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Non-food Brassicas for green chemistry purposes through a biorefinery approach

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Contents

PREFACE

Since the first applications of Biofumigation technique in agriculture was proposed during the first nineties', lot of work has been done and many results were achieved. At the same time, new unexplored frontiers need to be reached.

In this scenery, I had the privilege to carry out my activities with the major experts on this topic, working at the Research Center for Industrial Crops in Bologna (CREA-CIN) for more than five years with a driven group of researchers and technicians.

With the support of the University of Bologna, part of this work was carried out thanks to a grant from the Erasmus+ Programme at the Harper Adams University (Newport, Shropshire, UK), in which I learned from the great experience of its researchers in nematology and weed science.

The achieved results were presented at the 6th International Biofumigation Symposium held in Stellenbosch, South Africa.

Roberto Matteo

ABSTRACT

The valorization of Brassica oilseed crops, starting from the agronomical phase up to the exploitation of the derived products, was studied.

Camelina sativa (L.) Crantz applications in biorefineries is of deep interest, given the high added value of its oil and oilseed cake residue. Camelina was evaluated as a case study for the cultivation in the Po valley. In such conditions, camelina has shown to be potentially well adaptable and had a positive impact on the yields of the subsequent crop. Thus, studies on camelina management and its impact on soil properties deserve more attention.

Novel Brassica defatted seed meals (DSMs), containing different glucosinolates, were applied in the control of the southern root-knot nematode, *Meloidogyne incognita* (Kofoid & White Chitwood) in controlled glasshouse conditions. Among different Brassica DSMs, the best results in the reduction of *M. incognita* infestation were achieved by *Eruca sativa*, *Barbarea verna* and *Brassica nigra* DSMs. These first results open new perspectives for innovative bioactive molecules for biofumigation applied in cropping systems where nematode suppression is critical.

Finally, the effect of Brassica DSMs, applied in formulations with and without crude glycerin, on seed germination inhibition was evaluated. The most effective formulations were applied in *in vitro* and glasshouse trials to evaluate the germination inhibition of black-grass (*Alopecurus myosuriodes* Huds.). Both *in vitro* and *in vivo* experiments confirmed the effectiveness of Brassica DSMs formulated with glycerin, especially *B. nigra*, in germination inhibition. Among different advantages, the proposed formulations for weed control are completely bio-based, organic farming friendly, present a good fertilizing properties and a combined effect both on weeds and on soil borne pests and diseases.

1. INTRODUCTION

Context: legislation and society

The attention to a sustainable use of chemicals in the European Community starts from the Regulation (EC) n. 1907/2006 on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). In the Article 14 of this act, concerning Integrated Pest Management, it is clearly reported that "Member States shall take all necessary measures aimed at promoting low pesticide-input pest management, giving wherever possible priority to non-chemical methods".

In general, the last EU guidelines request a significant reduction in pesticide application in agriculture, with the aim of lowering the impact on the environment and human health. These pronouncements led to more restricted limits for the registration of new pesticides and the phase-out of conventional products characterized by a high environmental impact, as substances that deplete the ozone layer (UNEP, 2016), or for their biocidal properties (Rasmussen & Mac Lellan, 2001). So, it is possible to foresee that high impact chemical products in the European agriculture will be more and more controlled following the Directive 2009/128/EC on "Sustainable use of pesticides". In this legislative framework, the public opinion has raised some more relevant health and environment concerns in this last few years limiting the registration of potentially hazardous active compounds, as for glyphosate, which at the moment is waiting for a more depth study on its safety (Stokstad Erik, 2016). This new legislative framework should determine a structural change in the European production system. In agriculture, in addition to a more sustainable use of pesticides, all the other possible alternatives pass through a new revival of virtuous agronomic techniques such as i) rotation, ii) great attention to organic matter content in soils by the so-called soil conservation techniques, and iii) the use of natural biologically active compounds in plant management and defense. The common aim of all these approaches is to maintain a high productivity even with a lower application of chemicals through the improvement of soil fertility and plant health by environmental friendly physical, biological and agronomical techniques.

Brassica crops and Biofumigation: the glucosinolates-myrosinase system

In this ambit, twenty years of study have highlighted the benefits derived from the use of Brassicaceae in the so called Biofumigation technique. Biofumigation means "Pest and disease suppression by glucosinolate-containing plants arising specifically from the biofumigant properties of the glucosinolate (GL) hydrolysis products, particularly the isothiocyanates (ITCs) , released from incorporated tissues or rotation crops, notably the Brassicaceae" (Kirkegaard and Matthiessen, 2004).

The Brassicaceae is a family belonging to the order of Capparales, according to the Cronquist system (Cronquist,1981), but recently regrouped with other families in the Brassicales in accordance to the Angiosperm Phylogeny Group (2009). More than 300 genera and nearly 4000 species belong to this family, several of which are storically classified as edible plants in human diet, including Brassicas (cabbage, broccoli, cauliflower, rape etc.), Sinapis (mustard), Raphanus (radish), Eruca (rocket). The characteristic pungent taste of these plants is due to the glucosinolate-myrosinase (GL-MYR) system, a plant defensive tool able to release allelopathic compounds (Fahey *et al*., 2001), able to influence the growth, survival, and reproduction of several living organisms.

According to the classical theory, GLs are compartmentalized within the vacuole (Andréasson & Jørgensen, 2003), separated by the enzyme MYR, which instead, is confined on the endoplasmic reticulum or in specific myrosinic bodies linked to the cell membrane (Bones & Rossiter, 1996) (figure 1).

When a wound of the wall and the cell membranes occurs due to biotic or abiotic factors, the MYR enzyme come into contact with the GLs starting the hydrolysis reaction. In their native form, GLs are stable and marginally reactive sulfur- and nitrogen-containing plant secondary metabolites (figure 2). Glucosinolates contain a β-tioglucosidic bond, which in the presence of water and the endogenous enzyme MYR (ß thioglucoside glucohydrolase EC 3.2.1.147), is hydrolyzed, producing β-D-glucose, sulphate ion and a plurality of bioactive breakdown products such as ITCs and, to a lesser extent, nitriles, epithionitriles and thiocyanates, depending on the reaction conditions (Fahey et al., 2001; Bones & Rossiter, 2006). The damage in tissues may be caused by biotic and abiotic factors: hailstorms, insects or nematodes, or after cell death in the rhizosphere caused by the root tip growth (Rumberger & Marschner, 2003).

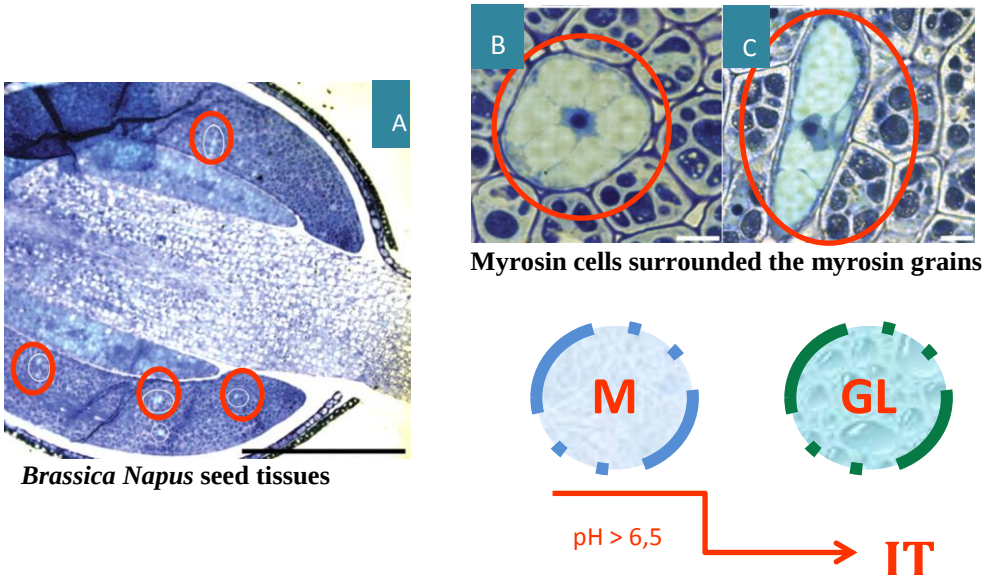

Figure 1. (A) Compartmentalization of the enzyme myrosinase (MYR) in myrosin cells in a Brassica Napus seed tissue section, stained with toluidine blue and observed under a light microscope. (B and C) Myrosin cell surrounded by myrosin grains visible as globular green vacuoles, containing MYR. The glucosinolates (GLs) are indeed dissolved in the cytoplasm of neighboring cells. It is only the simultaneous damage of at least three membranes which permits the initiation of the hydrolysis of GLs. Picture rearranged by Ahuja et al. (2011).

Even if a considerable number of additional suggested structures are concluded not to be sufficiently documented, more than two hundreds of different natural occurring GLs were recognized. The GLs can be classified according to their amino acid precursor and the amino acid side chain changes. In general it is possible to identify 4 categories: i) aliphatic and arylaliphatic; ii) hydroxylated apliphatic; iii) thiofunctionalized; iv) indole-type (Wathelet *et al*., 2004).

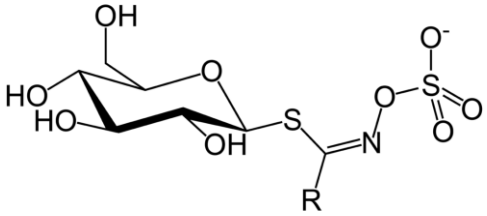

Figure 2. Glucosinolate structure.

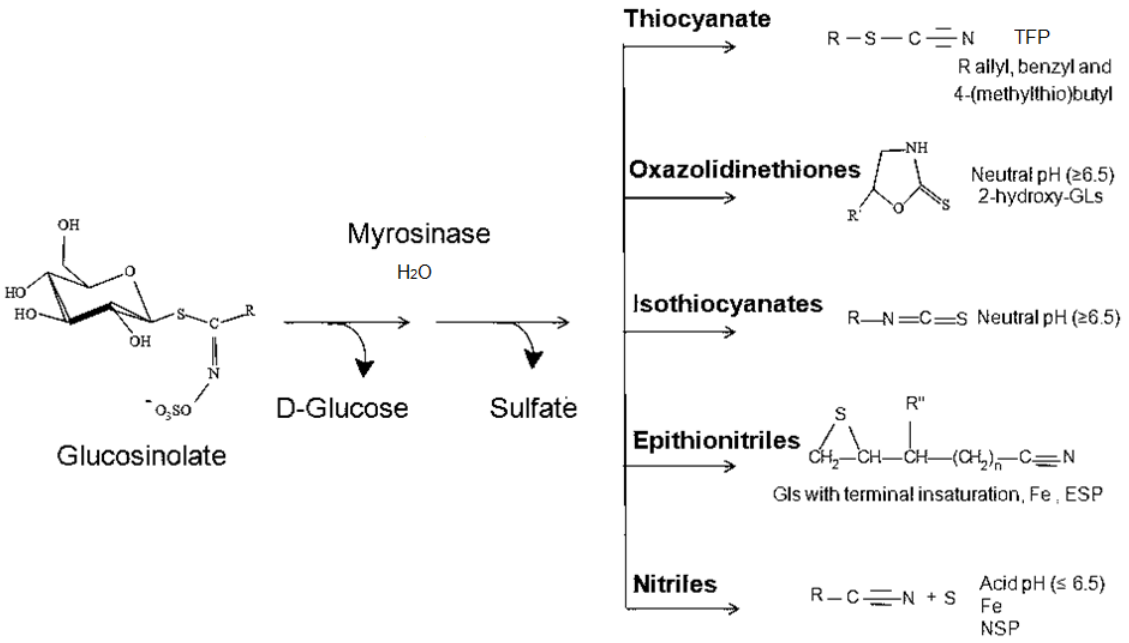

Fig. 3. Hydrolysis reaction via myrosinase.

The chemical and physical characteristics of GL derivative products depend on the nature of the starting GL and the different properties define the potential fields of application of these component of cruciferous materials. GLs with a hydroxilated structure in the side chain, for example, produce ITCs that are quickly converted to chiral oxazolidin-thiones which have been proposed as building blocks in fine chemistry (Gueyrard *et al.*, 2000). Other ITCs such as sulphorafane (4(R)-methylsulfynylbutyl ITC) produced by hydrolysis of glucoraphanin have relevant nutraceutical properties (Fimognari *et al.*, 2003), while allyl-ITC and some thiofunctionalized ITCs are characterized by a clear allelopathic activity towards some plant pests and pathogens.

Isothiocyanates and nitriles are the main reaction products and their ratio depends on the pH, the presence of ferrous ion (Fe^{2+}) and cysteine, besides the presence of EpithioSpecifier Proteins rather than Thiocyanate Forming Protein.

Also, ascorbic acid also in the vacuoles is important for regulating the MYR activity. In fact, ascorbic acid inhibits the enzyme at high concentrations, while, at low concentrations, does not alter the activity (Björkman , 1976; Bones & Slupphaug, 1989; Grob & Matile, 1980; Tani *et al.*, 1974).

In general, the hydrolysis products of thiofunctionalized GLs (glucoerucin, glucocheirolin and others) (Manici *et al*., 1999), some aliphatic (sinigrin) (Mayton *et al*., 1996), and arylaliphatic (nasturtin) have shown a greater biocidal activity compared to the hydrolysis products from hydroxylated aliphatic, both on fungi, nematodes (Lazzeri *et al*., 1993; Lazzeri *et al*., 2004) or weeds (Angelini *et al*., 1998). In particular, Allyl-ITC produced from 2-propenyl-GL (sinigrin) appears at the moment to be the most interesting one to contain soil borne fungi and pests, combining a high biological activity towards pathogens and a high volatility. These characteristics suggest the possibility of using this compound for soil borne pests and diseases control in answer to the widespread request for environmental friendly technical means for organic and conventional agriculture.

Nowadays, the biological properties of the GL-MYR system (Agerbirk & Olsen, 2012) and its application through Brassica plants and materials in the so-called biofumigation technique have been widely studied becoming practical in agricultural field (Kirkegaard *et al*., 1993).

Biofumigation technique: a wide range of applications

Almost thirty years of studies, carried out at the Research Centre for Industrial Crops (CREA-CIN) in Bologna, have highlighted the benefits derived from the use of *Brassicaceae* plants and materials by the Biofumigation technique. The applied research, aimed to a practical application in agriculture, has been directed toward the study of materials 100% based on vegetable biomasses with biofumigant properties, following all the main strategy of organic farming.

Their application year after year allows the soil incorporation of significant amounts of readily bioavailable organic matter with a C/N ratio of around 7/8, together with GL degradation products, mainly ITCs, that determine a natural containment of some widespread diseases. The main strategies of biofumigation are the following outlined.

i) Biofumigant green manure plants

The first proposed option is the intercrop cultivation of biofumigant green manures selected for rusticity, biomass yield and concentration of specific GLs in epigeal and/or hypogeal tissues. At flowering time, generally from 50 to 70 days after sowing time, the plants are incorporated by fine chopping. The residues has to be immediately incorporated in the soil to limit ITC volatilization which must occur directly in the soil.

