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Research Paper

Effectiveness of defatted seed meals from Brassicaceae with or without crude glycerin against black grass (*Alopecurus myosuroides* Huds.)

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ABSTRACT

Herbicide resistance has become an increasing problem, and at the same time pesticide usage is declining due to stringent EU pesticide legislation which aims to reduce the impact on environment and human health. For these reasons, new alternative integrated weed management approaches are becoming increasingly relevant. Formulations based on Brassica defatted seed meals (DSMs) and glycerin, have previously been shown to be effective in reducing the germination of lettuce seed.

In this work five DSMs, formulated with and without crude glycerin, were chosen for *in vitro* and glasshouse experiments: i) *Brassica nigra*, ii) *Brassica tournefortii*, iii) *Eruca sativa*, iv) *Rapistrum rugosum* and v) *Sinapis alba*. Black-grass (*Alopecurus myosuroides*), a weed demonstrating extensive herbicide resistance, was used as a target, and the germination inhibition caused on this weed by Brassica defatted seed meals was assessed.

In both *in vitro* and *in vivo* experiments, the most effective DSM for inhibiting germination of both lettuce and black-grass seeds was the sinigrin containing DSM, *Brassica nigra*.

The aim of the manuscript was to suggest a new high value application for Brassicas derived DSM as a co-products from the vegetable oil production chain. The proposed treatments could represent an interesting and 100% novel natural alternative to the conventional herbicides.

1. Introduction

Despite different land use contexts, from agriculture to urban settings, weed control remains a major problem. In particular, weed control in amenities such as parks and schools should be undertaken without threatening health and environment. At the same time, whilst herbicide resistance has become an increasing problem (Service, 2013), EU policy is demanding a significant reduction of pesticide usage. In fact, several high impact chemical products used in European agriculture are in phase out or under scrutiny following the Directive 2009/128/EC on "Sustainable use of pesticides", and the Regulation (CE) no. 1907/2006 (REACH) on Registration, Evaluation, Authorization and Restriction of Chemicals. In the Article 14 concerning Integrated Pest Management, the REACH clearly reports that "Member States shall take all necessary measures aimed at promoting low pesticide-input pest management, giving wherever possible priority to non-chemical methods".

At the same time, a wide range of fossil-based products could be substituted by bio-based products and materials derived from different biomasses such as energy crops, agricultural and forestry residues and waste (Maity, 2015).

The most abundant co-products of industrial vegetable oil production for bioenergy and green chemistry are defatted seed meals (DSMs) derived from seed defatting procedures. Among oilseed crops, biofumigant *Brassicaceae* crops have shown great potential within integrated pest management and organic farming solutions. In fact, some Brassica DSMs contain high level of glucosinolates (GSLs) and, after a patented procedure (Lazzeri et al., 2010), they proved to be suppressive against a variety of soil-borne fungal pathogens (Lazzeri et al., 2003), nematodes (Lazzeri et al., 2009; Ngala et al., 2015) and wire-worms (Furlan et al., 2010). In their native form, GSLs are stable and marginally reactive, while in the presence of water and the endogenous enzyme myrosinase (MYR) they are quickly hydrolyzed with the production of a series of bioactive breakdown products, mainly isothiocyanates (ITCs) and, to a lesser extent, nitriles, epithionitriles and thiocyanates, depending on the reaction conditions (Bones and Rossiter, 2006; Agerbirk and Olsen, 2012). Thanks to this natural process, *Brassicaceae* species have been widely studied for applications in the so called biofumigation technique (Kirkegaard et al., 1993). Since the inhibitory influence of aqueous extracts from parts of *Brassica oleracea* plants on the germination and

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growth of clover (*Trifolium repens* L.) and rye-grass (*Lollium* spp.) was first described by Campbell (1959), many other studies on the allelopathic effects of GSL degradation products were carried out. Angelini et al. (1998) reported a total inhibition of seed germination of different weeds by the hydrolysis products of glucoerucin and glucoraphanin. In the same study, seeds treated with the hydrolysis products of *epi*-progoitrin, mainly 5-vinyloxazolidine-2-thione, gave a high percentage of abnormal seedlings. The allelopathic potential of several species and cultivars of Brassica on wheat, in laboratory and field trials, has been reported by Mason-Sedun et al. (1986) and Mason-Sedun and Jessop (1988). More recent papers have shown the allelopathic effect of Rapeseed (*Brassica napus* L.) water extracts on *Phalaris minor* (Retz.), *Convolvulus arvensis* (L.) and *Sorghum halepense* (L.) (Aliki et al., 2014).

Furthermore, Brassica DSMs have shown a good synergy with crude glycerin (CG), an underestimated co-product derived from the biodiesel chain. D'Avino et al. (2015a) reported the first attempts to apply CG as an active ingredient for germination inhibition (GI), even if high doses were required for a significant inhibition activity. On the other hand, the DSMs, activated by a patent procedure (Lazzeri et al., 2010), were easily formulated with CG solutions, maintaining a high GSLs conversion rate and a sufficient retention capacity of the biologically active compounds. In this way, the synergic effect of CG and DSMs allowed a strong reduction of CG in the formulations.

Black-grass (*Alopecurus myosuriodes* Huds.), a major annual grass weed of winter cereals, was chosen as a potentially interesting target. Its winter annual growth habit is well adapted to winter cereal production, and recent crop management techniques such as earlier fall planting and intensive cereal rotation have led to rapid increases in black-grass populations, (Holm et al., 1997). One of the first cases of resistance to herbicides registered in Europe was in early 1980s, in Essex, UK (Moss and Cussans, 1985). Nowadays, herbicide resistance in black-grass populations is reported in a number of EU countries, including populations that demonstrate multiple resistance to a range of herbicides with different modes of action, including photosystem II inhibitors, ACCase inhibitors, and ALS inhibitors (Henriet and Marechal, 2009; Keshtkar et al., 2015).

