant products present in the leaf waxes undoubtedly have a significant detergent effect on the rate of water solubilization in a natural setting.

LABORATORY VERSUS FIELD EXPERIMENTS

The proposed mechanism for the release of *Conradina* and *Calamintha* allelochemicals appears to proceed via the leaching of allelopathic compounds from leaves and decaying litter. Frequent heavy rains, which begin in June and extend through October, define the growing season in the scrub and may play a major role in transport of allelotoxins. The impact of large raindrops may cause glandular trichomes to rupture, thereby releasing large quantities of monoterpenes from the leaves. Whether leaves or litter are more important will require more detailed quantitative studies in the field.

Another concern in all studies of allelopathy is the question of realistic bioassays (Hollis et al., 1982). Single applications of a higher concentration of an allelochemical are less realistic than pulses of lower concentration. Inhibition of growth may depend on regular exposure to an allelopathic agent (Blum and Rebbeck, 1989). A small but constant flux of allelochemicals into the soil, if continually taken up by target species, may have significant inhibitory effects on growth, even if high concentrations in the soil solution are never achieved (Williamson and Weidenhamer, 1990). While data are accumulating in the allelopathy literature on the concentrations of toxins in soil at a single point in time, the more important question is how much of an allelochemical is available over a period of time. Answering this question in the case of *Calamintha* and *Conradina* will require measurements of the total amounts of monoterpenes entering the soil during rainstorms through the growing season. This may require the development of new analytical methods, such as traps placed in the soil for an extended period of time followed by quantitative analysis. Environmental parameters such as temperature, nutrient limitation and plant density can significantly modify plant response to an allelochemical (Einhellig and Eckrich, 1984; Einhellig, 1987; Williamson et al., 1992a; Weidenhamer et al., 1989). These environmental factors, which may have no effect at ambient laboratory conditions, may strongly enhance or reduce inhibitory actions in the field.

Nutrient limitation is known to increase inhibition of growth by phenolic compounds (Glass, 1973; 1974; J.R. Harper and Balke, 1981). The effect of hydrocinnamic acid, a photochemical breakdown product of ceratiolin from the scrub endemic *Ceratiola ericoides*, is intensified by reduced levels of nitrogen and potassium (Williamson et al., 1992b). Since macronutrient levels in the Florida scrub are generally very low (Kalisz and Stone, 1984; Richardson, 1985), nutrient limitation may be an important factor in the phytotoxicity of scrub allelochemicals.

High temperature and low soil moisture increase phytotoxic effects of some phenolic allelochemicals (Einhellig and Eckrich, 1984; Einhellig, 1987). On sunny days, surface temperatures often exceed 50°C on the well-drained sands of the Florida scrub (Richardson, 1985), and hydric stress can occur during the dry season and during breaks in summer rains. Plant densities in the Florida scrub are low, and this may also contribute to enhanced phytotoxicity of scrub allelochemicals. At low densities of seeds and germinating seedlings, phytotoxic effects are increased (Weidenhamer et al., 1987, 1989). It is not known whether the effects of the monoterpenes from *Conradina* and *Calamintha* will also be intensified in the harsh scrub environment.

It is perhaps significant that the major monoterpene constituents of *Conradina* and *Calamintha* are also the most active in the bioassays conducted. The question of whether other compounds might be involved in the allelopathic effects must be considered. *Calamintha* contains a rich mixture of flavonoids in addition to the menthofuran monoterpenes (Hernandez, 1988; Hernandez and Fischer, 1988; Menelaou, 1990). As a result of the low biological activity of the flavonoids in laboratory experiments (Menelaou, 1990), their concentrations in the leaf soaks and mists were not determined. Furthermore, the biological activity of other unidentified polar constituents in the leaf washes of *Calamintha* is not known and requires further study.