Taking into account these common features, two different types of green manure plants were defined:

a. Biofumigant plants (*Brassica juncea* ISCI 99, ISCI20) containing high levels of sinigrin in the epigeal tissues. At chopping time, plants are able to release allyl-ITC characterized by a high volatility, a fundamental characteristic for biofumigation in the control of soil-borne fungi and wireworms. After plant incorporation, ALLYL-ITC persistence in soil is lower than 72 hours (D'Avino *et al.*, 2004). After this time the subsequent crop can be sowed or transplanted without any phytotoxic effect and benefiting of the organic matter effect both on physical and fertilizing properties. Furthermore, green manure residues can be considered as a pre-plant fertilization for the subsequent crops.

b. Catch crop plants (*Eruca sativa* sel Nemat, *Raphanus sativus* cv. Karacter). These plants have been selected for root content of thiofunctionalized GLs characterized by degradation products with low volatility. They play a catch crop activity in soil against some root and gall nematodes. More specifically *Meloidogyne incognita* larvae attack their roots determining ITCs release from the damaged cells. Thus nematodes feed a poisoned media and do not succeed in ending their reproduction cycle (Curto *et al.*, 2005[a]) in horticulture, floricultural and commodity crops.

ii) Biofumigant meals and pellets

During ripening, Brassica plants translocate GLs in their seeds at concentration from 8 to 10 times higher than those present in plant organs, becoming a fundamental basic material for biofumigation. In fact, after seed oil defatting, the residual meal contains a high level of GLs, whilst the MYR is almost completely deactivated. Therefore, the meals need to be reactivated by a patented procedure (Lazzeri *et al.*, 2010), able to optimize ITCs release in time. The formulated meals can be prepared both as pellets for mechanical distribution or as meals for manual distribution and are applied as pre-plant treatment. Meals and pellets are distributed on dry soil, incorporated and activated by light irrigation. As mentioned before, several studies (Lazzeri *et al.*, 2009), showed interesting effects in root nematodes and wireworm containment. In addition, their chemical composition, reported in Lazzeri *et al.* (2011), involves fertilizing and amendment properties.

iii) Liquids formulations

More recently, the hydrophobic nature of GL degradation products suggested the possibility of creating liquid products based on a vegetable oil in water emulsion admixed with small amount of biofumigant meals. The sinigrin hydrolysis is activated by the water in the emulsion, and, according to its hydrophobicity, the released ALLYL-ITC is solubilized in the oil fraction of the emulsion (Lazzeri *et al.* 2011). After emulsion distribution a thin microfilm of oil remains on plant organs, determining on some pathogens a physical suffocating action improved by the allelopathic effect of ITCs. It is interesting to report that alone vegetable oil in water emulsion is able to give a containment statistically equal to mineral oils; furthermore the addition of the biofumigant meals improve the efficacy of more than 30% (Rongai *et al*, 2008). Starting from these common features, two different types of liquid formulations were defined:

a. Liquid formulation for epigeal distribution. Made-up by a water-oil-meal suspension, it requires to be sprayed on the plants as a contact product with no systemic activity. For this application the formulated meals are micronized to avoid problems with nozzles. The results of some trials carried out during this research activity, against seven key-pests of Italian citriculture, were very positive. No toxicity on sprayed plants nor on larger insects, including bees and other useful ones, was noticed. In particular, the efficacy against mites, scales and whiteflies was substantial, with mortality percentages always higher than 80% and in some cases around 95%. The combinations of oil and DSM was particularly effective, with a clear additive action. The main action of oil in pest control is suffocation, and Najar-Rodríguez *et al*. (2008) recently suggested that the lipophilic properties of petroleum spray oils can rapidly penetrate and accumulate in some cells, including nerve cells, to cause death within minutes. A vegetable oil is not characterized by penetration capability and, for this reason, can only perform a contact action. At the same time, *B. carinata* DSM releases ALLYL-ITC . Previous studies (Nicetic *et al*., 2010) compared the mineral and canola oil effect on Black scale (*Saissetia oleae*) on olive trees, and the Citrus leaf miner *Phyllocnistis citrella* on lemons. This study found that both oils significantly reduced pest populations, but in general canola oil was less effective than the mineral one, requiring higher doses. This may depends on different tribologic properties between mineral and vegetal oils. Nevertheless, in Benfatto *et al*. (2015), the combined effect of oil and DSM caused a mortality rate that was not statistically different from paraffinic oils on pyriform and cottony cushion scale.

b. Liquid formulation for hypogeal distribution. This liquid formulation is distributed by drip or by minisprinkler, irrigation after a filtration of the suspension. For this application, in fact, the meals are ground at larger size to facilitate their separation by filtration, after ALLYL-ITC release, to avoid problems to the irrigation system. Gall nematodes are the main target of this product which present also fertilizing and biostimulant effects on root growth.

Herbicide potential of Brassicas in a biorefinery approach

Despite different land use contexts, from agriculture to urban settings, weed control is a major problem. In particular, weed control in amenities such as parks and schools should be undertaken without threatening health and environment, and at the same time, herbicide resistance has become an increasing problem. Besides the classical biofumigation technique, the potential of brassica bioactive compounds in weed control could be exploited with beneficial effects both on environment and human health. In addition, natural plant management and defense could play an important role in developing the bioeconomy sector with the aim of improving the circular economy even in food chain. In fact, a wide range of oil based products could be substituted by biobased products and materials derived from different types of biomasses as energy crops, agricultural residues and waste, forestry waste and residues and industrial and municipal wastes (Maity, 2015). For all these reasons, the substitution of conventional chemicals based on fossil sources by bio-based materials can provide several benefits for the health of workers and consumers and in general for the environment, depending on the application field and the substitution level.

In this background, due to the rise in global biodiesel production, and more generally of oilseed crops for non-food applications, the amount of defatted oilseed meals (DSMs) and crude glycerin (CG), the main co-products of seed defatting and oil esterification, respectively, have also steadily increased (Katryniok *et al.*, 2009). These products are generally considered as having a low added value and are mainly used in animal feeding or in the bioenergy chain. Therefore, the identification of high added value outlets should be explored in detail to improve the overall economic and environmental benefits of both co-products for the entire biodiesel chain.

The first by-products of the biodiesel chain are DSMs derived from seed defatting procedures. As previously discussed, some Brassicaceae derived DSMs are

characterized by a high level of GLs. After formulation of *B. carinata* DSMs following the patented procedure (Lazzeri *et al*., 2010), the allyl-ITC released from sinigrin was shown to be active for the containment of soil-borne fungi (Lazzeri *et al*., 2003), nematodes (Lazzeri *et al*., 2009) and wireworms (Furlan *et al*., 2010). Interestingly, some previous experiences showed an inhibitory effect on seed germination and plant growth caused by brassica derived compounds. Since the inhibitory influence of aqueous extracts from parts of *Brassica oleracea* plants on the germination and 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 GLs degradation products were carried out. Different allelopathic potential of some species and cultivars of Brassica on wheat in laboratory and field trials has been reported by Mason-Sedun *et al*. (1986) and Mason-Sedun & Jeppson (1988).

In addition Angelini *et al*., in 1998, showed how the hydrolysis products of glucoerucin and glucoraphanin, at a concentration of 10 mg ml^{-1} of native GL, completely inhibited the germination of *Chenopodium album* L., *Portulaca oleracea* L., in controlled conditions. In the same study, the hydrolysis products of epiprogoitrin, mainly 5-vinyloxazolidine-2-thione, gave a high percentage of abnormal seedlings even at a concentration of 1 mg ml^{-1} of native GL. Although previous studies have demonstrated the phytotoxicity of ITCs, their action modes are still poorly investigated. In Hara *et al*., 2010, the physiological responses of *Arabidopsis thaliana* treated with three exogenous ITCs is reported. High dose of methyl ITC, allyl ITC, and phenethyl ITC inhibited plant growth and induced severe bleaching in the rosette leaves and had an herbicidal effect by inducing oxidative burst-like responses.

More recent papers have shown the allelopathic effect of Rapeseed (*Brassica napus* L.) water extracts inhibited seed germination, root length, fresh root weight, shoot length and fresh shoot weight of *Phalaris minor* (Retz.), *Convolvulus arvensis* (L.) and *Sorghum halepense* (L.) (Aliki *et al*., 2014).

The second by-product derived from oil esterification or saponification processes, including in the biodiesel chain, is CG that contains glycerol and up to 20% of different impurities. Glycerol is an organic molecule characterized by a low toxicity when ingested, inhaled or upon contact with the skin; it is also readily biodegradable under aerobic conditions and highly stable in storage (Alexander *et al*., 2010). It is completely soluble in water and alcohol, while it is slightly soluble in many common solvents such as dioxane and insoluble in ether, chloroform and hydrocarbons (UNEP, 2002). Glycerol is one of known most versatile and valuable chemical substance, with several

thousand uses and applications (Pagliaro & Rossi, 2010). It can be used, in fact, in extremely different fields, from feed to chemicals, from biodegradable plastics to biolubricants (D'Avino *et al*., 2015[b]), or after transformation through different chemical synthesis approaches (Wolfson *et al*., 2009). Some applications require pure glycerol, while for some others the purity of CG is sufficient, and no further refining steps are needed. In the agricultural field, glycerol is used as an adjuvant in formulations, providing anti-evaporation characteristics and greater adhesion to the surface of plant tissues, and more generally for the treatment of crops, including spray solutions (Dissinger *et al*.,2009). Glycerol esters are applied in a patented formulation of conventional herbicides that play a humectant role and are able to improve the activity of a wide range of chemical active compounds (Krahmer *et al*., 2006) and are also present as an additive in a lemongrass-based natural product for weed management (Dayan *et al*., 2009).

Biobased product from Brassica crops: Camelina sativa *cultivated in the Po Valley*

In these last years there was a growing demand of new raw materials for new bio-refinery development and an increase of bio-products demand.

Furthermore, in 2010 worldwide biofuel production reached 105 billion liters, increasing by 17% from 2009 (Worldwatch Institute, 2011). Even the biofuel consumption in European transport continued to increase, reaching 13.6 Mtoe (millions of tons of oil equivalent) in 2011 (Eurobserv-ER, 2011), despite the energy production from dedicated industrial crops is a controversial subject and deep attention should be given to the production chain particularly at the cultivation level for what concerns the Mediterranean areas (Koçar & Civaş, 2013; Fontaras *et al*., 2012). Moreover, feedstock availability and cost, a failed valorization of co-products, greenhouse gas (GHG) emission, land use changes (LUC), and fuel vs. food/feed competition are also crucial aspects (Masoumeh *et al*. , 2017). To partially overcome these issues, in these last years the European communities took a series of decisions starting from the RED Directive on Renewable Energy Directive 2009/28/EC) on the sustainability of bio-energies amended in the Directive 2015/1513 which encouraged the use of residual biomass in energy production instead of dedicated crops. This decision aimed at decrease the environmental impact of bioenergy chain, led to a drastically decrease in several areas of oilseed crops cultivation for biodiesel, to benefit to new technologies as biogas or pyrolysis. Even if helpful, this approach underestimates the potential of the whole

valorization of the by-product obtained from this kind of biodiesel chain (such as defatted seed meals, glycerin) which could cover its entire environmental impact (http://www.chimicaverde.it/progetto-valso/) (fig. 4).

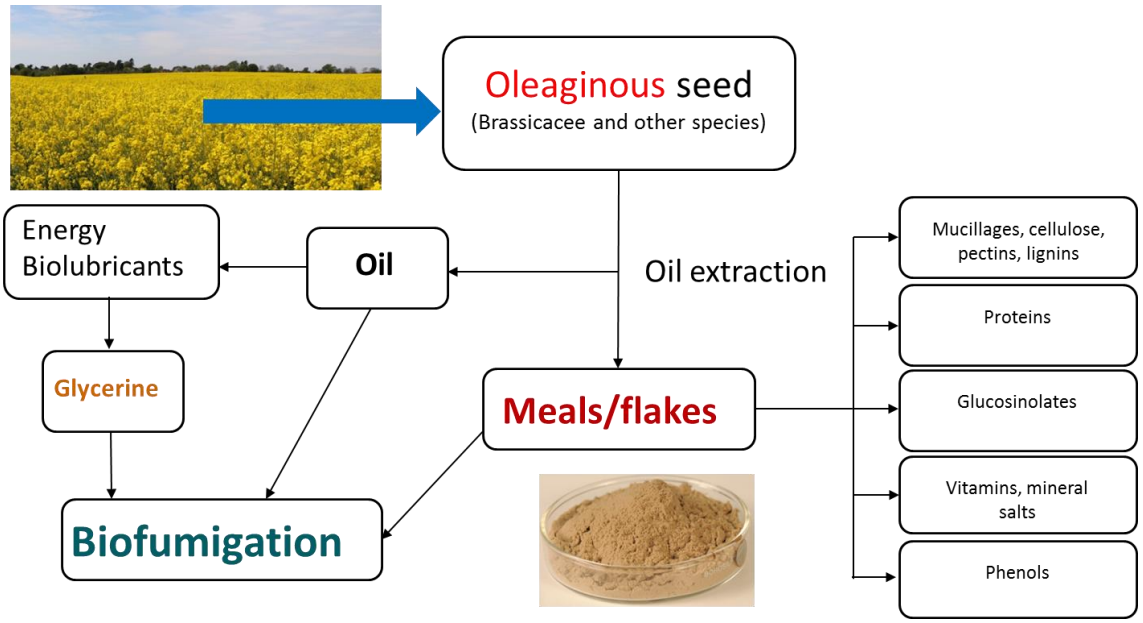

Fig. 4. Example of a valuable brassica oilseed crop exploitation.

Considering the alternatives in the biodiesel production, although the oil contents are similar between seed plants and microalgae, for example, there are significant variations in the overall biomass productivity and resulting oil yield and biodiesel productivity with a clear advantage for microalgae. In terms of land use, microalgae followed by palm oil biodiesel are clearly advantageous because of their higher biomass productivity and oil yield. At the same time, land use change for oilseed crops is reversible while it is not for microalgae, and a considerable investment in technological development and technical expertise is still needed before algal biodiesel becomes economically viable (Mata *et al*., 2010).

