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Soil Chemicals from Croftonweed (*Ageratina adenophora*) Are Phytotoxic

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Secondary plant metabolites may influence plant–plant interactions and plant invasions. Distinguishing such chemicals requires integrating varying chemical ecology approaches, information on the amounts and persistence of specific chemicals in nature, and measures of effects (e.g., phytotoxicity assays) to judge the importance of the chemicals (e.g., allelochemicals). The invasive plant croftonweed has caused substantial ecological and economic losses in China. We examined contents and degradation of croftonweed chemicals in the soil and their potential phytotoxic effects on conspecific and five allospecific plant species. Soils in which croftonweed was grown had four phytotoxins: DEHP, DBP, DTD, and HHO. All chemicals were detected in croftonweed-invaded soil, with contents ranging from 0.013 (for DEHP) to 0.353 (for DTD) μg g^{-1} of soil. All four compounds were degraded rapidly in 1 wk. Combinations of the chemicals inhibited seed germination or seedling growth of four of the six plants, including croftonweed itself, at mean contents found in the soil. The putative allelochemicals degraded rapidly in the soil, and the low levels of allelochemicals observed in the soil may be sufficient to affect seed germination and plant growth.

Nomenclature: DBP, dibuty1 phthalate; DEHP, bis(2-ethylhexyl) phthalate; DTD, amorpha-4, 7(11)-dien-8-one; HHO, 6-hydroxy-5-isopropyl-3,8-dimethyl-4a,5,6,7,8,8a-hexahydraphthalen-2 (1H)-one (HHO); croftonweed, *Ageratina adenophora* (Spreng.) King & H.E. Robins.

Key words: *Ageratina adenophora*, allelopathy, chemicals, croftonweed, phytotoxin.

Plants produce secondary metabolites; many of which are still of unknown functional importance. However, some secondary metabolites, such as root exudates, contribute to the invasiveness of some nonnative plant species (Ridenour and Callaway 2001; Silva et al. 2014; Vaughn and Berhow 1999). *Allelopathy* has been defined as all effects of plants on neighboring plants though the release of chemical compounds into the environment (Rice 1984). Here, we use the term *allelopathy* with the negative effect of allelochemicals released by invasive plant species on the growth or reproduction of another plant. The novel weapons hypothesis (Callaway and Ridenour 2004; Zangerl and Berenbaum 2005) posits that the invasive success of some exotic plants is partly due to their producing "novel" chemicals with phytotoxic effects (Inderjit et al. 2011; Prati and Bossdorf 2004). Allelopathic effects are influenced by the chemical, physical, and microbial components of the soil, which determine the fate of allelochemicals in the environment (Inderjit and Callaway 2003; Inderjit et al. 2008, 2010). Because the fates of soil chemicals depend on many variables, whether apparent allelopathy operates in any given habitat can be difficult to determine. Therefore, determining whether allelopathic effects exist requires considerable research into the chemical ecology and into methods of extraction, purification, quantification, and identification of potentially allelopathic chemicals (Dayan and Duke, 2014; Duke 2015; Hiradate et al. 2010).

Croftonweed (Asteraceae), a plant native to central Mexico, has invaded more than 30 countries and regions of tropical and subtropical zones. In the 1940s, it spread from Burma into the south Lincang (e.g., Cangyuan and Gengma) of China's Yunnan Province (Wan et al. 2010). The weed has subsequently spread widely throughout southwestern China, including Yunnan, Guizhou, Sichuan, Guangxi, and Xizang provinces and into Chongqing, continuing to spread eastward and northward at a speed of 20 km yr^{-1}. Croftonweed has caused serious economic losses to agriculture, forestry, and livestock, severely damaging the ecology and environment of China's native habitat, for example, exposure to, or consumption of, this weed makes