The aim of this work was to evaluate the GI activity on black-grass of different Brassica DSMs in formulation with and without CG. Before the evaluation of the effect of five Brassica DSMs on black-grass, a preliminary screening by *in vitro* and glasshouse trials on lettuce was set up to evaluate the antigerminative capability of twenty GSL containing DSMs from the CREA-CI collection. The proposed treatments could represent an interesting and novel natural alternative to conventional approaches in weed control.

2. Materials and methods

2.1. Materials

The CG was purchased from Cerealdocks S.p.A. (Vicenza, Italy), an industrial biodiesel company and its composition was previously published (D'Avino et al., 2015b). *Berteroa incana* (L.) DC., *Brassica oleracea* L., *Brassica rapa* L., *Brassica tournefortii* Gouan, *Eruca sativa* Mill., *Erysimum pseudorhaeticum* Polatschek, *Hesperis matronalis* L., *Lepidium campestre* (L.) W.T. Aiton, *Lepidium densiflorum* Schrad., *Lepidium sativum* L., *Lesquerella fendleri* L., *Limnanthes alba* Benth., *Raphanus sativus* L., *Rapistrum rugosum* (L.) All., *Camelina sativa* (L.) Crantz, *Cleome hassleriana* Chodat, *Reseda lutea* L., and *Sisymbrium officinale* (L.) Scop. DSMs derived from the CREA-CI *Brassicas* collection (Lazzeri et al., 2013) were defatted with hexane (1/3, W/V) overnight at room temperature (21 ± 1◦C). Before defatting, *Rapistrum rugosum* seeds were scarified with a grinder (Bühler-Miag, MLI-204) to remove the very corky no-GSLs containing silique. *Sinapis alba* L. DSM from the CREA-CI Brassicas collection, and *Brassica nigra* (L.) W.D.J. Koch DSM purchased by Agrium Italia S.p.A (Livorno), were produced after seed defatting by an endless screw press in which temperature was kept lower than 75 °C (Lazzeri et al., 2010). Lettuce (*Lactuca sativa* L. cv. Cosmic) was purchased from SAIS S.p.A. (Cesena, Italy), whilst *Alopecurus myosuriodes* Huds. black-grass, (population: Blackgrass Foxtail, Slender) was purchased from Herbiseed (Reading, UK).

2.2. Defatted seed meals characterization

All DSMs were produced by a patented procedure (Lazzeri et al., 2010) aimed at optimizing the enzymatic system that catalyzes GSL hydrolysis. The preparation details must be considered as commercially confidential and their property is of Agrium Italia S.p.A (Livorno). The DSMs were analyzed according to the following methods:

- Dry matter was determined by oven-drying the DSM at 105 °C for 12 h and evaluating the difference in weight before and after treatment;
- Residual oil content was determined by the standard Soxhlet extraction method using hexane as solvent;
- Nitrogen content was determined by the Kjeldahl method (Standard UNI, 1992), using a Tecator digestion system 20 and an automatic Büchi distillation unit (B-324);
- Glucosinolate content was determined following the ISO 9167-1 method (ISO 9167), with some minor modifications in the extraction phase, as described in Lazzeri et al., 2011.

All data are reported as mean ± standard deviation of four determinations.

2.3. In vitro trials: extract preparation and hydrolysis product GC–MS identification

2.3.1. Extract preparation

Water suspensions of each DSM (15 g L^{-1}) were kept under agitation on an orbital shaker, 0.22 g for 40 min, at room temperature (21 ± 1 °C). The suspensions were centrifuged at 3893*g*, for 30 min and filtered with filter paper (Filter-Lab, 1248).

2.3.2. Deactivation of defatted seed meals

A small batch of DSM was treated in sealed borosilicate glass containers by an autoclave (20 min, 120 °C) in order to deactivate the myrosinase enzyme and prevent GSLs hydrolysis to bioactive compounds. Extracts from deactivated DSMs were prepared as reported above.

2.3.3. Hydrolysis product GC–MS identification

Samples of the obtained aqueous extracts (500 µl) were mixed with ethyl acetate (LC–MS Chromasolv®) at a ratio of 1:1 and after agitation with a vortex for 3 min were centrifuged for 10 min at 1240*g*. One µL of the upper organic phase was than collected and injected in a Bruker GC451 Gas Chromatograph equipped with a HP-5 fused silica capillary column (30 m, 0.25 mm inside diameter, 0.25 µm film thickness, Scientific Inc, Folsom, CA) connected to a quadrupole mass detector Bruker SCION SQ Premium (Bruker Daltonics, Macerata, Italy). The oven temperature was set at 40 °C and maintained for 4 min, then it was programmed to rise from 40 to 220 °C at 10 °C min^{-1} and finally held at 220 °C for 4 min. Transfer line 280 °C, ion source 220 °C, split injection (1:20), carrier gas (Helium) 1 ml min^{-1} were applied. The mass spectrometer was operated in electron impact mode at 70 eV, scanning range 10–250 Mz, full scan acquisition mode. Compounds were identified by matching the recorded mass spectra with the NIST/EPA/NIH Mass Spectral Database (NIST11, GAITHERSBURG, MD) and by comparing retention time and spectra with reference standard compounds (Santa Cruz Biotechnology) analyzed in the same conditions.