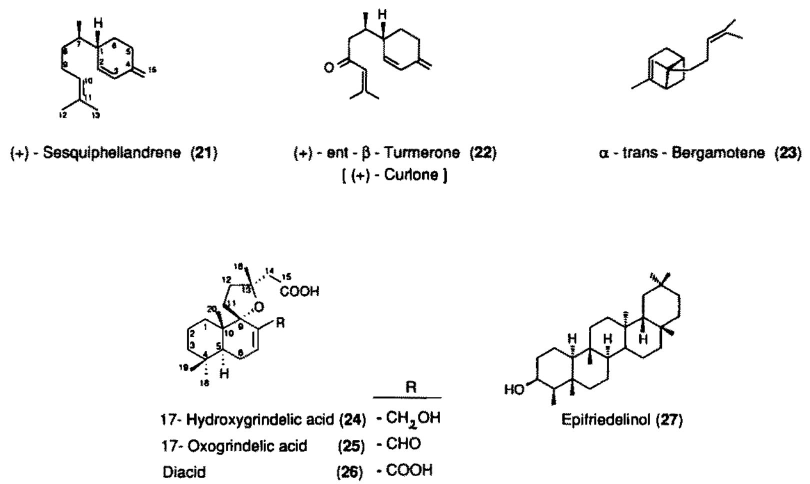
ALLELOPATHIC ACTIVATION IN FLORIDA SCRUB

The complexity of allelopathic actions in natural ecosystems is due to a multitude of events in a dynamic process of simultaneously occurring chemical transformations. After release of a secondary metabolite from leaves, litter, and/or the roots of a source plant into the environment, a cascade of spontaneous chemical, photochemical, and/or microbial events may follow. These often result in postrelease activations of nontoxic plant products into active derivatives with specific toxic effects for the target species, which can be enhanced by synergistic effects (Asplund, 1969; Fischer et al., 1988) and simultaneous reduction of autotoxicity (Fischer et al., 1988; Williamson, 1990). Ultimately, active compounds will be deactivated by microbial degradations. Two examples are given below.

Oxidative Activation of Diterpenes in Woody Goldenrod (Chrysoma pauciflosculosa)

The woody goldenrod (*Chrysoma pauciflosculosa*) of the family Asteraceae is a dominant shrub on open scrub sites in the Florida panhandle. In an earlier study, leaf washes of *Chrysoma* had significantly inhibited the germination of *Lactuca* in laboratory tests (Eleuterius, 1979). Therefore, this member of the immature scrub in the dunes of the Florida panhandle was included in our chemical and biological studies.

Aqueous extracts of fresh leaves of *C. pauciflosculosa* contain ca. 500 ppm of organic extractables (Menelaou et al., 1993). 17-Hydroxygrindelic acid **(24)** was a major constituent with the diterpene aldehyde **(25)** and the diacid **(26)** as well as the sesquiterpenes (+)-β-turmerone **(22)** [(+)-curlone], (+)-sesquiphellandrene **(21)**, and (−)-α-*trans*-bergamotene **(23)** being minor components (Figure 6). Once released into the environment, the moderately active allylic alcohol moiety in 17-hydroxygrindelic acid (HGA, **24**) is subject to light-mediated air oxidation to give first the more active aldehyde (OGA, **25**) and ultimately the diacid **(26)** (Figure 7), processes which seem to involve free radical intermediates (Menelaou et al., 1993). At a range of 36–144 μM (12–48 ppm) 17-oxogrindelic acid **(25)** reduced the germination and radicle growth of the two sandhill grasses, *Schizachyrium* and *Leptochloa*, but had no



(+) - Sesquiphellandrene (21)

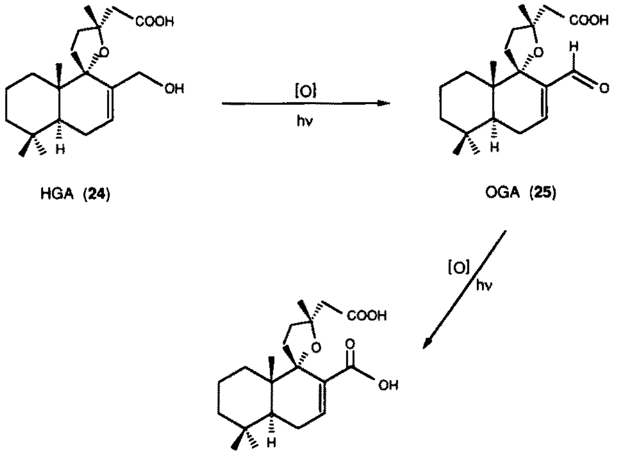
(+) - ent - β - Turmerone (22)
[(+) - Curlone]