Furthermore, the introduction of new non-food crops could bring an increased biodiversity as well as an environmental impact reduction achieved by replacing conventional refinery products with bio-based ones (D´avino *et al*., 2015[c]; Snell *et al*., 2015). Additional benefits to the economic and environmental sustainability of the supply chain can be derived from the use of by-products generated in the chain of bio-refinery (Dittenber & Gangarao, 2012), especially in Green Chemistry, increasing its economic value (Gasol, C. 2009; Pelletier, J. 2009). In fact, biobased products

generated substantial economic growth activity n 2014 (this industry contributed a total of $393 billion in value added to the U.S. economy) and American jobs (4.2 million American jobs are supported through direct, indirect, and induced contributions) (Golden *et al*., 2016). If compared to conventional ones, bio products can give clear environmental benefits due to their renewability, biodegradability, hypotoxicity and, usually, to the reduction of greenhouse gas emission (GHG) during their production and use. For these reasons, the economic exploitation of a "whole-use" of plant biomass is a remarkable tool for achieving improved economic and environmental sustainability (Bezzi *et al*., 2007) that can offer new chances not only limited to bioenergy crop systems (Venturi & Venturi, 2003).

Nevertheless, biorefineries often are not linked to the territory, where the production of the raw materials takes place, supplying main biomass component (viz starch for plastic, oil for lubricant, oil or biomass for energy) from the global market. This approach could suffer from the unstable availability and quality of raw materials, especially when defined chemical properties are needed. It is clear that in this historical context, even the agricultural sector have to play an essential role for a new green economy, that comprises the partial or total substitution of fossil oil based compounds by bio based materials. At the same, the production of biomasses, through a strong link with local agriculture, could also provide additional economic and environmental advantages for the production of bioenergy, biofuel and biobased products as its main pillars. In particular, starting from raw materials, a second generation biorefinery can produce several products with different uses (Kamm *et al*., 2006; Lazzeri & D'Avino, 2008) allowing several options for the final utilization and valorization of the entire biomass, whilst a third generation biorefinery- locally arranged and closely linked to the territory- thanks to the specificity of the agricultural productions, cannot be easily relocated.

Camelina (*Camelina sativa* (L. Crantz) oil could be taken as a significant case study. In recent years, in fact, it has drawn the attention from commercial ventures and airlines as a good source for alternative jet fuel, but this novel biofuel chain showed to be hardly sustainable (Cando *et al.*, 2016). In order to justify a biofuel production from camelina oil, a study carried out in Canada showed that yields below 1 ton ha^{-1} are unlikely sustainable, unless a smart co-products enhancement and diversification (viz. panel) (Mupondwa *et al*., 2016). Furthermore, the introduction in the crop rotation of oilseed crops (especially brassicas) could improve the yield and quality of cereals enhancing the soil fertility (Chen *et al*., 2015).

Camelina has gained a considerable attention in these last year mainly in Europe and U.S.A., in marginal lands from arid to cold areas, as a potential oilseed feedstock for advanced biofuels (i.e. aviation fuel) or even in nutraceutical and cosmetic sector. In addition a full valorization of camelina raw materials could produce a wide range of different bioproducts based on GLs containing DSM and oil (Shonnard *et al*., 2010; Kim *et al*., 2015). Camelina seeds produces an oil content ranging over 35% on dry matter, characterized by a 40% of α-linolenic acid (18:3n-6) an omega-3 fatty acid with interesting perspectives in human and animal diets. This composition determines unique properties both for industrial, food (Kirkhus *et al*., 2013), feed innovative applications (Hixson *et al*., 2014). and several potential application for the production of biobased products in bioenergy, cosmetic , agricultural, nutraceutical and other sectors. Camelina seeds, in addition to an interesting protein content, presents also different compounds, among which GLs, that make the resulting oilcake interesting for the production of high added value chemicals (fig. 4) (Matthäus & Zubr, 2000; Matthäus & Angelini, 2005). The growing interest is evidenced by the large increase in peer-reviewed publications containing the word ‘camelina’. Databases report 335 publications between 2013 and 2016, with 149 of them are published after 2015. Although the number of reports on nonfood type uses for camelina oilcake is limited, a range of applications has been explored (Berti *et al*., 2016). Some of the most interesting applications include, antibacterial compound source (Kumar & Pathak., 2016), soil fungicides (Ma *et al*., 2015), adhesives (Li *et al*., 2015), bio-oils (Boateng *et al*.,2010) and bio-herbicides (Cao *et al*., 2015).

2. AIMS OF THE RESEARCH

The aim of this thesis was to valorize Brassicas oilseed crops starting from the agronomical phase up to the exploitation of the derived products.
The activities were developed in three steps:
i) agronomical evaluation: characterization of a brassica oilseed crop (camelina) as a case study for the production in the Po valley (Bologna province, Emilia Romagna region), traditionally devoted to cereal, applying low environmental inputs at open field level in terms of a sustainable biorefinery approach.

ii) Pest control: evaluation of new Brassicaceae DSMs, containing different GLs, in the control of the southern root-knot nematode, *Meloidogyne incognita* (Kofoid & White Chitwood), and additionally determine their biostimulant effect on subsequent tomato plants. The southern root-knot nematode is a well-known endoparasite nematode that is found worldwide in sandy soils, where is responsible for severe damages to many profitable crops, such as tomato, pepper, eggplant, zucchini, melon, string bean, carrot, lettuce, beet, strawberry and others (Lamberti, 1979).

iii) Weed control: investigation of the potential effect on seed germination inhibition (GI) on lettuce (and other sensitive seeds) by Brassica DSMs derived from fatty-acid, biofuel and other bio-product chains applied in formulations with and without CG.
The most effective formulations were applied in further experiments to evaluate the GI activity on Black-grass. Black-grass (*Alopecurus myosuriodes* Huds.), is a major annual weed of winter cereals, resistant to seven classes of herbicides in eleven countries throughout Europe (http://www.weedscience.org). It was chosen among different weeds as a potentially interesting real target. In fact it is a widespread weed, and represents a serious problem in the western countries of Europe (Holm *et al*., 1997).

3 MATERIALS AND METHODS

3.1 Camelina cultivation in the Po Valley, yield by low input production systems and chemical characterization: material and methods

3.1.1 Cultivation techniques applied in the trials

A four -year experiment (2013-2016) (Bologna) was laid out as open field trial with a single plot design and a plot size of at least 1000 m^2. The cultivation site was located at Budrio (Bologna) in the Po Valley area (Emilia Romagna region. 44°30'N latitude; 27°00'E longitude, altitude 54 m a.s.l. and 0% slope). The area was characterized by flat land with a loamy soil with medium level of total nitrogen and organic matter content. The soil physical and chemical characteristics of the site are reported in Table 1.

Table 1.Physical and chemical soil characteristics of the field.

Parameter	**M. U.**	**methodology applied**	
Sand (0,05 - 2 mm)	%		9.0 - 9.1
Silt (0,02 - 0,05 mm)	%		55.9 - 56.2
Clay (< 0,02 mm)	g kg^{-1}		34.8 - 35.1
Type of soil			loamy
pH (H_2O 1:2,5 soil: water suspension)		McLean method	8.04 - 8.06
Organic matter	%	Walkley-Black method	1.81 - 1.91
Total nitrogen	g kg^{-1}	Kjeldhal method	1.59 - 2.89
Available phosphorus	mg kg^{-1}	Osen method	19 - 23
C/N ratio			4.3 -10.5
Cation Exchange	meq/100g	method $BaCl_2$, pH8,1	n.d.
Bulk density	g cm^{-1}		n.d.

Camelina was sown in autumnal on 5th October 2012, 16th October 2013, 20th October 2014, 30th September 2015, while harvest times were accomplished on 7th June, 29th May, 4th June and 8th of June in 2013, 2014, 2015 and 2016 respectively.

A rotation camelina-wheat-camelina was applied and the cropping techniques and mechanization methods were defined in relation to the specific characteristics of this area, according to the integrated production methods. In terms of effects on the

following cultivation the grain yield was assessed using, as a control plot each year, a classical rotation, in which in the last two years a succession of wheat-wheat was applied. No fertilizers were used during the trial. The adopted agro-techniques are reported in Table 2 while the meteorological data are reported in Table 3.

Table 2. Camelina crop management adopted in the trial.

Farming operations	Agronomic solution/practice
Soil preparation	Shallow ploughing (30 cm)
Pre-sowing processing	Rotating harrow
Pre sowing Fertilization	Organic fertilizers (12 Kg ha^{-1} of N)
Fertilizer landfill	Spike-tooth harrow
Sowing method	Sowing (rate of 12.5 kg ha^{-1} on 15 cm spaced rows using a plot drill for wheat)
Weeding	No chemical weeding
Chemical fertilization	No chemical fertilization
Mechanical Harvest	Plot thresher machine

Table 3. Meteorological data (monthly maximum and minimum temperatures and monthly rainfall) in the 2013, 2014, 2015 and 2016 growing seasons.

month	2012-13			2013-14		
	t max °C	t min °C	rainfall mm.	t max °C	t min °C	rainfall mm.
Oct	20.1	10.6	82.4	19.4	11.5	124.8
Nov	14.4	6.5	75.0	13.6	6.0	122.3
Dec	5.2	-1.3	27.8	10.1	-0.2	9.1
Jan	5.9	-0.3	63.8	9.8	2.4	127.9
Feb	7.1	-1.3	109.0	12.3	3.4	112.2
Mar	11.9	2.8	126.2	17.2	4.1	81.9
Apr	19.2	7.8	61.2	20.9	7.8	82.6
May	22.8	10.9	84.4	24.3	9.5	46.2

month	2014-15			2015-16		
	t max °C	t min °C	rainfall mm.	t max °C	t min °C	rainfall mm.
Oct	22.2	11.3	64.3	18.9	9.9	160.7
Nov	15.3	8.6	59.4	14.5	4.4	6.2
Dec	8.9	3.1	20.6	9.2	1.4	6.1
Jan	9.8	-0.8	12.0	10.0	-1.0	23.9
Feb	10.1	0.8	241.5	11.7	3.2	128,8
Mar	15.2	2.9	157.7	15.4	4.2	85.3
Apr	20.3	5.9	112.9	21.1	8.3	73.5
May	24.5	11.6	110.5	23.7	11.1	106.8

The results has been referred to a cultivation of plots at least of 2000 m^2 , applying a full mechanization of the cultivation techniques. This approach allows to consider the yield obtained as a real productivity evaluation of camelina in the Po Valley area over the years.

Three sample areas of 1 m^2 were randomly collected within each experimental plot, once reached seed maturity, to assess the productive characteristics (above- and below-ground biomass, seed yield, , and their qualitative characterization). Plant samples were ventilated in oven (70°C) overnight for dry weight determination and evaluation of moisture content. To evaluate potential seed yield, the plant samples were threshed by a fixed machine, using sieves suitable for small seeds. After sampling, the entire surface was mechanically harvested to assess the real seed yield at open field level.

3.1.2 Qualitative analysis

After harvesting, seeds were cleaned, partially dried, ground to 0.5 mm size and analyzed for their main components by the following procedures:

- Humidity content was determined by oven-drying the seeds at 105°C for 12 hours and calculated as the difference between the seed weight before and after treatment.
- Oil content was measured by NMR (Nuclear Magnetic Resonance) technique by an MQC benchtop NMR analyzer (Oxford Instruments) (ISO 5511). The quantitative determination was based on a specific calibration for camelina seeds defined by Soxhlet.
- Fatty acid composition was determined by extracting the oil from ground seeds by hexane and trans-methylated in 2NKOH methanol solution (Conte *et al.*, 1989). Fatty acid methyl esters (FAMEs) were evaluated by a gas chromatography-FID detector (Carlo Erba HRGC 5300 MEGA SERIES) on a capillary column Restek RT x 2330 (30 m x 0.25 mm x 0.2 µm) with oven temperature programming (170°C initial temperature for 12 min, followed by a gradient of 20°C/min to 240°C, for 3 min), helium as carrier gas at 1 ml/min and split mode 40:1. The detector and injector temperature was 260°C.Standards were used for identification of individual fatty acids. The internal normalization method (ISO 5508, 1998) was used to determine the fatty acid composition. The oil content was determined by the standard Soxhlet extraction method using n-hexane as solvent.
- Total content of (C-H-N) was determined using Elemental analyzer LECO CHN TruSpec according to the American Society for Testing Materials (ASTM D5373). The

protein content was expressed as percentage on dry matter and calculated from nitrogen using the conventional factor of 6.25.

3.2 Biofumigant effect of new defatted seed meals against the southern root-knot nematode, Meloidogyne incognita: material and methods

3.2.1 Defatted Seed Meals

The DSMs of thirteen Brassicaceae species *Barbarea verna* (Miller) Asch. (Land cress) sel. CIN 100, *Brassica carinata* A. Braun (Ethiopian mustard) sel. ISCI7, *Brassica nigra* (L.) W.D.J. Koch (Black mustard) sel. ISCI 27, *Brassica rapa* (L.) (Field mustard) cv. Silla, *Brassica tournefortii* Gouan (African mustard) sel. CIN 14, *Brassica oleracea* var. acephala (L.) (Cabbage), *Crambe abyssinica* Hochst ex. R.E. Fries (Abyssinian kale) cv. Mario, *Eruca sativa* Mill cv. Nemat (Rocket), *Lepidium densiflorum* Schrad. (Pepperweed), *Lepidium sativum* L. (Cress) sel. CIN 50, *Raphanus sativus* L. (Radish) cv. Boss, *Rapistrum rugosum* (L.) All. (Wild radish), *Sinapis alba* (L.) (White mustard) sel. Pira, were provided by the Brassicaceae seed collection of the Centre for Industrial Crops, Bologna (Italy), (Lazzeri *et al*., 2013). The Brassicaceae DSMs were compared to *Helianthus annuus* L. (Sunflower) DSM obtained from the industrial plant Italcol (Castelfiorentino, Firenze, Italy) as a non-biofumigant control.

The seed production of plants containing GLs started from the cultivation of selected germplasm as non-food oleaginous crops on plots of 100 m^2, inserted in a cereal rotation. All the cultivation phases from sowing to harvesting were performed by low impact cultivation techniques, with no irrigation or chemical treatment. After harvesting, seeds were cleaned, partially dried at 8% maximum moisture, ground and sieved to 0.5 mm and defatted by hexane. After oil extraction, all DSMs (including sunflower) were formulated in the laboratory to modulate the release over time of GL degradation products, in order to obtain the most effective biological activity (Lazzeri *et al*., 2010). The DSMs were characterized for: i) their main GL, type and amount, according to the ISO 9167-1 method (1992) with some minor modification as reported in Lazzeri *et al*. (2011); ii) nitrogen (N) content on dry matter, determined by the Kjeldhal method, using a Tecator digestion system 20 and an automatic Büchi distillation unit (UNI 22604:1992; UNI, 1992).

3.2.2 Nematodes

In all experiments, a population of *M. incognita* was used. It came from a sandy soil field located close to Ferrara, in North-East Italy, and was maintained on celery (*Apium graveolens* L. var. dulce cv. Vert d'Elne) in a glasshouse.

J2s were collected from egg masses extracted from celery plants by shaking chopped galled roots in 1% sodium hypochlorite for four minutes (Hussey & Barker, 1973), placed them in water and incubated 7 days at 24°C. The J2s, hatched in the incubation period, were collected, counted and finally used for inoculating the soil with four different concentrations.

