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Phytotoxic properties of *Drosophyllum lusitanicum* leaf extracts and its main compound plumbagin

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ABSTRACT

The aim of this work was to evaluate the phytotoxic properties of aqueous and hexane extracts from the insectivorous plant *Drosophyllum lusitanicum* (L.) Link using lettuce and bread wheat as model species. The results obtained confirmed that both germination and seedling growth bioassays were sensitive and able to detect the heterotoxicity potential of *D. lusitanicum* extracts. Aqueous and hexane extracts at several concentrations significantly inhibited the seed germination of lettuce and wheat, although wheat was less sensitive. The inhibitory effects of plumbagin, the major compound found in *D. lusitanicum* hexane extracts, were also evaluated. Comparing the results of the assays obtained with extracts and plumbagin it was postulated that plumbagin is the principal compound responsible for the phytotoxic effects of the extracts on lettuce but not on wheat. Therefore, although the phytotoxic potential of *D. lusitanicum* was demonstrated, further studies are needed to clearly specify the compounds responsible for the inhibitory effects and to ensure if the results obtained with the model species are reproducible to weed species in field conditions.

1. Introduction

Secondary metabolites produced and accumulated by plants can induce both inhibitory and stimulatory effects on organisms and may play roles in shaping plant and microbial communities (Pennacchio et al., 2005). Germination inhibitory compounds and allelochemicals are phytotoxic compounds produced by plants that aid them in both interspecific and intraspecific competitions (Meyer et al., 2007). The search for allelochemicals/phytotoxins is a growing research field, because these compounds have a great potential for controlling noxious weeds and could be used as herbicides in agriculture (Singh et al., 2003a).

Concerns about ecological, environmental, and health problems possibly associated with synthetic pesticides have increased interest in the development of new classes of environmentally safe herbicides (Dayan et al., 1999). The ability of a plant species to inhibit the germination of other plants is an untapped resource for weed control in crops that could revolutionize organic crop production. The study of the phytotoxic potential offers useful clues in the investigation of new models of natural herbicides that could be more specific and less harmful than the synthetic substances used in agriculture (Singh et al., 2003a; Bogatek et al., 2006; Hachinohe and Matsumoto, 2007). The natural plant products have a number of advantages over synthetic herbicides as they usually possess complex structures, exhibit structural diversity and hence are invaluable sources of lead compounds; have more chiral centers; have high molecular weight with no or low amount of halogens or heavy atoms; are environmentally benign as they degrade rapidly in the environment; and have novel target sites of action different from the synthetic herbicides (Dayan et al., 1999; Duke et al., 2000, 2002; Singh et al., 2003a).

Strategies for the discovery of compounds with phytotoxic properties are analogous to those for the discovery of compounds in the pharmaceutical industry and involve the screening of crude extracts and purified compounds for biological activity (Vyvyan, 2002). These initial bioassays must be quick, economical, and relevant to the system in question. The most widely used biological assays are seed germination and seedling growth studies (Vyvyan, 2002). The most commonly used species for the bioassays is *Lactuca sativa* L., a readily available sensitive species that germinates rapidly and uniformly. However, according to Dayan et al. (2000) both mono- and dicotyledons species should be used in assays to determine the potential selectivity of the agent.

Naphthoquinones and other related quinonoid compounds are one of the major natural product classes with varied biological activities (Akendengue et al., 1999). The naphthoquinone plum-

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bagin (5-hydroxy-2-methyl-1,4-naphthoquinone), which occurs in plants from the Plumbaginaceae and Droseraceae families, has been shown to exert anticarcinogenic (Sugie et al., 1998; Srinivas et al., 2004; Hsu et al., 2006; Kuo et al., 2006), antiatherosclerotic (Ding et al., 2005), antimicrobial (Lim et al., 2007) and insecticidal effects (Ganapaty et al., 2004; Gonçalves et al., 2008). Moreover, the antifeedant (Tokunaga et al., 2004) and allelochemical potentials of plumbagin were also described (Spencer et al., 1986; Kocacaliskan and Terzi, 2001; Rischer et al., 2002; Meyer et al., 2007).

Leaves of *Drosophyllum lusitanicum* (L.) Link (Drosophyllaceae), an carnivorous plant endemic to the western Iberian Peninsula and northwest Morocco, contain flavonoids (luteolin, leucocyanidin, leucodelphinidin), phenolic compounds and large amounts of plumbagin (Nahálka et al., 1998; Budzianowski et al., 2002; Grevenstuk et al., 2008). The insecticidal (Gonçalves et al., 2008) and antimicrobial (Gonçalves et al., 2009) activities of *D. lusitanicum* extracts have previously been described by our group, however, the phytotoxicity of *D. lusitanicum* extracts has not been evaluated yet. Thus, the present study describes the inhibitory effects of aqueous and hexane extracts from *D. lusitanicum*, and its main compound, plumbagin, on seed germination and seedling growth of two model species, lettuce (*L. sativa*) and wheat (*Triticum aestivum* L.).

2. Materials and methods

2.1. Plant material and extracts preparation

Healthy mature leaves of *D. lusitanicum* were collected in March 2005 from a population located in Algarve region (Portugal). The plant material was authenticated by Dr. A.I. Correia from the Botanical Garden of the University of Lisbon (Lisboa, Portugal) where a voucher specimen was deposited under the number LISU 206396.