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livestock ill (Wan et al. 2010). Most important, croftonweed can establish monocultures where diverse, native communities once flourished (Wang and Wang 2006). Several studies suggest that allelopathy has helped croftonweed become dominant in invaded plant communities. Foliar leachates and volatiles of croftonweed decreased the seed germination and seedling growth of some native plant species, such as, Chinese cabbage (*Brassica rapa* L. ssp. *Pekinensis*), rhodesgrass (*Chloris gayana* Kunth), white clover (*Trifolium repens* L.), and slenderleaf ixeris [*Ixeridium gracile* (DC.) Shih] (Inderjit et al. 2011; Song et al. 2000; Yu et al. 2004; Zhang et al. 2012; Zheng and Feng 2005). Field applications of activated carbon reduced the inhibitory effects of soil used to grow croftonweed on native plants (Tian et al. 2007). Two main putative allelochemicals that have phytotoxic effects on other plants were identified as DTD and HHO (Yang et al. 2006, 2008). Five phytotoxic compounds were purified from the root exudates of croftonweed, including DBP and DEHP (Yang et al. 2013). However, most empirical support of the allelopathic potential of croftonweed on native plants comes from experiments conducted in controlled environments.

To evaluate allelopathic potentials in soils more realistically, the effects of those soils should not be overlooked (Hiradate et al. 2010). Here, we present field and laboratory experiments addressing three objectives: (1) the quantitation of chemicals with a temporal pattern in soil, (2) the degradation of chemicals in the soil, and (3) the phytotoxic effects of chemicals at mean concentrations found in soil.

Materials and Methods

Preparations of Chemicals of Croftonweed. Four chemicals of croftonweed were selected and prepared in this experiment according to our previous studies (Yang et al. 2006, 2013). DBP and DEHP were purchased from Sigma Chemical Co. (St. Louis, MO). DTD and HHO were prepared (Yang et al. 2006) from leaves and shoots of croftonweed, collected from healthy, mature plants (BBCH [Biologische Bundesanstalt, Bundessortenamt, und Chemische Industrie] scale stage, 40 to 45), without flowers and seeds, in a natural field in Kunming, Yunnan Province, China. The fresh aerial plant parts (4 kg) were extracted with 20 L of distilled water three times (24 h $extraction^{-1}$) at room temperature (Rt). The extracts were filtered through four layers of cheesecloth and Whatman (No.1) filter paper (Maidstone, Kent, U.K.). After evaporation in vacuo, the residues (2 L) were decanted and extracted in *n*-hexane in a separating funnel. *n*-Hexane fractions were combined and dried using filtered, anhydrous Mg_2SO_4. The solvent was dried in vacuo at Rt. The residues were redissolved in 20 ml methanol. The volume was reduced to 3 ml using a flow of N_2. The solution was applied to a silica gel (200 to 300 mesh; 4 by 80 cm) column and eluted with a gradient consisting of a mixture of *n*-hexane/chloroform/ethyl acetate (EtOAc) to increase polarity. Fractions of 30 ml were combined to form seven fractions according to the similarity of the retention factor on thin-layer chromatography. According to our previous results (Yang et al. 2006), the second fraction contained one major compound and other minor components, which were finally purified by recycling preparative high-performance liquid chromatography (RP-HPLC) to yield a colorless, solid form of DTD (Rt, 16.5 min; 195 mg). RP-HPLC was performed with a JAI LC-9201 HPLC (Japan Analytical Industry Co., Ltd., Tokyo, Japan) and a GS-310 column (21.5 by 300 mm) and MeOH : H_2O (80 : 20 v/v) as the mobile phase at a flow rate of 5 ml min^{-1}. After purification two times with silica gel column chromatography, the product was eluted with chloroform : EtOAc (3 : 1, v/v), and HHO (53 mg) as yellow oil was isolated from the fifth fraction.