To classify nematode species, the females were extracted from galled roots of potted tomato plants and identified by their perineal pattern (Jepson, 1987). The morphological classification was furthermore confirmed by TaqMan® real-time PCR analyses performed by FERA laboratory of Sand Hutton, York (United Kingdom).

3.2.3 Experiment description

The experiments were set up in a glasshouse located in Bologna, Italy (44°46"58'N; 11°33'79" E, 54 m a.s.l.), at 22 ± 2 °C with a 12h photoperiod, in pots containing 500 cm^3 of sandy-loamy soil, which had previously been placed in a sealed container, in a layer of 30 cm, and steamed at 90°C for 60 minutes.

Four experiments, one for each initial *M. incognita* density (30, 120, 250 and 1000 J2s 100 cm^{-3} of soil), were carried out consecutively over a period of 6 months with the same methodology, assuring homogeneous environmental and agronomical conditions.

The inoculation of each pot was done with 50 ml of water suspension of J2s directly into the soil by a micropipette. A filter paper (Filter-lab 1300/80 Barcellona, Spain) was inserted on the bottom of the pots to prevent the leaching of infested soil particles by irrigation during the experiment. Two days after the J2 inoculation, the infested soil of each pot was carefully mixed with 0.9 g of each DSM, corresponding to the dose of 2.5 ton ha-1, usually applied in the field, then each pot was moistened with 30 ml of water.

For each DSM treatment, 6 pots were used to verify the nematicidal effect directly in the uncultivated soil, and 9 pots cultivated with a host plant (*Solanum lycopersicum* L. cv. UC82 - ISI Sementi, Parma – Italy), to verify the DSM effect on the root infestation and on the vegetative vigor.

For each population density (30, 120, 250, 1000 J2 100 cm^{-3}) one experiment was organized in a completely randomized experimental design. The DSM treatments were compared with a non-biofumigant DSM as *Heliantus annuus* and an untreated control.

The nematicidal effect was verified seven days after DSM incorporation: *M. incognita* J2s were extracted from the soil (6 pots per treatment) by Baermann trays (Barker, 1985; Tacconi & Ambrogioni, 1995) and the number of J2s 100 cm^{-3} of soil in each pot was counted.

One seedling of tomato cv. UC82 20-25 cm high with at least more than four leaves was transplanted into each of the 9 remaining pots for each treatment. The roots of each seedling were gently washed under cool running water before transplanting with the aim of eliminating the seedbed peat and exposing the roots to the infested soil. After transplanting, the soil was kept wetted by spraying water. A moisture content of approximately 10% (w/w) was achieved and soil moisture was maintained throughout the experiments by a drip irrigation system.

The tomato plants were grown in a glasshouse for 21 days, after which roots were inspected for galls and plant vigor was assessed.

The Gall Index (GInd) was calculated as a weighted average of root infestation rated on a 0–5 scale (Lamberti, 1971): 0 = no galls, 1 = 1–5 galls, 2 = 6–20 galls, 3 = more than 20 galls, 4 = root system reduced and root physiology altered by some large galls, 5 = root system completely destroyed. Vigor was also rated using 0–5 classes, whose range was calculated in each trial by dividing 5 by the difference in height between the tallest and the shortest plant.

Not all accessions were tested on each population density: the most effective accessions and those currently marketed as either catch crop or green manure (*B. verna*, *B. carinata*, *E. sativa*, *R. sativus*, *S. alba*, *B. nigra*) represented the criterion of choice.

3.2.4 Statistical analysis

In all experiments, the data of J2s 100 cm^{-3} were specified as the mean of 6 repetitions ± standard error (SE), those of GInd and vigor as the mean of 9 repetitions ± SE; the data of J2, GInd and vigor were processed by analysis of variance (ANOVA) followed by the least significant difference (LSD) test. The statistical analysis was performed by means of ARM7® software.

3.3 Inhibition of sensitive seed germination through novel formulations based on defatted brassica oilseed meals and crude glycerin: material and methods

3.3.1 Materials

CG was purchased from Cerealdocks S.p.A. an industrial biodiesel plant (Vicenza, Italy). *Brassica carinata* A. Brown and *Brassica nigra* L. DSM, produced after seed defatting by an endless screw press under controlled conditions, were purchased from Agrium Italia S.p.A. (Livorno, Italy). DSMs of *Barbarea verna* (Miller) Asch., and *Crambe abyssinica* Hochst came from the CRA-CIN Brassicaceae collection and were defatted with hexan at lab level (Lazzeri *et al.*, 2013); *Helianthus annuus* L. DSM were purchased from Italcol S.p.A. (Castefiorentino-Firenze, Italy) after a pressure/hexane extraction process. The standard allyl-ITC was obtained from Aldrich Chemical (Milwaukee, Wi, USA). Lettuce (*Lactuca sativa* L. cv. Cosmic) was purchased from Tozer Seeds, purslane (*Portulaca oleracea* L.) from B&T seeds (France), garden cress (*Lepidium sativum* L.), cucumber (*Cucuminus sativus* L.), and sorghum (*Sorghum vulgare*, Pers.) were purchased from Florsilva Ansaloni (S. Lazzaro di Savena, Bologna, Italy); tomato (*Solanum lycopersicum* L. cv. UC82) from ISI Sementi (Parma, Italy).

3.3.2 Crude glycerin characterization

Crude glycerin was paper filtered to remove impurities and analyzed for some minor components by the following procedures:

•density was measured by gravimetric analysis;

•water and glycerol content were determined at the Experimental Station for the Industries of Oils and Fats, Milan Italy (SSOG) by respectively using Karl Fischer colorimetric titration and HPLC determination. An HP1100 (Hewlett Packard, Waldbronn, Germany) system equipped with an HP1047A refractive index detector and an Aminex HPX-87H column (7.8 mm x 300 mm, BioRad, Richmond, USA) was used. The mobile phase used was 0.01 N sulfuric acid at a flow rate of 0.8 mL min^{-1}. The injection volume was 20 µL. An external six points calibration curve was constructed analyzing standard dilutions of 99.5% glycerin from 10 to 25 mg mL^{-1}.

- Determination of ashes content was performed according to the British and European Standard (BS EN 14775, 2009)

- The presence of heavy metals, such as Cadmium (Cd), Chrome (Cr), Lead (Pb) and Copper (Cu), were determined at the Research Institute for Agroindustry (Modena, Italy) by Inductively Coupled Plasma Spectrometry; samples were prepared according

to AOAC 922.02 (1990):and the analysis was performed according to AOAC 985.01 (1988).

3.3.3 Defatted oilseed meal characterization

The DSM were analyzed for their main components by the following methods:

- moisture content was determined by oven-drying DSM at 105°C for 12 hours and humidity was calculated as the difference between the weight before and after treatment;
- nitrogen content was determined by the Kjeldhal method, using a Tecator digestion system 20 and an automatic Büchi distillation unit (B 324).
- GL content was determined following the ISO 9167-1 method (1992) with some minor modification as reported in Lazzeri *et al*. (2011).

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

All DSMs were formulated by a patented procedure (Lazzeri *et al*., 2010) aimed at restoring the enzymatic system that catalyzes GL hydrolysis, that proved to be almost completely deactivated during oil extraction procedure (Lazzeri *et al*., 2011). The formulation details must be considered as confidential information and will not be extensively reported.

3.3.4 Determination of allyl isothiocyanate entrapment in crude glycerin/water solutions

270 µmol of pure allyl-ITC were dissolved in 100 mL of water, or in the same volume of a 5%, 10%, 15% concentration of CG. Tubes were immediately closed with gas–tight Teflon caps and placed in agitation for 30 minutes at room temperature. A volume of 100 µL was collected for allyl-ITC basal determination and tubes were left open under continuous stirring for 30 min before a second sampling, to simulate the applicative preparation methods. allyl-ITC was measured according to Zhang *et al*. (1992). Briefly, 100 µL of allyl-ITC solution were added to 900 µL of 100 mM potassium phosphate buffer (pH 8.5), 900 µL of methanol and 100 µL of 80 mM 1,2 benzenedithiol in isopropanol. The vials were heated for 2 hours at 65°C, cooled at room temperature and absorbance at 365 nm was measured against a paired blank containing all the ingredients except allyl-ITC.

3.3.5 Evaluation of allyl isothiocyanate release from defatted seed meals

To evaluate sinigrin conversion to allyl-ITC, suspensions of CG at a concentration of 10% supplemented with *B. nigra* DSM at 1 and 3% (w/v), and of CG at 20% with *B. nigra* DSM at 4% were prepared in a 100 ml screw Pyrex flask. The suspensions were kept under continuous stirring at room temperature and sampled at different times, until the detected allyl-ITC amount reached a plateau. For the analyses, 1 ml of mixture was withdrawn and transferred into a 10 ml vial that was closed by a screw plug and a pierceable septum. The vial was kept in agitation for 5 minutes and then sampled by the headspace technique following the procedures reported in De Nicola *et al*. (2013). Calibration curves with several dilutions of pure allyl-ITC in 10% of CG as such or with 1 and 3% of sunflower DSM, and in 20% of CG with 4% of sunflower DSM were performed.

3.3.6 Bioassay protocols

The formulations were prepared by adding DSM to CG/distilled water solutions. The suspensions were mixed every 5 minutes and after 20 minutes, the time required to reach the higher GL hydrolysis, were ready for distribution.

3.3.6.1 In vitro trials – Petri dish preliminary experiments

The tested suspensions were prepared by mixing CG/water solutions *B. carinata*, *B. nigra*, *B. verna*, and *C. abyssinica* DSM. The suspensions were paper filtered and immediately utilized in the trials. Solutions at different concentrations of CG were also tested. A trial with sunflower DSM, which does not contain GLs, and another one with distilled water only were used as controls.

Ten lettuce seeds (chosen as a seed particularly sensitive to loss of germinability) were placed in a 9 cm diameter Petri dish on a paper filter and then treated with 1.8 ml of the different solutions. Each Petri dish was immediately sealed with parafilm (Parafilm M®) and incubated in the dark at 20 ± 1°C. After 72 h., the effects on GI of CG alone and CG/DSM suspensions were evaluated, counting the number of germinated seeds in each Petri dish. For result evaluation, a seed was considered as germinated when the hypocotyl was longer than one mm.

3.3.6.2 In vivo trials – Pot experiments

The experiments were performed in a glasshouse at 22 ± 2°C. At least three pots (height 9 cm and diameter 9.5 cm) containing 350 g each of a mixture of 50% of sandy-loamy soil (clay 13%, silt 18%, sand 69%) and 50% of peat (Floradur®, raised-bog-peat with $CaCl_2$ pH ranging from 5 to 6.5) were arranged for each trial. Seeds were sown at one cm depth on dry soil, treated only once with 40 ml of the different solutions per pot, a dose lower than field capacity.

Trial 1 –Effects of crude glycerin on lettuce and purslane seed germination and plant growth

The effect of solutions with different concentrations of CG (5%, 10%, 20%. 30% w/v) were tested on lettuce and purslane. Each pot was sown with 25 seeds and the solution was poured on the soil. Six pots were prepared for each different concentration. After 30 days, the number of germinated seeds, the length of seedlings, stems and root biomass weight on dry matter (DM) were determined.

Trial 2 – Crude glycerin and defatted seed meals combined effect on lettuce seed germination

In each pot, 25 lettuce seeds were sown. Four experiments were prepared using a fixed concentration of CG (10% w/v). This solution was poured as such and after the addition of 1%, 2% and 3% (w/v) of *B. nigra* DSM. After treatment, pots were evaluated every 7 days to assess the seed GI trend over time.

Trial 3- Crude glycerin and defatted seed meals combined effects on cress, cucumber and sorghum seed germination

Seeds of three different species: garden cress (20 seeds per pot), cucumber (5 seeds per pot), and sorghum (5 seeds per pot), were compared for their sensitivity to the CG/DSM system. After sowing, each pot was treated with a solution of CG 20% plus 4% in weight of *B.nigra* DSM; doses of CG and DSM were higher than those applied in previous experiments due to the bigger size of cucumber and sorghum seeds. The number of germinated seeds was counted 30 days after sowing. To evaluate the residual toxicity of the applied treatments, two 15 cm tall tomato plants were transplanted in each treated pot where no seeds had germinated. The untreated pots were used as control. Eleven weeks after tomato transplanting, the plants were evaluated for number of leaves, plant height and biomass, both epygeal and hypogeal, and humidity.

3.3.7 Statistical analysis

The comparison between the allyl-ITC trapping ability of water and CG/water solutions was performed in triplicate, and statistical analysis was performed on the absorbance at 365 nm by Student's test: water vs 5%, 10%, 15% CG (Sigma Plot 10.0- SPSS, Chicago, IL, USA). $P<0.05$ was considered statistically significant.

In *in vitro* trials the effects on lettuce GI were expressed as percentages of germination reduction referred to untreated seeds (Abbott, 1925, formula 2). Three Petri dishes per treatment were assessed; data were subjected to analysis of variance (ANOVA of arcsine square root transformed percentages) by Statistica 8.0 (Statsoft, Inc). Significant differences among treatments were determined by Tukey's multiple range test ($P\leq0.05$).

In in vivo trial 1, six pots per treatment were assessed. Probit analysis (Finney, 1971) was performed by EPA program (EPA Probit Analysis Program Version 1.5) to assess the concentration causing the death of half of the seeds (LC50), reporting correlated 95% confidence intervals. Stem and root length and biomass weight on DM per plant were measured at the end of the trial. When the mean number of plants per pot was lower than three, the length and biomass evaluation was considered not applicable (n/a) for consistency. The values are expressed as the mean per pot ± standard error (SE).

In trial 2, four pots per treatment were assessed. The results are expressed as the mean of seed germination ± SE referred to the untreated control by Abbott's formula.

In trial 3, three pots per treatment were assessed. The results are expressed as seed germination per pot ± SE. The GI trend of treated and untreated pots was graphically compared.

3.4 Inhibition of black-grass seed germination through novel formulations based on brassica defatted oilseed meals and crude glycerin: material and methods

3.4.1 Materials

The CG was purchased from Cerealdocks S.p.A. (Vicenza, Italy), an industrial biodiesel plant. *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., *Sinapis alba* L., and *Sisymbrium officinale* (L.) Scop. DSMs derived from the CREA-CI Brassicales collection (Lazzeri *et al*. 2013) were defatted by 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-GLs containing silique. *Brassica nigra* (L.) W.D.J. Koch DSM, was purchased by Agrium Italia S.p.A (Livorno), produced after seed defatting by an endless screw press in which temperature was kept lower than 75°C. Lettuce (*Lactuca sativa* L. cv. Cosmic) was purchased from SAIS S.p.A. (Cesena, Italy), whilst black-grass, (population: Blackgrass Foxtail, Slender) was purchased from Herbiseed (Reading, UK).