Samples of fresh and dried (at 45 °C in a ventilated drying oven) plant material were used to prepare aqueous extracts. Fresh material was divided into small pieces (±1.5 cm) and dried material was powdered using a blender. The extracts were obtained by extracting different quantities of fresh (2.5, 5 and 10 g) or dried (2.5, 5, 10, 20 and 30 g) plant material with 100 ml of distilled water in a shaker over 24 h at room temperature. The mixtures were then vacuum filtered (Whatman filter paper No. 1). Aliquots of the extracts obtained from fresh and dried plant material at different concentrations (w/v) were stored at −20 °C until tested. The concentrations of aqueous extracts are presented as a percentage (%, w/v) of the plant material weight per water volume used for extraction.

Fresh plant material of *D. lusitanicum* was extracted with *n*-hexane (Riedel-de Haën, Buchs, Switzerland) in a Soxhlet apparatus over 8 h. The extract obtained was concentrated in a rotary-evaporator under reduced pressure at 50 °C until dry. The stock solution of the hexane extract (100%) was obtained by resuspending the crude extract in *n*-hexane (10 mg ml^{-1}). This solution was stored at −20 °C and was later diluted with *n*-hexane to obtain the different concentrations to be tested.

2.2. Phytotoxic effects of the plant extracts

Seeds of *L. sativa* cv. Capitata and *T. aestivum* cv. Tâmega were surface-sterilized for 20 min in 20% (v/v) sodium hypochlorite (NaClO) solution, and rinsed several times with sterile distilled water. To test the phytotoxicity, 500 μl of the extract at different concentrations were added separately over Whatman filter papers No. 1 in Petri dishes (9 cm Ø). Then filter papers were moistened with 2 or 2.5 ml (for lettuce or wheat, respectively) of distilled water, and 20 seeds were evenly placed into the treated filter paper. For the hexane extract the filter paper was only moistened after the evaporation of the solvent. Controls were prepared in a similar way with pure solvent, allowing evaporation, or distilled water only. Seeds were incubated at 25 ± 2 °C in darkness.

In the tests with lettuce seeds, three concentrations (2.5, 5 and 10%, w/v) of aqueous extract prepared with fresh and dried plant material and nine concentrations of hexane extract (0.5, 1, 2.5, 5, 10, 25, 50, 75 and 100%, v/v) were assayed. For wheat seeds, three concentrations (10, 20 and 30%, w/v) of aqueous extract prepared with dried plant material and five concentrations of hexane extract (10, 25, 50, 75 and 100%, v/v) were assayed. These concentrations were selected from preliminary assays. For each extract and concentration 10 repetitions with 20 seeds were performed.

In another experiment seeds of lettuce and wheat were exposed to hexane extract at 25 or 100%, respectively, for 3, 6 and 24 h and then transferred under sterile conditions onto filter papers containing only distilled water where they remained until the end of germination (5 days). The experimental conditions were like those mentioned above, and they were repeated 5 times for each species.

2.3. Phytotoxic effects of plumbagin

The quantification of plumbagin in the hexane extract (1.0 μl) was performed by the external standard methodology on an Agilent (Agilent Technologies, Little Falls, DE, USA) 6890 Series gas chromatograph interfaced to an Agilent 5973 *N* mass selective detector as described by Grevenstuk et al. (2008). The content of plumbagin in each concentration of the hexane extract tested was calculated. Then solutions of plumbagin (from *Plumbago indica*, Sigma, Steinheim, Germany) in *n*-hexane, in a range of concentrations (from 0.02 to 10.63 mM) close to those observed in the extracts were prepared. The effect of those solutions on lettuce (0.02, 0.04, 0.08, 0.17, 0.33, 0.66, 1.33, 2.66, 5.31, 7.97 and 10.63 mM) and wheat seeds (0.66, 1.33, 2.66, 5.31, 7.97 and 10.63 mM) was evaluated as described for the hexane extract. As control *n*-hexane only was used. For each plumbagin concentration and species 5 repetitions with 20 seeds were tested.

2.4. Data collection and statistical analysis

Seed germination was assessed daily, and seedling (root and shoot) growth and biomass were measured after 5 days. Data were subjected to one-way ANOVA followed by the comparison of multiple treatment levels with the control applying the Dunnett test at 5% level. For the germination results the ANOVA was performed for data at 5 days only. The analysis was performed using the SPSS statistical package for Windows (release 15.0; SPSS Inc., Chicago, IL, USA). The values presented in the figures and tables are the means of the repeated experiments.

3. Results and discussion

Although the use of solvent extractions for an increased yield of phytochemicals from plant tissue extracts is common in the literature, certain extracted chemicals may not be released into a natural environmental under average field conditions. However, water-soluble allelochemicals such as phenolics have been identified in several species (Singh et al., 2003b; Batish et al., 2006a, 2007). Therefore, in this work, both hexane and aqueous extracts from *D. lusitanicum* were evaluated for their phytotoxic properties under laboratorial conditions.

Aqueous extracts obtained from both fresh and dried plant material had an inhibitory effect on germination of lettuce seeds, although the dried extract was more effective (Fig. 1A). The fresh extract did significantly reduce the germination of lettuce seeds at