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Phytochemistry 64 (2003) 493–497
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

7,8-Benzoflavone: a phytotoxin from root exudates of invasive Russian knapweed

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Received 8 January 2003; received in revised form 26 March 2003

Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Root exudates from *Acroptilon repens* (Russian knapweed) were found to be phytotoxic and the phytotoxin in the exudate was identified as 7,8-benzoflavone (α-naphthoflavone), (**1**), not previously known as a natural product. In tests on growing seedlings both **1** and its isomer 5,6-benzoflavone (**2**) were phytotoxic. Flavone, a structural analog of **1** and a known granular leaf and stem exudate of other plant species, was also phytotoxic and more potent than **1** or **2**.

Keywords: *Acroptilon repens*; Asteraceae; *Centaurea repens*; Compositae; Russian knapweed; 7,8-Benzoflavone; α-Napthoflavone; Flavone; Phytotoxin; Allelochemical

1. Introduction

Roots of the Northwest US invasive weed *Centaurea maculosa* Lam. (spotted knapweed) exude (±)-catechin. (−)-Catechin (but not its enantiomer) proved to be potently phytotoxic to many plant species and to also inhibit seed germination (Bais et al., 2002). Although *C. maculosa* was itself not affected by (−)-catechin, the closely related species *Centaurea diffusa* Lam. (diffuse knapweed) was susceptible. We are currently examining root exudates from related species to determine if they also exude phytotoxic allelochemicals.

Russian knapweed, *Acroptilon repens* (L.) DC, is another Eurasian-derived noxious invader of the western US (Goslee et al., 2001 and refs therein). It was formerly a part of *Centaurea* (Whitson et al., 1992), a complex genus now known to be polyphyletic (Garcia-Jacas et al., 2001). Russian knapweed has long been suspected to produce allelochemicals. Early work focussed on sesquiterpene lactones, which were not potent enough as phytotoxins to be considered ecologically important (Stevens, 1982). Polyacetylenes and thiophene derivatives are also known *A. repens* root chemicals and one of the polyacetylenes (among several isolated from *A. repens* roots) proved to be phytotoxic (Stevens, 1986). This compound could also be isolated from soil around the plant roots in concentrations apparently sufficient to have a significant effect on the surrounding plant community (Stevens, 1986).

As was done in the case of *C. maculosa* (Bais et al., 2002), we elected to directly bioassay root exudates from *in vitro* grown plants of *A. repens* for the presence of phytotoxic components and to isolate and determine structures for any which proved to be biologically active.

2. Results and discussion

Root exudates of in vitro-grown *A. repens* plants were assayed for effect on the phenotypic response of various plant species, including *Gaillardia aristata* Pursh, *Linaria dalmatica* (L.) Mill, *Centaurea. diffusa*, *C. maculosa* and the model plant *Arabidopsis thaliana* (L.)

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0031-9422/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00276-0

Heynh. All the species showed mortality on the seventh day after addition of root exudates from *A. repens*. Plants showed wilting symptoms prior to senescence with reduced shoot and root differentiation after administration of the root exudates. Hexane extracts of freeze-dried medium in which *A. repens* had been grown were subjected to HPLC analysis and collected fractions were used for bioassay. Essentially all the phytotoxicity was confined to a single compound and no activity remained in the extracted medium. The active component was identified as 7,8-benzoflavone (α-naphthoflavone), **1** (see Section 3). Phytotoxicity of **1**, along with structural analogs 5,6-benzoflavone (**2**) and flavone (**3**), were assayed in more detail for their effect on test plant phenotypic responses in terms of shoot and root differentiation inhibition. Generally, the phytotoxic activities were in the range of 50–250 μg/ml except for **3**, which was active at 50 μg ml^{-1}. For comparison purposes, a concentration of 250 μg ml^{-1} for all three tested flavones showed a distinct plant inhibitory profile (Fig. 1). All plants except *A. repens* showed mortality on the seventh day after the addition of **1** and **2** but flavone **3** was also active against *A. repens* (Fig. 1). The addition of 100 μg ml^{-1} of **1** to the roots of *Arabidopsis thaliana, C. diffusa*, *C. maculosa* and *L. dalmatica* led to a condensation of the cytoplasm characteristic of cell death, which showed **1** to be a potent rhizotoxin (Fig. 2). The naphthoflavone **1** was exuded from *A. repens* roots at concentrations as high as 180 μg/ml during a 30-day course. This concentration was therefore higher than the MICs for **1**. The root exuded concentration did not show any autotoxicity to *A. repens* itself. The phytotoxic potency of **1** is a little less than that observed for (−)-catechin (Bais et al., 2002), but **3** appears to be approximately as potent. One of the North American native species (*G. aristata*) was found to be resistant to **1** and **2**, although not to flavone **3** (Fig. 1). In the field, populations of *G. aristata* are also resistant to knapweed invasion, one of the few native species to exhibit such resistance (R.M. Callaway, University of Montana, private communication).