3.4.2. Defatted oilseed meals preparation and characterization

All DSMs were formulated by a patented procedure (Lazzeri *et al*., 2010) aimed at restoring and optimizing the enzymatic system that catalyzes GL hydrolysis. The formulation details must be considered as confidential information and will not be extensively reported. The DSMs were analyzed according to the following methods:

- moisture content 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 (UNI 22604, 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.

3.4.3. In vitro trials: extract preparation and hydrolysis product GC-MS identification

Water suspensions of each DSM (15 g/L) were kept under agitation on an orbital shaker, 140 RPM for 40 minutes, at room temperature (21 ± 1°C). The suspensions were centrifuged at 4500 RPM, for 30 minutes and filtered with filter paper (Filter-Lab, 1248). Samples of 500 µL of aqueous solution were extracted after agitation with a vortex for 3 min in ethyl acetate (LC-MS Chromasolv®) at a ratio of 1:1. Finally the samples were centrifuged for 10 min at 3000 RPM. One µL was collected from the upper organic phase and injected in a Bruker GC451 Gas Chromatoghraph equipped with a HP-5 fused silica capillary column (30m, 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. Mass spectra were identified by matching the recorded mass spectra with the NIST/EPA/NIH Mass Spectral Database (NIST11, GAITHERSBURG, MD).

A small batch of DSM was treated in sealed borosilicate glass containers by an autoclave (20 minutes, 120°C) in order to deactivate the MYR enzyme and prevent GL hydrolysis to bioactive compounds. The deactivated DSMs were analyzed as above.

3.4.4 Preliminary in vitro trials: lettuce germination inhibition

Preliminary *in vitro* trials on lettuce were set up to evaluate the antigerminative capability of twenty GL containing DSMs from the CREA-CIN collection. The experimental conditions in the trials were those applied in D'Avino *et al*. 2015[a] (§3.3). The *in vitro* trials were carried out following the UNICHIM protocol (UNICHIM, 2003) with minor modifications. Ten lettuce seeds were placed in each Petri dish containing a filter paper (Filter-Lab, 1300/80, diam. 90mm) with 1.8 ml of DSM extract. The Petri dishes were kept in the dark at 20 ± 1°C. After 7 days, the number of germinated seeds was counted. Each treatment was replicated five times. The extracts from deactivated DMSs were applied as control in the same experimental conditions in order to verify that the observed inhibition was due to the GL hydrolysis products activity. At the end

of the trial, besides the number of germinated seeds, the epicotyl and primary root lengths of lettuce seedlings, treated with deactivated DSMs, were measured.

3.4.5 Preliminary glasshouse trial: lettuce germination and growth inhibition

The preliminary glasshouse experiment was performed in Bologna, Italy (44°31'N 11°21'E) at CREA-CIN. In this experiment, depending on the efficacy, the seed availability and the feasibility of the defatting process, five DSMs were chosen for further glasshouse trials on lettuce: i) *Brassica tournefortii*, ii) *Brassica nigra*, iii) *Eruca sativa*, iv) *Rapistrum rugosum*, and v) *Sinapis alba*. The DSMs were applied, formulated with and without CG, in pots. The plastic pots (high 9 cm, diameter 9,5 cm, 250 ml) were filled with 200 g of a mixture of 50% of sandy-loamy soil (clay13%, silt 18%, sand 69%), 50% of peat (Floradur®, raised-bog-peat with $CaCl_2$, pH ranging from 5 to 6.5) and 710 mg of each DSM were mixed to the soil. In each pot, 20 lettuce seeds were sown 1 cm deep in the dry soil. The experiment was carried out in a glasshouse under controlled conditions at 22±2°C. The pots were watered with 24 ml of tap water, a dose lower than soil field capacity, or treated only once with the same amount of a CG solution at 10% (9.2 ml L^{-1} of soil).The number of germinated seeds was measured 27 days after sowing. The epygeal and the root biomass production (dry matter) were measured to assess the effect on the following plant growth. Each treatment was replicated three times.

3.4.6 Black-grass in vitro trial

A Petri dish trial was set up preparing an extract as above reported. Since the black-grass seeds need more time to germinate and, as other weeds, have a long dormancy, a different approach to the *in vitro* experiment was necessary. For these reasons, further modification to the protocol were defined: 150 blackgrass seeds were soaked in the extracts for 24h and kept in a growth chamber (Cooled Incubator MIR-154-PE, Panasonic Healthcare Co., Ltd, Jp), in the dark, at 20±1°C. The day after, 20 seeds were surface sterilized with sodium hypochlorite 5% and rinsed 2 times with sterile distilled water, and then placed in each Petri dish. The Petri dishes were kept in the dark, in the same growth chamber, at 20°C. The number of germinated seeds was counted after 14 days.

3.4.7 Black-grass glasshouse trial

Further glasshouse experiments were performed in Newport, Shropshire, UK (52°46'N 2°25'W) at Harper Adams University, Newport, Shropshire, UK. The effect on black-grass germination of five different DSMs formulated with and without CG was evaluated: i) *B. tournefortii*, ii) *B. nigra*, iii) *E. sativa*, iv) *R. rugosum*, and v) *S. alba*. The DSMs were applied in a pot experiment, mixed into the soil before sowing. Each pot (c.ca 300 ml volume) used in this trial was filled with 200 g of a mix of 50% Horticultural Silver Sand (silica sand, CEM-SPEC LTD, Harby, Leics., UK) + 50% John Innes N°.2 soil-based compost for potting plants (J. Arthur Bower's products, William Sinclair Horticulture Ltd, Lincoln, UK). Three doses for each DSM were applied: i) 1.4; ii) 2.7; and iii) 5.5 g L^{-1} of soil. Fifty blackgrass seeds were sown in each treated pot. The same three doses of DSMs were applied in formulation with CG. In these pots 28 ml of a 10% CG solution was poured into the soil after seed sowing. The pots were kept in a glasshouse under controlled conditions, with light 16 hour a day (SON-T 400 Watt bulb with ignitor) and a temperature of 22±2°C. The amount of CG used was based on a previous experience reported in §3.3 (D'Avino *et al*., 2015[a]). Each treatment was replicated three times. After 8 days the number of germinated seeds was counted and plant heights and the dry matter yield by each pot were measured.

3.4.8 Statistics

All the experiments were organized in a randomized experimental design. In *in vitro* and *in vivo* trials, the effects on both lettuce and black-grass germination were expressed as germination inhibition (GI) percentages, a calculation of the germination reduction referred to the untreated control according to the Schneider-Orelli's formula (Püntener, 1981). Germination inhibition percentages, stem and root lengths and biomass weight measured in the trials were subjected to ANOVA and Tukey's post hoc test performed with R software (R version 3.00.00, The R Foundation for Statistical Computing) and $P<0.05$ was considered statistically significant. The values are expressed as mean per Petri dish/pot ± standard error (SE).

Data collected from the black-grass glasshouse trials are expressed in percentages of reduction:

i) GI (Schneider-Orelli formula);

ii) Height reduction (HRED) = (mean plant H in treated pot –mean plant H in the untreated control) / mean plant H in the untreated control)*100

iii) Dry matter reduction (DMRED) = (mean DM per plant in treated pot –mean DM per plant in the untreated control)/ mean DM per plant in the untreated control)*100

The measured parameters GI, HRED, and DMRED were subjected to Factorial ANOVA performed with R software (R version 3.00.00, The R Foundation for Statistical Computing), considered as a result of the following separate factors: i) type of DSM, ii) DSM concentration and iii) CG. F pr.<0.001 was considered statistically significant.

4. RESULTS AND DISCUSSION

4.1 Camelina cultivation in the Po Valley, yield from low input production systems and chemical characterization: results

All cultivation phases (soil preparation, sowing, harvesting) were performed by common full commodity crop mechanization, that was possible thanks to the large dimension of the growing plots, at least higher than 1000 m^2.

Camelina confirmed the good adaptability in autumnal and spring sowing, at harvesting time, after full maturity, even if revealed a consistent difference between the potential and the effective yield. In fact, the potential seed yield, obtained from small experimental plots and harvested by hand, resulted in a mean yield of 0.8±0.2 ton ha^{-1}, whilst the real yield, obtained at open field level, sometimes resulted around the half of the potential data. The highest effective seed yield was observed in the camelina grown in 2015 and 2016, during the 3rd and the 4th year of cultivation. None relevant decrease in camelina yields during the four year of cultivation was recorded, despite a very intensive rotation camelina-wheat on the same plots (tab.4). The oil content ranged around 40% on dry matter.

Table 4- Seed yield (expressed as dry matter)and oil content in the trial, with oil and crude protein content (expressed as % on dry matter).

Year	**Seed yield t ha^{-1}**		**Oil content**	**Crude protein**
	potential	**effective**	**% on D.M.**	**% on D.M.**
2013	0.9±0.3	0.5	40.2	4.5
2014	0.9±0.2	0.4	39.4	4.0
2015	0.6±0.1	0.5	38.4	4.7
2016	0.8±0.1	0.5	38.9	3.5
Average	**0.8**	**0.5**	**39.2**	**4.16**
d.s.	*0.2*	-	*0.8*	*0.57*

The fatty acid composition and GL contents were comparable to the highest found in the literature (Schuster and Friedt, 1998; Russo and Reggiani, 2012).

Seed and oil yield were higher than those obtained in other experiments were spring sown was adopted. This was probably due to damages by frost in the initial vegetative growth stages (Angelini, 2012). The oil and protein seed content was statistically steady during the four years of cultivation.

The Low amount of GLs, ranging around 30 μmoles g^{-1} of seed on DM, was characterized by a profile with three main GLs:

i) Glucocamelinin, (10-(methylsulfinyl)decylglucosinolate) as predominant GL;

ii) Glucoarabin, (9-(methylsulfinyl)nonylglucosinolate);

iii) 11-(methylsulfinyl)undecylglucosinolate (minor) with significant content variation among years and environments (Table 5).

All these GLs are characterized by an extra Sulphur atom in their side chain R, a characteristic that seems generally correlated to the allelopathic effect of GL degradation products on some soil-borne fungi (Manici *et al*., 1997; Ma *et al*., 2015), and bacteria (Kumar & Pathak., 2016). Furthermore, Gls from camelina can produce ionicthiocyanates, proved to be effective as a bio-herbicide against redroot pigweed (*Amaranthus retroflexus* L.) and wild oat (*Avena fatua* L.) (Cao *et al*., 2015).

Table 5. Glucosinolate content and fatty acid composition of the oil obtained from camelina seed in each year of cultivation

year	GLs	Oil - Fatty acids (%)												
	(µmoli / g)	(C16:0)	(C18:0)	(C18:1)	(C18:2)	(C18:3)	(C20:0)	9- (C20:1)	(C20:2)	(C20:4)	(C22:0)	(C22:1)	(C24:0)	(C24:1)	**Others**
2013	24,8	6,0	2,6	14,9	18,0	37,0	0,0	14,0	2,0	1,5	0,3	2,7	0,0	0,0	0,7
2014	30,0	5,7	2,7	15,0	16,7	36,6	1,2	14,5	2,0	1,6	0,3	2,6	0,0	0,6	0,7
2015	31,6	6,3	2,5	17,3	17,7	36,3	1,3	12,8	1,6	1,3	0,0	2,4	0,0	0,4	0,5
2016	27,5	6,4	2,8	16,0	17,5	38,6	1,1	12,7	1,5	1,4	0,0	1,9	0,0	0,4	0,0
average	**28,5**	**6,1**	**2,6**	**15,8**	**17,4**	**37,1**	**0,9**	**13,5**	**1,8**	**1,4**	**0,2**	**2,4**	**0,0**	**0,3**	**0,5**
d.s.	*3,0*	*0,3*	*0,1*	*1,1*	*0,5*	*1,0*	*0,6*	*0,9*	*0,2*	*0,1*	*0,2*	*0,4*	*0,0*	*0,2*	*0,3*

(C16:0) Palmitic (C18:2) Linoleic 9-(C20:1) Gadolenic (C22:0) Behenic (C24:1) Nervonic
(C18:0) Stearic (C18:3) Linolenic (C20:2) Cis-11,14-eicosadienoico (C22:1) Erucic
(C18:1) Oleic (C20:0) Arachidic (C20:4) Arachidonic (C24:0) Lignoceric

.

The fatty acid composition was characterized by a mean in the four trial years of 57,5%±1,2 of polyunsaturated fatty acids with a 32% ± 1,2 % of monounsaturated fatty acids and of saturated fatty acids 10,1 ± 1,0%. The rate between Ω 6 and Ω 3 gave data ranging around of around 0.60. Besides the use as a raw material for jet fuel and biodiesel, camelina oil, due to its characteristic fatty acid composition, has found a wide range of application even in health and body care products. Furthermore, camelina DSM, from cold press oil extraction, contains residual oil and an interesting protein content, making it an appropriate animal feed integration (Ye *et al*., 2016).

Table 6. Total residue express as dry matter yield (DMY), carbon and nitrogen contents expressed as percentages on DMY.

Year	Total residues DMY (t ha^{-1})	Carbon content (% on DMY)	Nitrogen content (% on DMY)
2013	3.4±1.2	42.6±1.3	1.2±0.2
2014	4.2±0.2	45.4±1.5	0.9±0.1
2015	2.1±0.7	46.6±2.3	1.0±0.1
2016	3.4±1.0	45.7±2.6	0.7±0.3
Mean	3.3	45.1	0.9
s.d.	0.9	1.7	0.2

In addition it was determined even the epygeal and hypogeal as two additional biomasses that could play a role in the environmental balance of the entire cultivation phase. Camelina confirmed the low amount of total residues, epygeal and hypogeal (Tab 6), that ranged around 3 ton ha^{-1} as a mean value of the four years. Even if the hypogeal biomass ranged around 10% of the total residues, but it could be useful to improve the system fertility if incorporated into the soil after harvesting.

This means that in this trial, an average amount of 28 Kg ha^{-1} of organic nitrogen and 1500 Kg ha^{-1} of carbon remained incorporated into the soil each year by incorporating the residues. In fact, it is worthwhile to emphasize that carbon and nitrogen yield of root apparatus are surely underestimated for the difficulties of sampling roots one meter deep underground. Anyway, we have to consider with attention the amount of organic nitrogen which can be of benefit to the following crop fertilization, considering even its slow release in soil.

The differences in the comparison between the grain yield of wheat of the wheat-wheat and the camelina-wheat-camelina rotations were significant (Fig. 5).