Contents of Chemicals in the Soil. *Quantitative Method Development of Chemicals.* To quantify the contents of chemicals from croftonweed in soil of infested fields, we developed an effective extraction method, referencing Blair et al. (2009), with different solvents. Extraction efficiencies were examined using two solvents (methanol and EtOAc). DTD, HHO, DBP, and DEHP were individually added to 30 g of soil from site I (described below) in petri dishes, followed by the addition of 1 ml water to maintain moist conditions. Treatments included a control (without no chemicals added), and three concentrations (15, 75, and 375 μg g^{-1} soil) of each chemical. Soil samples were sealed with Parafilm (Bemis, Neenah, WI) and stored in the dark at ambient temperature for 24 h before analyses. Soil samples were extracted with 100 ml solvent (methanol or EtOAc) three times with occasional vortex. Each extraction time was 2 h. The extracts were centrifuged at 1,500 × *g* for 15 min, followed by filtering with 0.45-μm nylon filters. Subsequently, all samples were dried with N_2 before resuspension in 1 ml of methanol. All solvents (Fisher Chemical Co., Pittsburgh, PA) were HPLC grade.

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HPLC was performed with L-2000 HPLC (Agilent Eclipse Plus, Palo Alto, CA) with a C_{18} column (4.6-mm by 150-mm inner diameter, 5 μm) at 20 C. The mobile solvent system consisted of methanol and water (70 : 30, v/v) at 1 ml min^{-1}, with a UV detector wavelength of 254 nm. Aliquots (10 μl) were injected into the HPLC system, and compounds were quantified with external standard peak area.

Quantitation of Chemicals. To quantify the four chemicals in the invaded soil of croftonweed, we collected soil samples once a month from March to December in 2011. The study was carried out at Chunhe Town in Hongta District, Yuxi, Yunan Province, China (24.7°N, 102.87°E), located in an evergreen, broad-leaved–deciduous mixed forest, with average elevation of 2,000 m (range, 1,993 to 2,016 m). The average annual precipitation and temperature in the study area were 952 mm and 16.5 C. The area is a subtropical, hot, and arid valley climate with pronounced wet and dry seasons. Two sites were selected in the study area, where the rhizosphere soil was sampled in a previous study (Niu et al. 2007), and were classified by croftonweed coverage. The two sites had similar elevation, topography, gradient, and soil types and have been supporting croftonweed more than 30 yr. Site I, an evergreen, broad-leaved forest, was dominated by *Machilus pingii* W.C. Cheng ex Y.C. Yang, *Machilus nanmu* (Oliv.) Hemsl., paper-mulberry [*Broussonetia papyrifera* (L.) L'Hér. ex Vent.], *Cyclobalanopsis plaucoides*, and Chinese alder (*Alnus cremastogyne* Burkill), with a very low density of croftonweed (coverage $<10\%$). Site II was a deciduous broad-leaved forest, with alder (*Alnus cremastogyne* Burkill) and tea seed pomace (*Camellia oleifera* Abel) as the dominant species in the tree layer and a dense croftonweed population (coverage $>90\%$) in the understory layer. In site II, four soil cores (5 cm diam, 10 cm depth) were collected directly under the randomly selected croftonweed plants; 40 g of soil were retained from each soil core. All soil samples were sieved (2 mm) and cooled immediately in individual plastic bag. Soil samples were stored at −20 C until analysis.

Degradation of Chemicals in the Soil. The soil from sites I and II were sampled once only for determining the time consumed in chemical degradation. We investigated the degradation of DBP, DEHP, DTD, and HHO in the soil from sites I and II. Four chemicals at the concentration of 200 mg L^{-1} dissolved in EtOAc were prepared, and 1 ml aliquot of each chemical was added into petri dish (6 cm diam) and dried. Subsequently, 30 g of soil was added and mixed in each petri dish. Thus, the initial concentration of these chemicals in each petri dish was 6.67 μg g^{-1}. To remove the disturbance of any background chemical content in the soil at site II, the same soil samples were extracted and analyzed immediately. The background concentrations of DBP, DEHP, DTD, and HHO were 0.30, 0.05, 0.24, and 0.04 μg g^{-1}, respectively, and were not detected at site I. All treated soil samples were sealed with Parafilm and kept in darkness at 25 C in the growth chamber for 7 d. Then, extraction and quantification were determined as previously described. The experiment was conducted in a completely randomized design with five replications for control and each treatment.