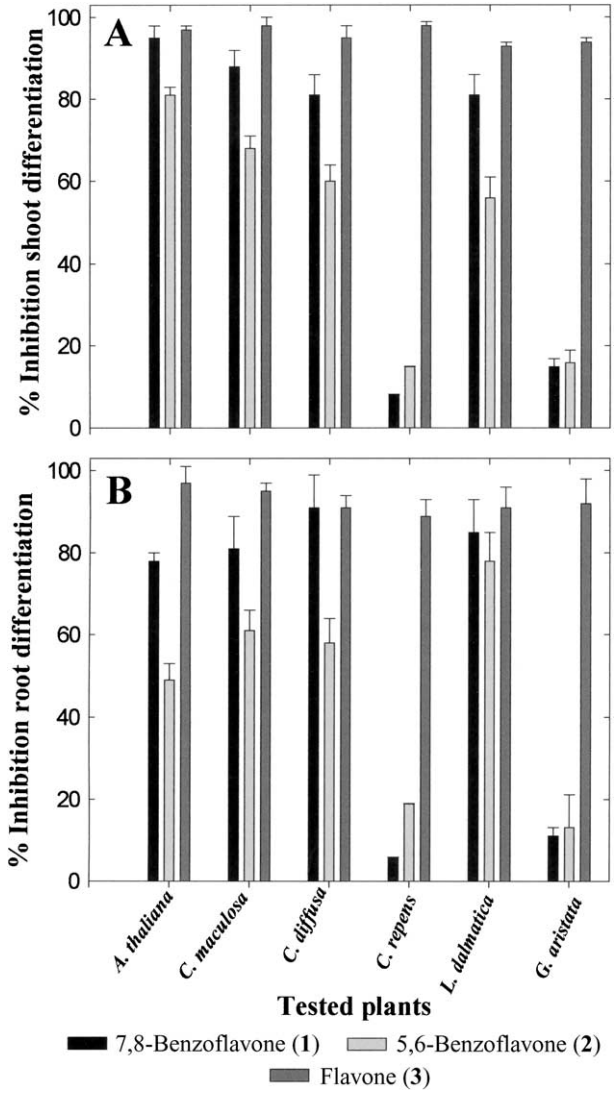
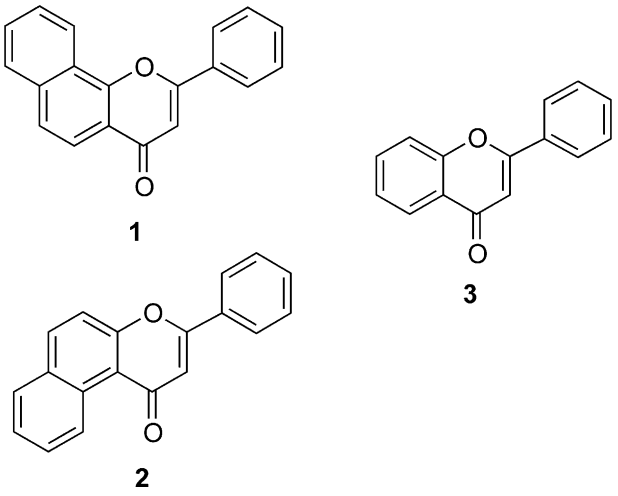