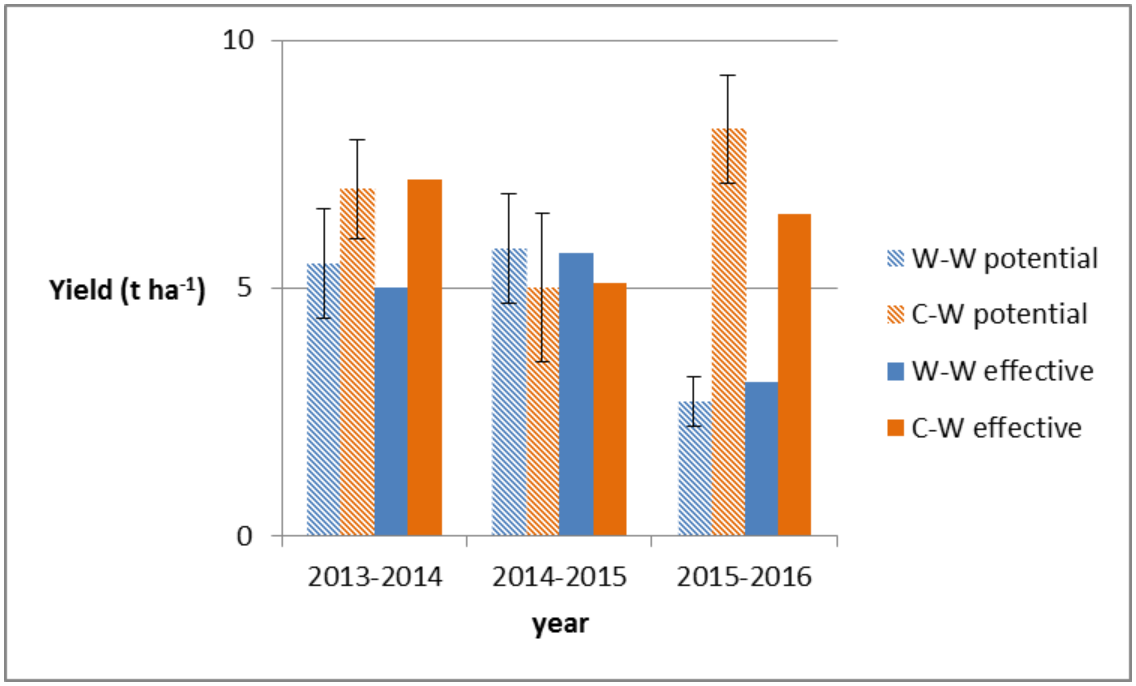

Figure 5. Grain production of wheat in the last three year of the trial expressed as potential (transparent columns) and effective yield (full columns).

Despite the very intensive type of rotation and the low cultivation inputs, the introduction of camelina in this cropping system allowed a costant yield of wheat in its rotation. In fact, the depletion in wheat production in the camelina-wheat rotation plot was statistically irrelevant during the years. At the same time, the grain obtained from the wheat-wheat plot in the last year of the experimentation was much lower and of worst quality in terms of organoleptic characteristics (author communication, data not shown) compared to the grain obtained from the camelina-wheat plots, confirming the effect of the camelina cultivation on soil health (Chen *et al*., 2015; Gesch, *et al*., 2015).

4.2 Biofumigant effect of new defatted seed meals against the southern root-knot nematode, **Meloidogyne incognita*: results***

All the production phases of DSMs, from sowing to seed defatting, were rather simple. The only drawback occurred during the harvest of *B. tourneforti*, *R. sativus*, *L. densiflorum* and *R. rugosum* that required hand harvesting due to their high seed scattering.

All the tested DSMs contained different types of GLs, at the same or higher doses than those of *B. carinata* currently marketed as biofumigant amendments.

The DSMs inserted in the experiments can be considered as representative of the main classes of GLs:

i) Alkenyl GLs: sinigrin (*B. carinata*, *B. nigra*), gluconapin (*B. rapa*);

ii) Thio GLs: glucoerucin (*E. sativa*), glucoiberin (*B. tourneforti*), glucoraphanin (*B. oleracea*), glucoraphenin (*R. sativus*), glucocheirolin (*R. rugosum*);

iii) Hydroxy GLs: sinalbin (*S. alba*), epiprogoitrin (*C. abyssinica*);

iv) Aromatic GLs: gluconasturtin (*B. verna*), glucotropaeolin (*L. sativum*)

Each DSM contained a predominant GL that in some cases was alone or almost alone, such as in B. carinata, E. sativa , B. verna , B. nigra , S. alba and R. rugosum. The other accessions showed a mixture of GLs with purity ranging from 82 to 97%. All the DSMs showed a high content of GLs that ranged from 98 (B. carinata) to 172 µmoles gr-1 of DSM (S. alba) (Table 7).

The results of the four glasshouse experiments regarding the DSM effectiveness on M. incognita J2s, assessed seven days after the DSM soil incorporation, are reported in Tables 8-11.

Table 7. Chemical characterisation of the tested defatted seed meals: main glucosinolate (GL) content (type and amount) and nitrogen content.

Defatted seed meals	Side chain R of Main GLs	Trivial name	main GL (% on total content)	GL content (µmoles g^{-1}±SE)	N content (% DM)
Brassica carinata	2- Propenyl	Sinigrin	98	92.8 ± 1.5	5.4 ± 0.4
Eruca sativa	4- Methylthio-butyl	Glucoerucin	91	120.9 ± 1.3	6.1 ± 0.5
Barbarea verna	2-Phenyl-ethyl	Gluconasturtin	100	125.0 ± 8.5	4.2 ± 0.3
Brassica nigra	2- Propenyl	Sinigrin	99	147.4 ± 4.0	6.9 ± 0.6
Brassica rapa	3-Butenyl	Gluconapin	93	146.2 ± 1.8	6.4 ± 0.7
Brassica tourneforti	3-Methyl-sulfinyl-propyl	Glucoiberin	83	110.5 ± 4.8	5.5 ± 0.5
Brassica oleracea	4-Methyl-sulfinyl-butyl	Glucoraphanin	82	103.1 ± 6.8	6.1 ± 0.5
Sinapis alba	P- Hydroxy-Benzyl	Sinalbin	100	172.2 ± 3.5	6.5 ± 0.4
Raphanus sativus	4-Methyl-sulfinyl-butenyl	Glucoraphenin	97	140.3 ± 9.3	8.6 ± 0.6
Crambe abyssinica	(2R)-2-hydroxy-3-butenyl	*Epi*-progoitrin	97	103.4 ± 9.6	4.7 ± 0.5
Lepidium densiflorum	4OH-3-5-Dimethoxy-Benzyl*	Unknown	72	201.1 ± 8.3	4.8 ± 0.4
Lepidium sativum	Benzyl	Glucotropaeolin	99	148.2 ± 12.1	6.1 ± 0.5
Rapistrum rugosum	3-Methyl-sulfonyl-propyl	Glucocheirolin	100	32.0 ± 5.2	24 ± 0.9
Heliantus annuus	-	-	-	not present	5.2 ± 0.4

*GL identified in Pagnotta *et al*., 2017 (in press).

Table 8. Number of *Meloidogyne incognita* juveniles in 100 $cm^3$of soil (J2s 100 cm^{-3}) 7 days after the defatted seed meal (DSM) application in a soil inoculated with 30 J2s 100 cm-3, compared to untreated control and non-biofumigant DSM of sunflower.

Treatment	**J2s 100 cm^{-3} soil (N°)**	
Untreated control	8.26 ± 0.21[1]	a[2]
Barbarea verna (Land cress)	1.54 ± 0.08	b
Brassica tourneforti (African mustard)	1.49 ± 0.12	b
Brassica oleracea var. acephala (Cabbage)	1.35 ± 0.11	b
Heliantus annuus (Sunflower)	1.00 ± 0.11	b
Brassica rapa (Field mustard)	0.94 ± 0.11	b
Rapistrum rugosum (Wild radish)	0.47 ± 0.20	b
Brassica carinata (Ethiopian mustard)	0.35 ± 0.13	cd
Raphanus sativus (Radish)	0.20 ± 0.13	cde
Brassica nigra (Black mustard)	0.08 ± 0.08	de
Crambe abyssinica (Abyssinian kale)	0.00 ± 0.00	e
Eruca sativa (Rocket)	0.00 ± 0.00	e
Lepidium densiflorum (Pepperweed)	0.00 ± 0.00	e
Lepidium sativum (Cress)	0.00 ± 0.00	e
Sinapis alba (White mustard)	0.00 ± 0.00	e
ANOVA[3] F Values		
Replicate F	2.744*[4]	
Replicate Prob (F)	0.0254	
Treatment F	38.572**[5]	
Treatment Prob (F)	0.001	
LSD (P=0.05)	0.119[6]	
Standard Deviation	0.103[6]	
CV	49.4	

[1]Mean values ± Standard Error. All means and standard errors are reported as log transformed data.

[2]Means followed by the same letters are not significantly different (P = 0.05) according to Least Significant Difference's Test (LSD).

[3]ANOVA on log transformed data.

[4]Statistically significant at $P \leq 0.05$ (*).

[5]Statistically significant at $P \leq 0.01$ (**[5]).

[6]LSD and Standard Deviation descriptions are reported in log transformed data units.

Table 9. Number of Meloidogyne incognita juveniles in 100 cm3 of soil (J2s 100 cm-3) 7 days after the defatted seed meal (DSM) application in a soil inoculated with 120 J2s 100 cm-3, compared to untreated control and non-biofumigant DSM of sunflower.

Treatment	**J2s 100 cm^{-3} soil (N°)**	
Lepidium densiflorum (Pepperweed)	86.86 ± 0.21[1]	a[2]
Untreated control	30.13 ± 0.46	b
Crambe abyssinica (Abyssinian kale)	3.94 ± 0.17	c
Heliantus annuus (Sunflower)	2.40 ± 0.13	cd
Sinapis alba (White mustard)	2.14 ± 0.34	cde
Raphanus sativus (Radish)	2.14 ± 0.34	cde
Rapistrum rugosum (Wild radish)	1.38 ± 0.07	def
Brassica oleracea var. acephala (Cabbage)	0.92 ± 0.15	defg
Brassica rapa (Field mustard)	0.91 ± 0.18	defg
Brassica carinata (Ethiopian mustard)	0.76 ± 0.40	efg
Barbarea verna (Land cress)	0.35 ± 0.16	fg
Eruca sativa (Rocket)	0.19 ± 0.09	g
ANOVA[3] F Values		
Replicate F	1.794	
Replicate Prob (F)	0.1294	
Treatment F	37.113**[4]	
Treatment Prob (F)	0.0001	
LSD (P=0.05)	0.262[5]	
Standard Deviation	0.227[5]	
CV	38.66	

[1]Mean values ± Standard Error. All means and standard errors are reported as log transformed data.

[2]Means followed by the same letters are not significantly different (P = 0.05) according to Least Significant Difference's Test (LSD).

[3]ANOVA on log transformed data.

[4]Statistically significant at P ≤ 0.01 (**).

[5]LSD and Standard Deviation descriptions are reported in log transformed data units.

Table 10. Number of Meloidogyne incognita juveniles in 100 cm3 of soil (J2s 100 cm-3) 7 days after the defatted seed meal (DSM) application in a soil inoculated with 250 J2s 100 cm-3, compared to untreated control and non-biofumigant DSM of sunflower.

Treatment	**J2s 100 cm^{-3} soil (N°)**	
Untreated control	53.63 ± 0.43[1]	a[2]
Heliantus annuus (Sunflower)	13.84 ± 0.92	b
Sinapis alba (White mustard)	6.93 ± 1.02	b
Brassica carinata (Ethiopian mustard)	1.30 ± 0.37	c
Raphanus sativus (Radish)	0.70 ± 0.05	c
Eruca sativa (Rocket)	0.24 ± 0.15	c
Barbarea verna (Land cress)	0.08 ± 0.08	c
Brassica nigra (Black mustard)	0.08 ± 0.08	c
ANOVA[3] F Values		
Replicate F	5.660**[4]	
Replicate Prob (F)	0.0006	
Treatment F	22.599**[4]	
Treatment Prob (F)	0.0001	
LSD (P=0.05)	0.384[5]	
Standard Deviation	0.326[5]	
CV	57.1	

[1]Mean values ± Standard Error. All means and standard errors are reported as log transformed data.

[2]Means followed by the same letters are not significantly different (P = 0.05) according to Least Significant Difference's Test (LSD).

[3]ANOVA on log transformed data.

[4]Statistically significant at P ≤ 0.01 (**).

[5]LSD and Standard Deviation descriptions are reported in log transformed data units.

Table 11. Number of Meloidogyne incognita juveniles in 100 cm3 of soil (J2s 100 cm-3) 7 days after the defatted seed meal (DSM) application in a soil inoculated with 1000 J2s 100 cm-3, compared to untreated control and non-biofumigant DSM of sunflower.

Treatment	**J2s 100 cm^{-3} soil (N°)**	
Untreated control	412.70 ± 0.07[1]	a[2]
Heliantus annuus (Sunflower)	139.13 ± 0.10	b
Sinapis alba (White mustard)	88.87 ± 0.08	c
Raphanus sativus (Radish)	48.53 ± 0.15	d
Brassica carinata (Ethiopian mustard)	33.18 ± 0.18	d
Brassica nigra (Black mustard)	14.05 ± 0.24	e
Eruca sativa (Rocket)	4.30 ± 0.25	f
Barbarea verna (Land cress)	1.44 ± 0.08	g
ANOVA[3] F Values		
Replicate F	1.169	
Replicate Prob (F)	0.3436	
Treatment F	138.403**[4]	
Treatment Prob (F)	0.0001	
LSD (P=0.05)	0.182[5]	
Standard Deviation	0.154[5]	
CV	10.09	

[1]Mean values ± Standard Error. All means and standard errors are reported as log transformed data.

[2]Means followed by the same letters are not significantly different (P = 0.05) according to Least Significant Difference's Test (LSD).

[3]ANOVA on log transformed data.

[4]Statistically significant at P ≤ 0.01 (**).

[5]LSD and Standard Deviation descriptions are reported in log transformed data units.

All DSMs caused a significant reduction in M. incognita populations when compared to the untreated control, except for *L. densiflorum* in the experiment performed with an inoculum of 120 J2s cm^{-3} soil.

The DSMs of *B. verna*, *E. sativa*, *B. nigra*, *B. carinata* and *R. sativus* proved to be the most effective at the highest population density too. Even the DSM of *H. annuus*, that does not contain GLs, caused a decrease in J2s confirming the positive effect due to the incorporation of organic matter in nematode containment even if the observed reduction was generally lower than the least efficient DSM containing GLs.

The results regarding root infestation and the vigor of tomato plants in the four experiments at increasing levels of inoculation (30, 120, 250, 1000 J2s cm^{-3} soil) are shown in Figures 6-9. The best performances at all population densities were achieved by DSMs of *E. sativa* (max. and min. GInd = 0.6-1.7; max. and min. vigor = 3.0-4.7), *B. verna* (max. and min. GInd = 0.1-2.3; max. and min. vigor = 2.7-4.1) and *B. nigra* (max. and min. GInd = 0.6-2.1, max. and min. vigor = 2.7-4.2).