Phytotoxic Effects of Chemicals. An assay was conducted to characterize the phytotoxic effects of the four chemicals on allospecifics of five native plants and conspecifics and croftonweed. Upland rice (*Oryza sativa* L.), slenderleaf ixeris, white clover, Chinese cabbage, and mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.] were selected to determine the phytotoxicity of chemicals in the rhizosphere of croftonweed. All plant seeds were surface-sterilized with 0.3% hydrogen peroxide and placed on two sheets of filter paper in petri dishes (9 cm diam), 30 seeds $dish^{-1}$ were used for upland rice and slenderleaf ixeris and 50 seeds $dish^{-1}$ for the other four species. The four compounds were diluted with EtOAc to form a combined test solutions. DEHP (0.01 mg L^{-1}), DBP (0.16 mg L^{-1}), DTD (0.35 mg L^{-1}), and HHO (0.05 mg L^{-1}) were prepared based on the arithmetic mean concentration (= *Sum of detected concentration of every sample* (our results from soil samples)/10), as described in our "Results" below. The test solutions, dissolved in 2 ml EtOAc, were added to petri dishes and dried. The filter paper in each dish was moistened with 2 ml of distilled water. Ethyl acetate without chemicals was used as a control. Thereafter, 1 ml of water was added to maintain sufficient moisture, as needed. Petri dishes were sealed with Parafilm and kept in a growth chamber at 25 C with 10 h of light. The incubation period was 3 d for upland rice, 10 d for croftonweed, and 7 d for the other four plant species. The number of germinated seeds, the length of the primary roots and shoots, and the biomass of seedlings were measured. All experiments were conducted in a completely randomized design with four replications for each treatment.

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Statistical Analyses. The data obtained from extraction efficiencies and content investigation of four chemicals in the soil were analyzed for variation using the ANOVA procedure in SAS software (version 6.12; SAS Institute, Cary, NC). Multiple comparisons of mean values were based on Fisher's protected LSD test. For the test on soil degradation and the phytotoxic effects of chemicals, the normality of all data was detected. However, some data failed normality: the degradation of four allelochemicals in the soils of sites I and II; the germination rate of slenderleaf ixeris, white clover, Chinese cabbage, and croftonweed; the root length of the upland rice; and the fresh weight of the slenderleaf ixeris, white clover, and croftonweed. Therefore, the normal data were analyzed with a *t* test and compared with the initial content or control, whereas the non-normally distributed data were analyzed using the Mann-Whitney *U* test. All data are expressed in percentages converted into quasinormal distributions by inverse sine transformation during analysis.

Results and Discussion

Contents of Chemicals in the Soil. It has been well documented that, before the ecologically relevant effects can be investigated for some putative allelochemicals, analytical chemistry must be used for compound detection and quantification, particularly from natural soil matrices (Blair et al. 2009; Inderjit and Weiner 2001). Thus, quantifying compounds from a complex sample matrix (i.e., soil) requires the development of a protocol that efficiently extracts the compounds of interest, for example, the recovery efficiency should approach 100% if practically possible (Blair et al. 2009). The extraction efficiency of MeOH and EtOAc was about 60 to 80% and 80 to 100%, respectively (Figure 1). Most recoveries from EtOAc were between 80 and 100%; therefore, the EtOAc extraction was used in all soil work.

All chemicals were detected, but their content fluctuated with seasons; among which, the DTD and DBP presented a similar temporal pattern: low levels during summer, but higher in spring, fall, and winter. The mean $\pm$ SE concentrations of DTD (0.353 ± 0.130 μg g^{-1}) and DBP (0.163 ± 0.060 μg g^{-1}) tended to be greater than that of HHO (0.049 ± 0.009 μg g^{-1}) and DEHP (0.013 ± 0.008 μg g^{-1}) (Figure 2). A relatively high content of DEHP, but not HHO, was found, which was not detected in November and December. Our data indicate these chemicals remain in soils in low