Fig. 1. Effect of 7,8-benzoflavone (**1**), 5,6-benzoflavone (**2**) and flavone (**3**) on shoot (A) and root (B) differentiation of different weeds on the seventh day after treatment. Compounds were dissolved in CH_3OH and administered (250 μg ml^{-1}) to the media containing 10-day-old seedlings. Twelve-well aseptic plates were subsequently incubated under a 16 h light and 8 h dark photoperiod in an incubator. The data represent the percent inhibition relative to the untreated control in shooting and rooting efficiency response in various tested seedlings (values are mean ± S.D, $n = 10$).



A search of the literature and various databases failed to reveal any references to 7,8-benzoflavone **1** as a natural product although it has been used in a number of biological studies. For example, it is an activator of cytochrome P450 (Lee et al., 1997), is an inhibitor of benzopyrene-caused DNA damage (Baird and Diamond, 1976; Wu et al., 2001) and binds to adenosine receptors (Ji et al., 1996).

Flavone **3** has only rarely been encountered as a natural product and always as a component of a so-called

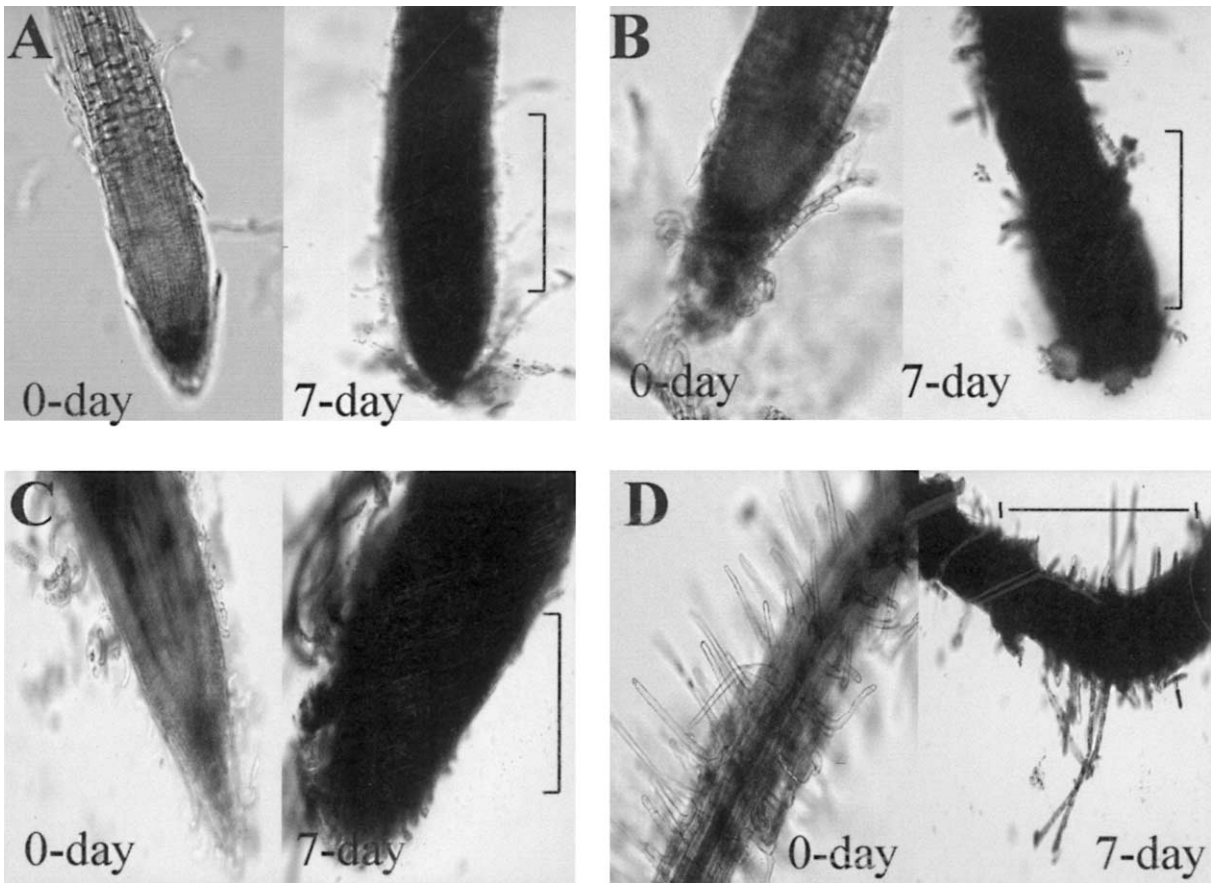


Fig. 2. Effect of 7,8-benzoflavone (**1**, 100 μg ml^{-1}) over a 7-day time course on roots of *Arabidopsis. thaliana* (A), *C. diffusa* (B), *C. maculosa* (C) and *L. dalmatica* (D) depicting cytoplasmic precipitation, a clear signature of cell death and rhizotoxicity (inset).

farinose (granular or mealy) exudate on leaves, particularly of species in the family Primulaceae (Hegnauer, 1969; Harborne, 1971), and in a few others as well (Freeman et al., 1981). Exudation of flavone **3** by these species could be of ecological importance as a result of its phytotoxicity.