In some cases, the effectiveness of the DSMs was related to the rate of J2 inoculum: in the experiment with the lowest rate (30 J2s 100 cm^{-3} soil) some accessions such as S. alba, *R. sativus*, *E. sativa* and *L. densiflorum* were found to be effective in nematode control (GInd = 0.8-0.9), all the other accessions gave intermediate results, with a GInd between 1.2 and 1.8, and a vigor class of about 3.3 (Figure 6).

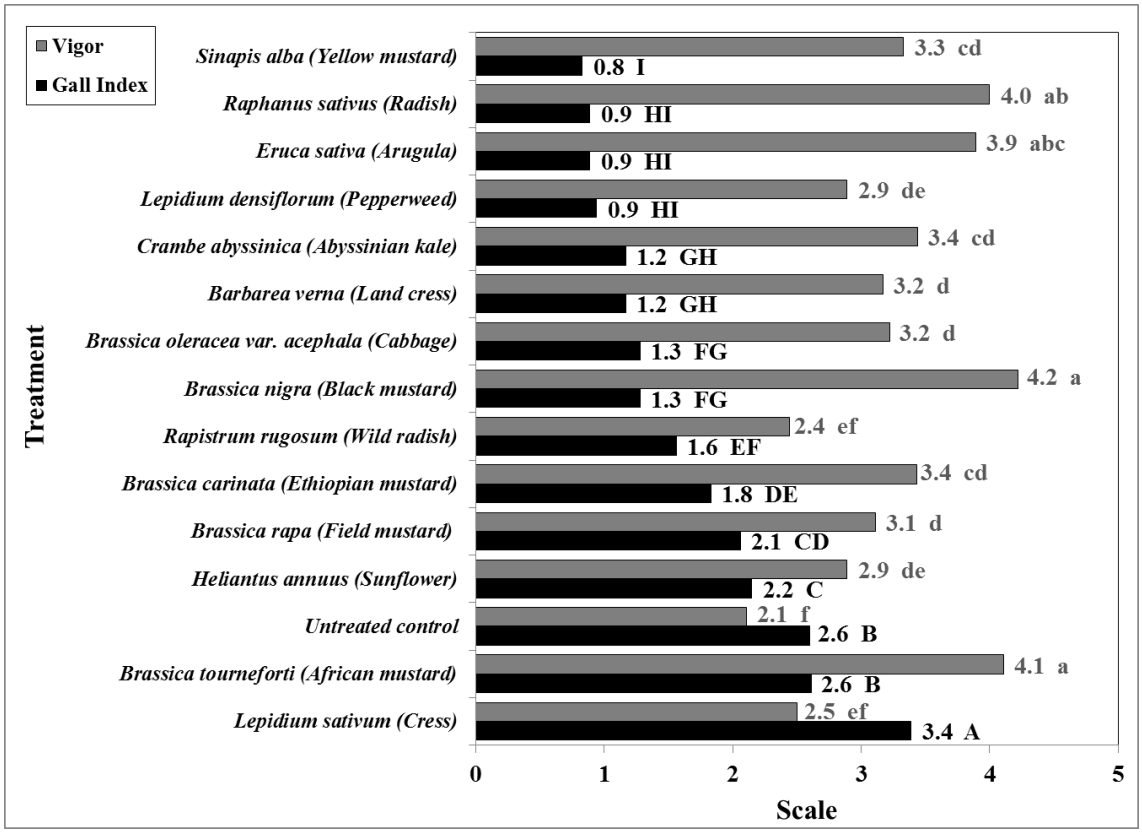

Figure 6. Gall index and vigor of tomato plants, 21 days after transplanting, at inoculation of 30 *Meloidogyne incognita* juveniles in 100 cm^3 of soil (J2 100 cm^{-3} soil). ANOVA and LSD test ($P \leq 0.05$). Means followed by the same letter do not differ statistically.

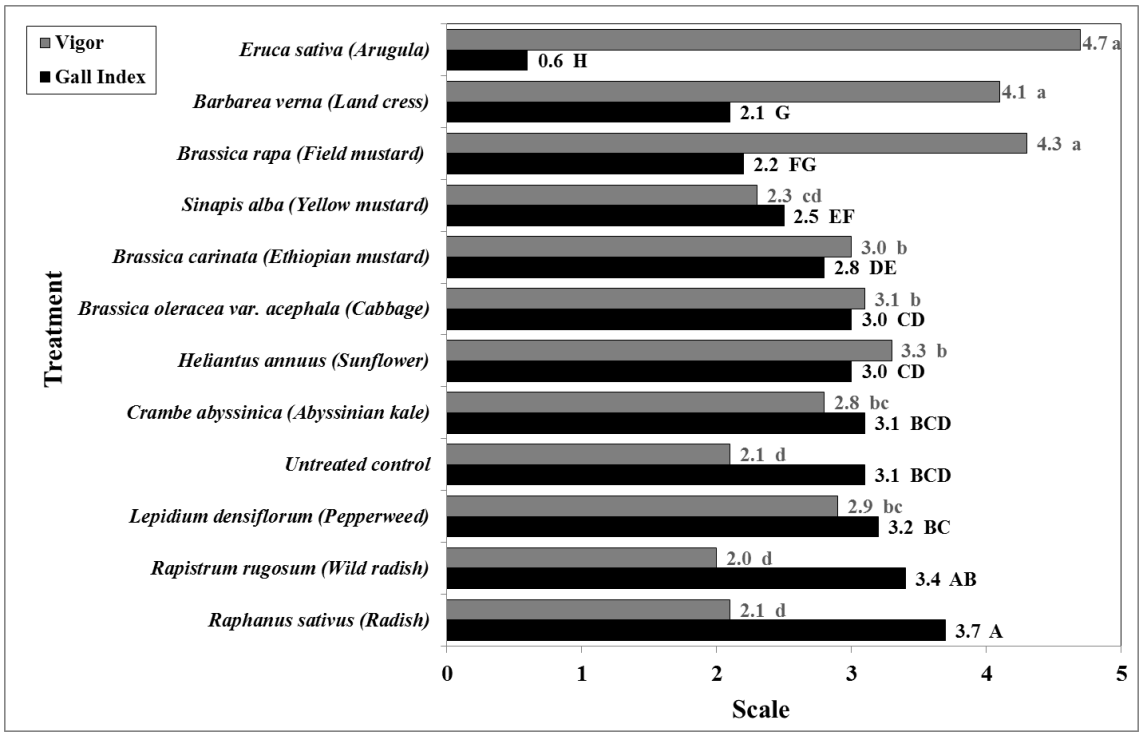

Figure 7. Gall index and vigor of tomato plants, 21 days after transplanting, at inoculation of 120 *Meloidogyne incognita* juveniles in 100 cm^3 of soil (J2 100 cm^{-3} soil). ANOVA and LSD test ($P \leq 0.05$). Means followed by the same letter do not differ statistically.

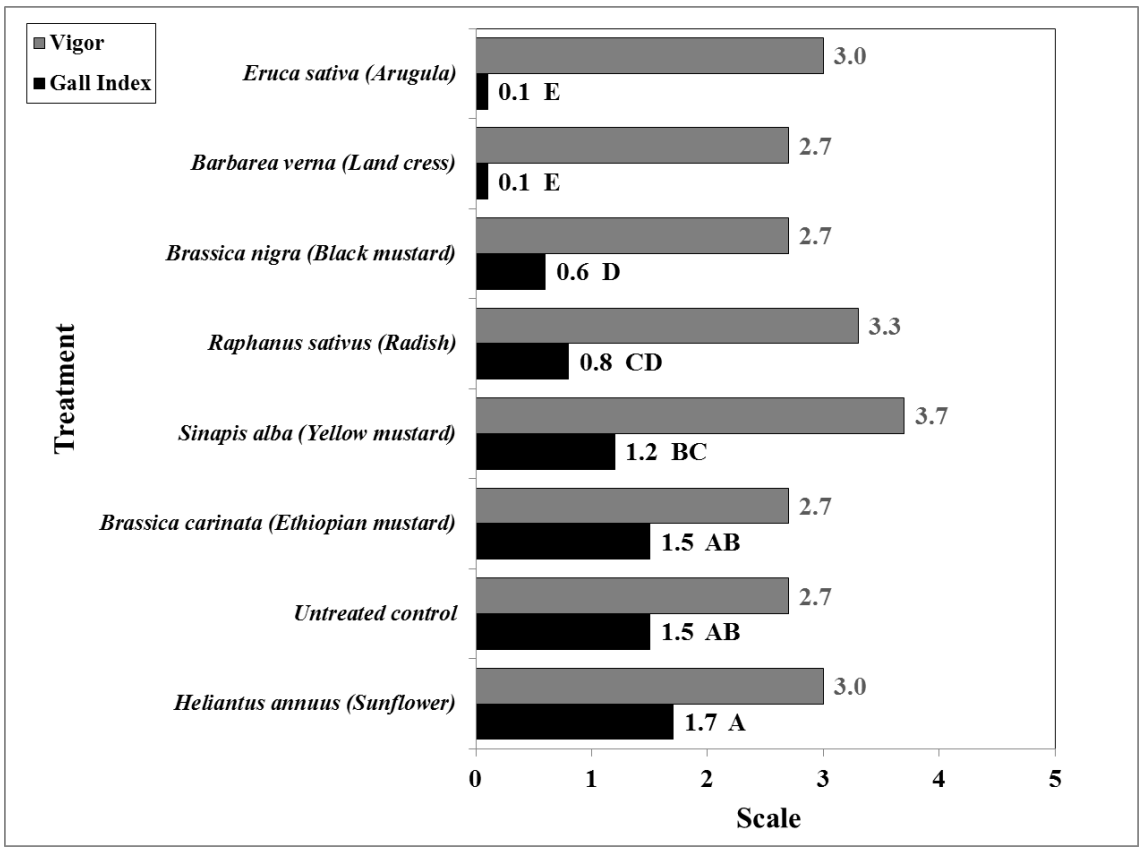

Figure 8. Gall index and vigor of tomato plants, 3 weeks after transplanting, at inoculation of 250 *Meloidogyne incognita* in 100 cm^3 of soil (J2 100 cm^{-3} soil). ANOVA and LSD test ($P \leq 0.05$) showed statistical difference among treatments only in gall index values, while no statistical differences were found in vigor index. Means followed by the same letter do not differ statistically.

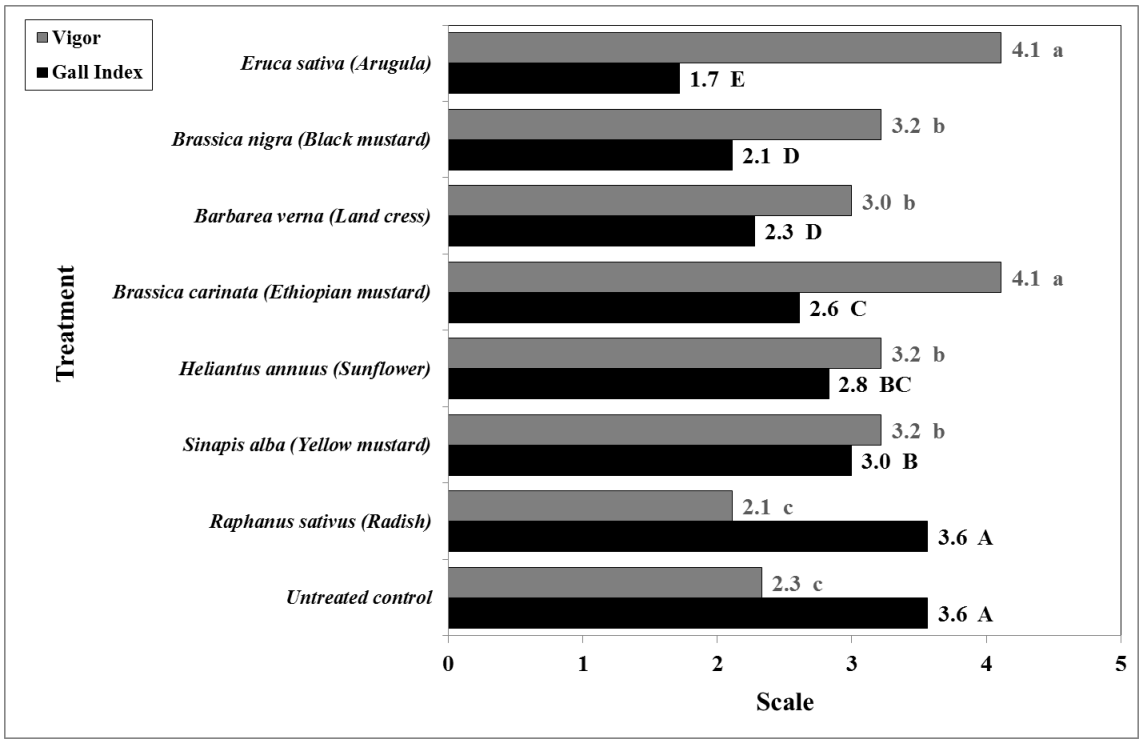

Figure 9. Gall index and vigor of tomato plants, 3 weeks after transplanting, at inoculation of 1000 *Meloidogyne incognita* juveniles in 100 cm^3 of soil (J2 100 cm^{-3} soil). ANOVA and LSD test ($P \leq 0.05$). Means followed by the same letter do not differ statistically.

Generally *R. sativus*, *B. carinata* and *S. alba* applied in all the experiments gave unsteady results in the GInd and vigor classes (Figures 7-9). The DSM from *B. tourneforti* and *L. sativum*, gave results lower than the untreated control even at the lowest population density (Figure 6) and therefore they were excluded from the subsequent experiments. *Brassica oleracea*, *C. abyssinica*, *L. densiflorum*, *R. rugosum* and *B. rapa* were eliminated after the experiment using population density of 120 J2s 100 cm^{-3} soil (Figure 7), because their results were sometimes either unsatisfactory or not statistically different from the untreated control and/or the sunflower DSM.

In addition, all DSMs, including sunflower, gave a clear biostimulant activity on the growth of tomato plants, evaluated as plant height at the end of the experiments, about 20 days after the seedling transplant.

The results of the glasshouse experiments confirmed the effectiveness of *Brassicaceae* DSMs in the control of *M. incognita*. These results were correlated in most cases, but not always, to the nematicidal effect (LD50) of the ITCs released from isolated GLs hydrolyzed by pure MYR. In fact, *E. sativa* DSM, containing glucoerucin as main GL, showed in this study a high biofumigant effect of its degradation products at every population density, in the same time confirming the very low LD50 of its GL (0.02 mM) (Lazzeri *et al*., 2004). The effects on *M. incognita* growth cycle of *E. sativa* green manure application had been reported in glasshouse studies (Curto *et al*., 2005[b]; Curto *et al*., 2012) and in open field trials (Curto *et al*., 2006), in which the *M. incognita* life cycle was impeded and finally inhibited in *E. sativa* roots. Following these results, this accession is currently cultivated as nematicidal catch crop and/or green manure.