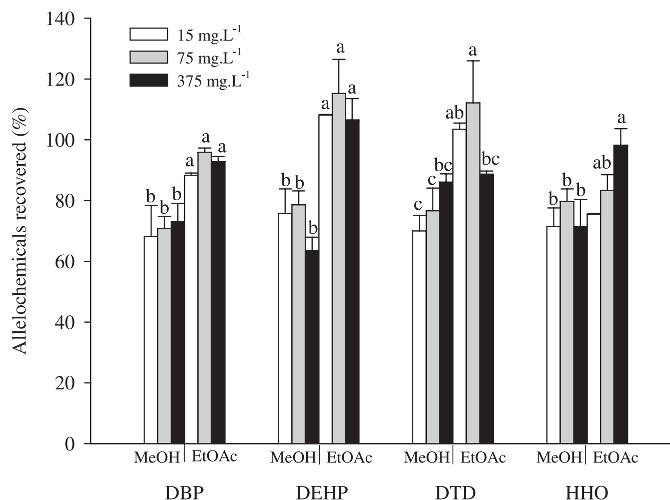

Figure 1. Comparison of extraction efficiencies from MeOH and EtOAc for four chemicals from soil. Bars are mean $\pm$ SE. Bars with different letters within the same chemical indicates significant differences within each treatment at $P < 0.05$ (Fisher's protected LSD test).

concentrations, which fluctuated with the seasons. The chemicals were present in very low concentrations or not detected during some months, especially from May to October. Some factors could explain the fluctuation in detecting chemicals in the soils below fields infested with croftonweed. As described above, at site II, the zone at which we tested the soil, located in a subtropical, subhumid climate area, had an average 18 and 27 C minimum and maximum temperature, respectively, and the mean monthly precipitation was 137.6 mm from May to October, which is obviously higher than those of the other months. It could be speculated that the levels of test chemicals decreased with gradually enhanced rainfall and temperature, which is similarly reported in the apigenin-4′-(*O*)methylin exudates of gum rockrose (*Cistus ladanifer* L.) (Chaves and Escudero 1999). However, the static concentration of a compound in the soil is less important than the rate of bioaccumulation in the receiving plant from a steady state or nearly steady state concentration that is maintained by a constant influx from the donor plant (Duke 2010). Thus, some rainfall events under comparative high temperature could decrease the quantitative levels of chemicals in the soil, but this does not mean the chemicals from croftonweed produced less ecological effect on the surrounding plants. Meanwhile, the chemicals were sometimes released at concentrations below minimum detection levels (e.g., HHO in November and December), which could be associated with the season when the plants may not be actively exuding this chemical. Besides, as discussed below, the compounds are

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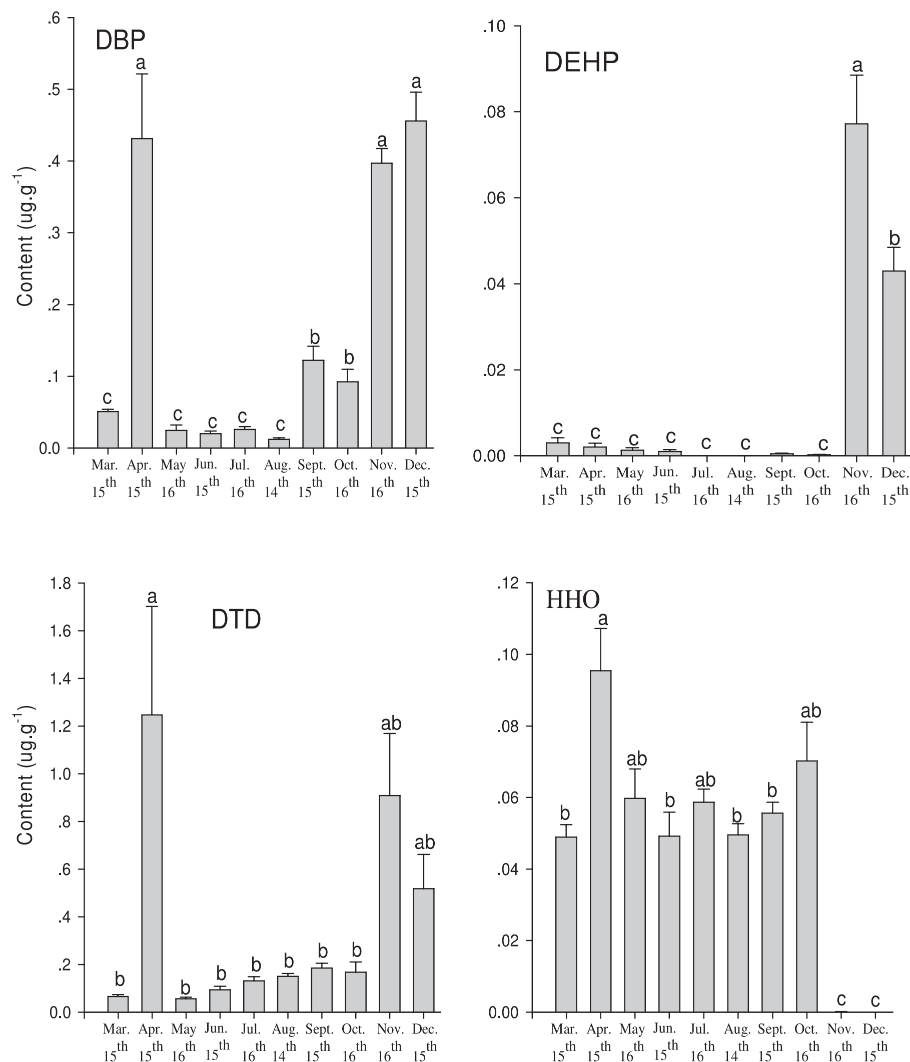