We could find no reports on phytotoxicity of **1** or **3**, but both were shown (along with a variety of other tested chemicals) to be inducers of microsomal ecdysone monooxygenase in fall armyworm larvae (Yu, 1995, 2000). Flavone **3** was reported to be an active antimicrobial component of *Primula malacoides* (50 μg/ml against *Mycobacterium tuberculosis* var. *hominis* H37) (Weller et al., 1953).

Our results do not necessarily contradict those of Stevens (1986) who found a polyacetylene to be a root phytotoxin of *Acroptilon repens*. That study involved direct extraction of roots rather than analysis of root exudates. An extensive modeling study based on relationships among *A. repens* invasiveness, plant soil texture and allelopathy resulted in postulation of an important role for allelopathy, although the chemical nature of any allelochemical(s) was not studied (Goslee et al., 2001). It is certainly possible that *A. repens* invasiveness involves more than one phytotoxin, but final judgement will await growing season analysis of rhizosphere samples for the presence of **1**. Nevertheless, the present work again demonstrates that secondary metabolite exudation by plant roots (Bais et al., 2002) can involve unique metabolic processes. It also suggests a possible ecological role for the known leaf farinose exudations of flavone **3** (Freeman et al., 1981; Harborne, 1971).

3. Experimental

3.1. Materials

Seeds of plant species were from the collections of Professor R. Hufbauer, Department of Bioagricultual Sciences and Pest Management, who also identified these species in the field. Seeds of *Acroptilon repens* (L.) DC, *Centaurea maculosa* Lam., *Centaurea diffusa* Lam. *Gaillardia aristata* Pursh (Asteraceae) and *Linaria dalmatica* (L.) Mill. (Scrophulariaceae) were obtained from natural populations in Larimer or Routt Counties, CO. Seeds of *Arabidopsis thaliana* were purchased from Lehle Seed Company, TX. The 5,6- and 7,8-benzoflavones **2** and **1** and flavone **3** were obtained from Indofine Chemical Co., Hillsborough, NJ.