α - trans - Bergamotene (23)

		R
17- Hydroxygrindelic acid	(24)	- CH_2OH
17- Oxogrindelic acid	(25)	- CHO
Diacid	(26)	- COOH

Epifriedelinol (27)

FIG. 6. Sesqui-, di-, and triterpenes from *Chrysoma pauciflosculosa*.



HGA (24)

OGA (25)

(26)

FIG. 7. Photochemical allelopathic activation of diterpenes of *Chrysoma pauciflosculosa*.

significant effects on the germination and slight stimulatory effects on radicle growth of *Rudbeckia hirta*. At a concentration range of 60–357 μM (20–120 ppm) the diterpene alcohol **(24)** was less active in reducing germination and radicle growth of the same target species and the diterpene diacid **(26)** gave no significant activity at comparable concentration.

The *C. pauciflosculosa* sesquiterpenes had only minor effects on the germination and radicle growth of the four target species (Menelaou, 1990). *Schizachyrium* radicle growth was significantly stimulated by 10^{-4} M solutions of (+)-ent-β-turmerone **(22)** and (−)-α-*trans*-bergamotene **(23)**, but *Leptochloa* was not affected by any of the three sesquiterpenes. *Rudbeckia* radicle growth was reduced to about 80% of the control by 10^{-4} M solutions of (+)-ent-β-turmerone and (−)-α-*trans*-bergamotene. Lettuce germination was inhibited by aqueous saturated (65% of control) as well as 10^{-4} and 10^{-5} M solutions (49% and 77% of control) of (+)-ent-β-turmerone and was stimulated significantly by a 10^{-4} M solution of (−)-α-*trans*-bergamotene.

In the assays of mixtures of oxogrindelic acid **(25)** with the three sesquiterpenes, (+)-ent-β-turmerone, (+)-sesquiphellandrene and (−)-α-*trans*-bergamotene, there was no indication of synergistic or antagonistic effects of a diterpene aldehyde–sesquiterpene mixture. This suggested that the three sesquiterpenes do not seem to be significantly involved in allelopathic effects of *C. pauciflosculosa*.

Ceratiolin Cascade in Florida Rosemary (Ceratiola ericoides)

Although unrelated to the theme of allelopathic terpenes, the following example demonstrates the complexity of chemical events in allelopathic actions. The biologically inactive dihydrochalcone, ceratiolin, is leached from fresh leaves and litter of *Ceratiola* and degraded to hydrocinnamic acid (HCA), a selective toxin for native Florida grasses and pines (Tanrisever et al., 1987; Williamson

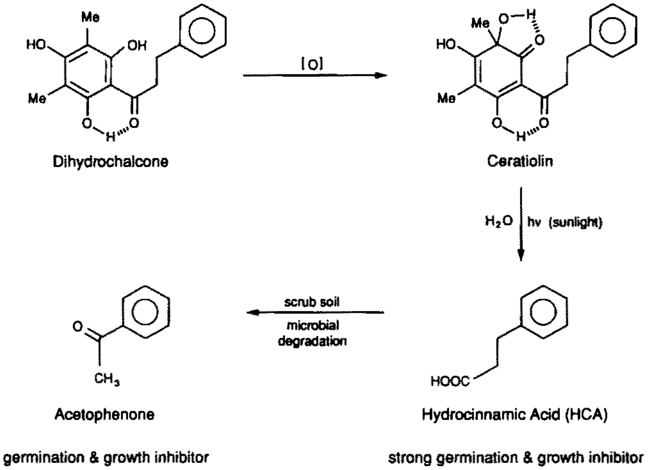


FIG. 8. Photochemical allelopathic activation in the Florida scrub: the ceratiolin cascade.