Figure 2. Content of four chemicals from croftonweed in the soil at site II. Bars are mean ± SE. Bars with different letters within the same chemical indicate significant differences within each treatment at $P < 0.05$ (Fisher's protected LSD test).

quickly dissipated in soil by microorganisms, and evaporation of these chemicals always occurs (Inderjit et al. 2010).

Soil Degradation of Chemicals. After 7 d, the soil content of the four compounds decreased relative to their initial content. The degradations of the DEHP, DTD, and HHO were more than 70% in site I soil and 80% in site II soil (Figure 3). However, DBP degraded more slowly at both sites (Figure 3). Although DEHP, DTD, and HHO could degrade rapidly in soil within 1 wk under our test conditions, the time consumption of chemicals released into the soil by plants could vary across the growing season, by developmental stage of the plants in the field or both (Cantor et al. 2011) and could also be influenced by outside environmental factors, e.g., climate, soil biology, and especially precipitation and degree days (Dayan 2006; Marushia and Holt 2008; Wang et al. 2012). For example, studies have demonstrated that, in soils in which an allelochemical was present, the soil microbial community adapted to using that chemical as an energy source or as a carbon source for mineralization, resulting in its enhanced degradation (Gimsing et al. 2009; Kong et al. 2008). Interestingly, our previous results found that soil biota could markedly reduce allelopathic effects of croftonweed (Zhu et al. 2011). Whether the soil biota could increase the degradation of the test chemicals and their interaction mechanisms in soil is worthy of our further study.

Phytotoxic Effects of Chemicals. Chemicals of croftonweed exhibited inhibitory effects at different levels on both allospecifics and conspecifics receptors. Our results indicate that treatment with different compounds led to a decrease in germination of slenderleaf ixeris (by 15%; Mann-Whitney U test,

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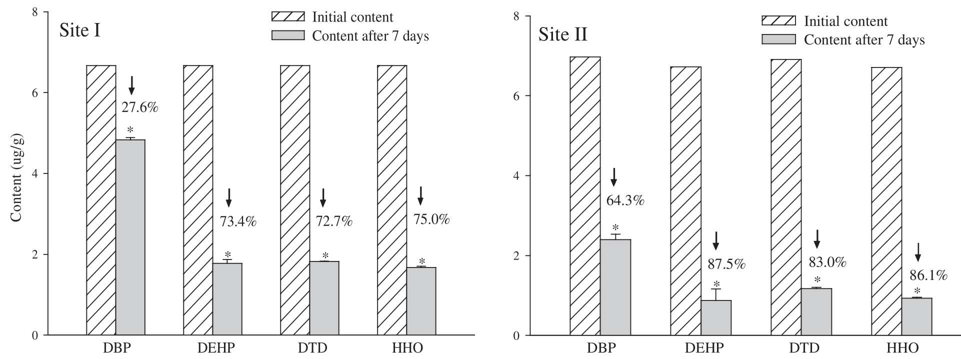

Figure 3. Degradation of four chemicals after 7 d in soil at two study sites (sites I and II). Error bars represent 1 SE, and asterisks denote significant differences ($P < 0.05$) between content of initial soil sample and the soil sample after 7 d.