3.2. Culture conditions

Seeds were washed in running tap water and surface sterilized using sodium hypochlorite (0.3% v/v^{-1}) for 10–15 min, followed by 3–4 washes in sterile distilled water. Seeds were inoculated on static MS (Murashige and Skoog, 1962) basal media in petri dishes and allowed to germinate for 10 days until roots and shoots emerged. They were then incubated under 16 h light and 8 h dark at 25±2 °C. The light intensity in the growth chamber was 24 μmol m^{-2} s^{-1}. Ten-day-old seedlings were transferred to 5-ml 12-well aseptic plates with 2 ml of liquid MS basal media. Plant cultures were maintained on an orbital platform shaker set at 90 rpm

3.3. Plant growth inhibitory bioassays

Root exudates were collected from the *Acroptilon repens* plants, were filter sterilized to avoid collecting root border cells and cellular debris, and were found to be phytotoxic. For final tests the exudates were autoclaved (120° C for 30 min at 15 lb pressure), and administered at different concentrations (1–3 ml v/v) over the test plant seedlings as were collected HPLC-eluants (see later). Ten-day-old seedlings of *A. repens*, *C. maculosa, C. diffusa, Arabidopsis thaliana, L. dalmatica* and *G. aristata* were placed on MS basal medium in petri dishes after initial surface sterilization. Petri dishes were kept under a 16 h light and 8 h dark photoperiod in an incubator. Length of shoots, number of shoots and length of the primary root of the treated and untreated plants were measured and were denoted as units for shoot and root differentiation. Similar procedures were used to test phytotoxicity of 5,6- and 7,8-benzoflavones **2** and **1** and flavone **3**.

3.4. Extraction and HPLC isolations

Filter-sterilized growth media exudates (2 ml) were extracted with 5 ml hexane, vortexed and stored for 24 h at 4 °C. The supernatant was transferred with a Pasteur pipette to a separate test tube, and 1 ml of hexane was added. The supernatant was further concentrated by freeze-drying, and the weighed powder was re-dissolved in absolute MeOH (500 μl) for HPLC analyses.

Compounds in the root exudates were subjected to purification by gradient elution on a reversed phase 5 μm, C_{18} column (25 cm × 4.6 mm) (Supelco Co) using a Summit Dionex pump and injection system with detection at 210 nm. Mobile phase Solution A was double distilled water and Solution B was MeOH. A multi-step gradient was used for all separations with an initial injection volume of 50 μl and a flow rate of 1 ml min^{-1}: 0–10 min 5% B, 10–15 min 20% B, 15–25 min 20% B, 25–40 min 80% B, 40–60 min 100% B, 60–70 min 100% B, 70–80 min 5% B. Major peaks were collected and bioassayed for phytotoxicity, with toxicity showing only for a 44 min eluant. This eluant was collected from several injections, concentrated under vacuum at 30° C and repurified by reinjection and collection.

3.5. 7,8-Benzoflavone (*1*) identification

The ^{1}H and ^{13}C NMR spectra (Varian INOVA 500 and 75 MHz in $CDCl_3$ and CD_3OD, respectively) and EIMS of the active compound were identical to those of commercial **1** and nonidentical to those of **2**. EIMS *m/z* (rel. int.) 272 (95), 244 (15), 170 (100), 114 (55); ^{1}H NMR ($CDCl_3$): δ 6.93 (1H, *s*), 7.56 (3H, *m*), 7.68 (2H, *m*), 7.74 (1H, *d*, $J=8.5$), 7.90 (1H, *m*), 7.99 (2H, *m*), 8.14 (1H, *d*, $J=8.5$), 8.55 (1H, *m*); C^{13} NMR (75 MHz in CD_3OD): δ 108.1, 121.0, 121.1, 123.6, 125.4, 127.1, 127.8 (2), 129.0, 129.6, 130.6 (2), 131.1, 132.9, 133.3,137.8, 155.3, 165.3, 180.5.

Acknowledgements

This work was supported by funds from the Invasive Weeds Initiative of the State of Colorado, the Colorado State University Agricultural Experiment Station, and the Departments of Chemistry (F.R.S.) and Horticulture and Landscape Architecture (J.M.V.). J. M. V. is a National Science Foundation Early Career Development Faculty Fellow (MCB-0093014).

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