et al., 1992a). As shown in Figure 8, decomposition of ceratiolin in aqueous solution is a light-dependent process, suggesting that in the scrub, formation of the active HCA represents a novel photochemical process of allelopathic activation (Tak and Fischer, unpublished results). As soil microbes metabolize and degrade organic plant products (Blum and Shafer, 1988) and as HCA is released from living and decaying foliage of *Ceratiola* and transported in aqueous soil solution at toxic levels (Jordan, 1990), further degradation was anticipated. HCA spiked in nonsterile scrub soil at ambient temperature disappeared after about 60 hr. After 12 hr, acetophenone, which is also inhibitory toward target grasses, was detected in soil extracts, reaching a maximum after 60 hours and decreasing to nondetectable levels after 144 hr (Jordan, 1990). In sterile soil after application of HCA, acetophenone was not detected. Therefore, the degradation process must be a microbial, rather than an air-oxidative decomposition of HCA (Jordan, 1990). In contrast to the photochemical activation–microbial degradation process of the ceratiolin cascade, allelopathic activation of the dihydrochalcone phloretin from apple roots (*Pirus malus* L.) involves formation of the active *p*-hydroxydihydrocinnamic acid exclusively by microbial action (Boerner, 1960).

CONCLUSIONS AND FUTURE STUDIES

Our studies in the Florida scrub have provided insight into new mechanisms of release and action of allelopathic agents that might be used as models for studies of other natural ecosystems. Chemical, microbial, or spontaneous decomposition of nontoxic plant products into potent phytotoxic derivatives provides possible mechanisms for specific, direct toxic effects toward a target species. In *Ceratiola ericiodes*, the nontoxic plant product ceratiolin is only the precursor, and its photochemical decomposition product HCA, which is mainly formed in the litter, acts as the specific allelotoxin for sandhill target grasses.

As emphasized earlier in this issue (Langenheim, 1994), synergistic allelopathic effects, exemplified by the dramatic increase in inhibition of germination caused by mixtures of monoterpenes can be expected. Bioactivity enhancements due to synergism of terpene mixtures could dramatically increase biological activities at concentrations one to two orders of magnitude lower than single component activities (Asplund, 1969; Fischer et al., 1988). Furthermore, observation of highly selective phytotoxicity of *Calamintha* terpenes with selective germination inhibition of different target species represents another useful guide for future studies. Nonpolar cutical waxes and resins can possibly function as fixatives to prevent the loss of allelopathic volatiles by rapid evaporation. This property as a slow-release solvent for organic volatiles could be facilitated by a wide host of constituents in plant leaf waxes: triterpenes, long-chain fatty acids, hydrocarbons, alcohols, esters, and fats. Enhanced long-term retention of active components undoubtedly plays a significant role in the retention of allelopathic plant volatiles.

Our collaborative study of the Florida scrub has made substantial progress in the understanding of the intricacies of plant–plant interactions in this highly endangered natural ecosystem. We have provided evidence that terpenes, in general, and monoterpenes, in particular, play a major role in the allelopathic action in the endemic scrub mints *Calamintha ashei* and *Conradina canescens*. Furthermore, our findings on the water solubility of a series of potent phytotoxic monoterpenes has opened new doors for future ecological studies of this group of compounds. We hope that this new knowledge forces change not only in relation to their allelopathic actions in natural ecosystems but also changes our thinking of this major group of natural products in general ecological terms.

Many significant questions remain to be answered before the original hypothesis of allelopathic effects by the four scrub endemics *Conradina*, *Calamintha*, *Chrysoma*, and *Ceratiola* can be confirmed or refuted. Crucial to this assessment will be long-term bioassays and field studies that must address the effects of terpenes and other compounds on the growth of native sandhill species under conditions comparable to the harsh environment of the Florida scrub. Application regimes that mimic the periodic release of these compounds during the rainy season in the Florida scrub will ultimately be required to ascertain the allelopathic properties of these shrubs.

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