$Z = -2.323$, $P < 0.05$) and white clover (18.5%; $Z = -2.191$, $P < 0.05$) seeds. There was, however, no obvious influence on germination of upland rice, Chinese cabbage, and mouse-ear cress (Figure 4A). Root length of upland rice ($Z = -2.309$, $P < 0.05$), but not the other plants, was reduced after exposure to these chemicals (Figure 4C). The biomass of slenderleaf ixeris and white clover was reduced substantially ($P < 0.05$) (Figure 4E). Moreover, these compounds also inhibited germination ($Z = -2.477$, $P < 0.05$) and reduced the biomass ($Z = -2.165$, $P < 0.05$) of croftonweed itself (Figures 4B, 4D, and 4F).

In the bioassay, we used a combination of the four chemicals at the mean concentration detected in soil, which represented, in part, the actual amount of chemicals that receptor plants could contact directly. Therefore, it is feasible to check the effects of chemicals at their net concentrations on the receptor plants in petri dishes without soil. Interestingly, a mixture of the four chemicals led to more-significant fitness reductions in test plants, when compared with that of a single compound (Yang et al. 2006, 2013). Our data demonstrated synergistic effects of the chemicals will occur when applied together (Leão et al. 2010). Still unrevealed are the mechanisms likely involved in the enhanced phytotoxic activity of the croftonweed chemicals from synergistic effects, but it has been demonstrated that the compounds present in mixtures have differential binding to soil particles and extend their soil bioavailability (Tharayl et al. 2006, 2008). Instead of single dose of a chemical in soil, as used in our experiment, allelopathy presumes the allelochemicals in the soil are in a constant flux between input from the donor plant, uptake by the receiving plants, and degradation, which strengthens the allelopathic potential of croftonweed.

The biological activity of the allelochemicals produced by croftonweed may be a "double-edged sword," which was observed in our results of allospecific and conspecific phytotoxic effects. More than half of the test species, including the croftonweed, used in this study were sensitive to the chemicals, with reductions in either seed germination or seedling growth. However, previous study showed that the soil biota in the invaded site had a greater inhibitory effect on native plant species than it does on croftonweed. Soil biota in the native plant site inhibited the growth of native plant species but did not inhibit croftonweed (Niu et al. 2007). Thus, we surmise that inhibiting effects of the croftonweed chemicals in the field would tend to have stronger allospecific interactions rather than conspecific interactions. Furthermore, the phytotoxic effects of the chemicals on conspecifics were found and the bioassay dose was based on the content of chemicals in soil directly under croftonweed plants; that is, marked autotoxic effects could be seen from the rhizosphere soil of croftonweed, which was consistent with the field tests (Yu and Ma 2006). Plant allelochemicals may serve dual roles as allelochemicals and autoinhibitors under natural conditions. The chemicals of croftonweed may also function as a form of territoriality, reducing the intensity of intraspecific competition and maximizing the fitness of the dominant members of a population (Schenck et al. 1999). We found that the population density of croftonweed in the invaded field was about 10 plants m^{-2} (G Yang, personal observation). Thus, our autotoxic experimental results clearly showed that the chemicals could help regulate the field population of croftonweed at an optimal density.

Our results clearly indicated that chemicals were present in soils under croftonweed plants and that they were phytotoxic at low concentrations, which is consistent with previous work in vitro and in sand cultures (Yu et al. 2004; Zheng and Feng 2005). Firstly, the chemicals were consistently present, in very low concentrations, in field soil samples. Secondly, the chemicals were obviously degraded in soil.

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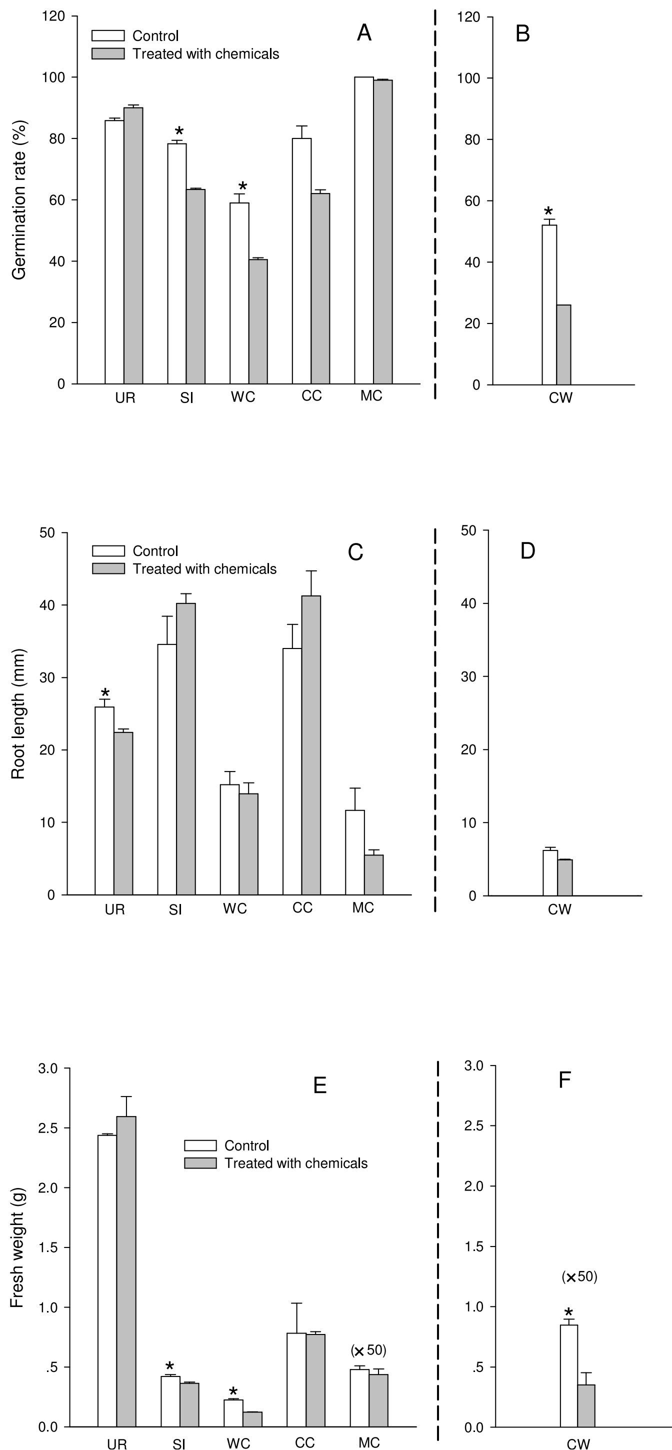

Figure 4. Phytotoxic effects of four chemicals from croftonweed on six test plants, including croftonweed. (A, C, and E) Effects of the chemicals on seed germination, root length, and fresh weight of allospecifics. (B, D, and F) Effects of the chemicals on seed germination, root length, and fresh weight of conspecifics. Magnifications of ×50 in panels E and F show the fresh weights of mouse-ear cress and croftonweed are magnified 50 times. Error bars represent 1 SE, and asterisks denote significant differences ($P < 0.05$) between treatments and the control of each receptor plant. Abbreviations: UR, upland rice; SI, slenderleaf ixeris; WC, white clover; CC, Chinese cabbage; MC, mouse-ear cress; SS: croftonweed.

Thirdly, the chemicals were phytotoxic to most of the test plants, including to itself. Taken together, our results support the notion that the tested chemicals were putative allelochemicals and which, as an autoinhibitor, could potentially influence the plant population ecology in areas in which croftonweed is growing.

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