Other DSMs gave positive results in this study. Particularly *B. verna* and *B. nigra* DSMs showed high nematicidal effectiveness. That was a particularly significant result, considering that, to the best of our knowledge, these DSMs had never been investigated in nematode control. The degradation products of gluconasturtin and sinigrin, the two main GLs of these crops, respectively, had also proved to be effective in previous in vitro bioassays, achieving the LD50 at concentration of 0.01 mM of gluconasturtin (*B. verna*) and 0.3 mM of sinigrin (*B. nigra*) (Lazzeri *et al*., 2004).

Furthermore, in other studies, the allyl-ITC from sinigrin showed a strong anti-hatching effect on *M. incognita* eggs masses under controlled conditions (Mocali *et al*., 2015), using *Brassica juncea* L. Czern DSM (Yu *et al*., 2007). This result was also confirmed in this study with other sinigrin-containing DSMs such as *B. nigra* and *B. carinata*. To continue, *B. juncea* DSM was seen to be the most nematode suppressive in the control of *M. incognita* and *Pratylenchus penetrans* in laboratory experiments (Zasada *et al*.,

2009) and very effective in decreasing the gall number, egg masses, eggs and egg-hatching of *M. incognita* in tomato plants (Oliveira *et al*., 2011). The suppressive power of the degradation product of sinigrin had also been observed in *B. carinata* DSM, which reduced the GInd and significantly increased the yield of a melon crop in rotation field trials on *M. incognita* management in organic horticulture (Curto *et al*., 2008).

DSMs containing GLs previously characterized for their low nematicidal activity, such as *B. tourneforti* (glucoiberin) and *B. rapa* (gluconapin) (Lazzeri *et al*., 2004), gave little or no effect in the present study, even at the lowest population density, confirming the importance of GLs profile in the DSMs for a significant nematicidal impact.

Sinapis. alba (sinalbin) was effective only at 30-120 J2s 100 cm^{-3}, *C. abyssinica* (epi-progoitrin) at 30 J2s 100 cm^{-3} and *R. sativus* (glucoraphenin) at 30-250 J2s 100 cm^{-3}. The low efficacy of these three DSMs agreed with the low activity observed in *in vitro* trials of the isolated sinalbin (LD50 0.8 mM), epi-progoitrin (LD50 0.9 mM) and glucoraphenin (LD50 1.2 mM), their main GLs respectively (Lazzeri *et al*., 2004). A similar result was obtained by Meyer *et al*. (2011) in a pot experiment on pepper using *S. alba* DSM alone or mixed with *B. juncea* DSM. Finally, it is interesting to observe how the three DSMs that gave the best performances in these trials *E sativa*, *B. verna*, and *B. nigra* contained, respectively, glucoerucin, gluconasturtin and sinigrin that in *in vitro* trials gave the lower LD50, ranging around 0.021, 0.013 and 0.022 mM respectively.

It is still unclear why *L. sativum* was less effective in nematode control, especially considering its high content of glucotropaeolin that when applied as isolated compound gave an LD50 of 0.015 mM (Lazzeri *et al*., 2004). A possible hypothesis could be the limited ability of *Lepidium* spp. DSM in releasing corresponding ITCs. In fact, as observed in Burow *et al*. (2007), the autolysis in *L. sativum* seeds produces thiocyanate and nitrile together with the ITC, this is due to the presence of a thiocyanate-forming protein.

In some cases, the different efficacy of the same DSM treatment applied on different population densities have to be evaluated with great attention. If confirmed, these differences could suggest a low correlation between effectiveness and level of infestation, an aspect that may impact significantly on the experimentation with these DSMs. On the contrary, the relative effectiveness of the various DSMs can help in choosing those best suited to different levels of *M. incognita* infestation: in the event of high infestations, only DSMs of *E. sativa*, *B. verna* and *B. nigra* could be applied

successfully, while for low-medium infestations less potent DSMs, such as *B. carinata*, *R. sativus* and *S. alba* could be used.

It should be noted that DSMs contain several other different compounds so their activity in the soil may also relate to biostimulant and fertilizer effects of each one, in synergy with their biofumigant action. The effect of all DSMs on plant vigor seems to be partially related to the action of the organic matter imcorporation. In fact, high vigor was also observed in plants grown in pots treated with sunflower DSM. On the contrary, the reduction of the GInd on tomato roots was exclusively due to *Brassicaceae* DSMs and absent in sunflower DSM, which never gave a GInd significantly different from the untreated control.

4.3 Inhibition of seed germination through novel formulations based on defatted brassica oilseed meals and crude glycerin: results and discussion

4.3.1 Crude glycerin characterization

As reported in Table 12, the CG was characterized by a glycerol content of around 80% and a residual water content of around 14%.The remaining components were mainly inorganic (ash 4%), except for a minor amount of methanol residual from trans esterification. Concentrations of Cd, Cr, Pb, and Cu were much lower than Italian limits for soil amendment (Dlgs. 75, 2010), which are 1.5, 0.5, 140 and 150 mg kg^{-1}, respectively.

Table 12. Crude glycerin (CG) characterization (mean ± standard deviation).

	Unit	**CG**
Density at 20°C	g cm^{-3}	1.23 ± 0.03
Glycerol	%	80.2 ± 0.6
Water	% w/w	14.7
Ashes	% w/w	4.21 ± 0.19
Cadmium (Cd)	mg kg^{-1}	<0.01
Chrome (Cr)	mg kg^{-1}	0.02 ± 0.00
Lead (Pb)	mg kg^{-1}	0.05 ± 0.00
Copper (Cu)	mg kg^{-1}	0.58 ± 0.03

4.3.2. Defatted oilseed meal characterization

Brassica nigra DSM contained the highest amount of GLs among the tested DSMs (129 µmol g^{-1}). However, the DSMs used in these studies differentiated not only in GL concentration, but also in their GL chemical composition, which can both affect seed GI. In fact, *B. nigra* and *B. carinata* contained mainly sinigrin (98% and 97% of the total GL amount, respectively), while *B. verna* contained 99% of gluconasturtin (phenyl-ethyl-GL) and *C. abyssinica* 96% of Epi-progoitrin ((2S)-2-hydroxybut-3-enyl-GL). Taking into account the amount of DSMs applied in the treatments, the differences in nitrogen and oil content among different DSMs were probably too low to be responsible for modifying the GI. The differences in oil content confirmed the relation with the extraction method. Total phenolic content resulted not significantly different,

with the exception of *C. abyssinica* that contained around half of the phenolic compounds, a characteristic that can reduce its biological activity (Table 13).

Table 13. Defatted seed meal characterization (mean ± standard deviation)

DSM		*B. carinata*	*B. nigra*	*C. abyssinica*	*B. verna*	*H. annuus*
Moisture	%	4.3 ± 0.3	6.1 ± 0.4	5.5 ± 0.3	8.9 ± 0.6	7.5 ± 0.0
Nitrogen	% DM	5.7 ± 0.0	6.7 ± 0.1	4.8 ± 0.2	4.2 ± 0.1	5.0 ± 0.1
GLs	μmol g^{-1}	90.3 ± 2.0	129.0 ± 2.5	106 ± 2.5	105.9 ± 2.0	Absent

DSM, Defatted oilseed meal; DM, dry matter; GL, glucosinolate

4.3.3. Determination of allyl-isothiocyanate entrapment in crude glycerin/water solutions

The solutions containing CG (at least 10%) showed an allyl-ITC retention capacity significantly higher than water alone ($P < 0.05$). In fact, after 30 min of continuous stirring in open vials, the allyl-ITC retention in water was about 20% of the initial allyl-ITC, while in 10% CG solution it was about 45% (Fig. 10), confirming the different release of allyl-ITC from DSM in water and in crude glycerin solutions. The solutions at 10 and 20% CG in water did not significantly interfere with the enzymatic activity of MYR and consequently with the release of GL degradation products from formulated DSMs. Indeed, after just 20 min of continuous stirring, all the tested combinations of CG and DSMs, showed a sinigrin conversion to allyl-ITC that was higher than 85% in agreement with the results obtained in water (De Nicola et al., 2013). Therefore, these results showed that trans-formation of starting GL to allyl-ITC was not inhibited by CG and/or DSM, at least for solutions with 20% CG and 4% *B. nigra* DSM, which were the higher concentration tested in this paper.

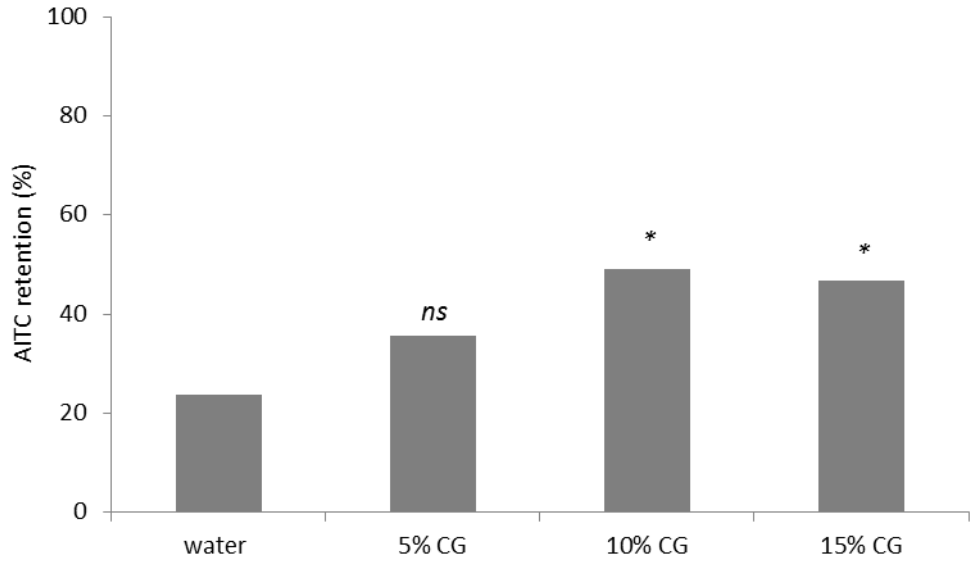

Figure 10. Allyl isothiocyanate (AITC) retention in water and crude glycerin (CG) solutions. Data are expressed as relative % retention after 30 min of shaking in open vials compared to allyl- isothiocyanate basal concentration in closed vials. Statistical analysis on the absorbance at 365 nm data was performed by Student's test (*, $P<0.05$; ns, no significant difference): water vs CG.

4.3.5. In vitro *trials – Petri dish experiments*

The *in vitro* trials clearly showed the biological inhibition of lettuce seed germination, both by CG alone and after the addition of DSMs obtained from *Brassicaceae* species (Table 14). In detail, CG alone showed an inhibitory effect with a clear dose/effect, reaching total inhibition, according to Tukey's test, at 3% concentration. Among DSMs alone, *B. nigra* exerted the highest GI: close to 90% at a concentration of 1.5% in water, while at 1% no difference compared to the untreated control were recorded. *Brassica carinata* had a similar trend to *B. nigra*, even though a slightly lower GI was observed, probably linked to the lower GL content in the meal. *Brassica verna* DSM determined a GI of 23 and 50% at 1 and 1.5%, respectively. On the contrary, *C. abyssinica* DSM did not exert any inhibition activity even at 1.5%, as sunflower DSM. Taking into account the phenolic content in sunflower DSM (Table 13), these results suggest that differences between DSMs in GI are probably due to the different types of GL, which produce hydrolysis products with different biological activities. In addition, the results showed

the synergistic effect of CG and DSM: while CG alone at concentrations of 1 and 2% did not show any inhibition activity, combining 1% CG with *Brassicaceae* DSMs the effect on GI was observed and, furthermore, was markedly higher than applying DSMs alone. Interestingly, GI of CG at 1% supplemented with 1% *B. verna* meal was higher than 80%, suggesting a particularly high synergistic effect. At the same time, the combination of CG and *B. nigra* DSM at 1.5% caused total inhibition. Summarizing the discussion of the results reported in Table 14, the highest GI in synergy with CG was obtained after the addition of DSMs in this order: *B. nigra*> *B. verna*> *B. carinata*> *C. abyssinica*, whereas *H. annuus*, a DSM that contains phenols but no GLs, did not determine any effect on GI.

Table 14. Effect on lettuce germination of different formulation after 72h exposure. Data are expressed as percentages of germination inhibition compared to untreated control (Abbott's formula). Different letters indicate a significant difference between treatments applying ANOVA and Tukey's test ($P \leq 0.05$) of square root arcsine transformed percentages.

DSMs	DSM content (% w/v)	CG content (% v/v)	GI (%)
No DSMs	-	2.0	0.0 e
	-	2.5	13.8 de
	-	2.7	65.5 abc
	-	3.0	96.7 a
Heliantus annuus	1.5	1.0	0.0 e
Crambe abyssinica	1.5	0.0	0.0 e
	1.0	1.0	0.0 e
	1.5	1.0	66.7 abc
Barbarea verna	1.0	0.0	23.3 cde
	1.5	0.0	50.0 cd
	1.0	1.0	83.3 ab
	1.5	1.0	86.7 ab
Brassica carinata	1.0	0.0	6.7 de
	1.5	0.0	23.3 bcde
	1.0	1.0	16.7 cde
	1.5	1.0	70.0 abc
Brassica nigra	1.0	0.0	0.0 e
	1.5	0.0	89.7 a
	1.0	1.0	28.3 cd
	1.5	1.0	100.0 a

DSM, Defatted oilseed meal; CG, crude glycerin; GI, germination inhibition

Table 15. Total mortality of lettuce and of purslane seeds at different doses of crude glycerin (CG).

CG content (% w/v)	Mortality (%)	
	Lettuce	**Purslane**
0	4.7	13.0
5	10.0	17.3
10	29.0	42.7
20	86.0	80.7
30	95.3	86.0

4.3.6. In vivo *trials – pot experiments*

Brassica nigra DSM was chosen for pot trials due to the results of *in vitro* trials and its agronomic characteristics in terms of rusticity and mechanization of the main cultivation phases, that make it a more valuable non-food industrial crop than *B. verna*. The CG doses that caused GI in *in vivo* trials were typically higher than those in *in vitro* because of (i) open air conditions, which facilitate the air dispersion of volatile molecules, and (ii) the presence in soil of organic substances and dwelling organisms that probably decrease the bioavailability of active ingredients. The *in vivo* trials confirmed that the model for assessing the effects of CG/DSM formulations on GI applied to lettuce could be extended to other seeds, although, as expected, it was necessary to use different concentrations in relation to weed seed size and characteristics.

Trial 1– Crude glycerin effect on lettuce and purslane seed germination and plant growth

As shown in Table 15, the application of growing doses of CG was clearly correlated to GI both on lettuce and purslane. The LC50 values were very similar on both lettuce and purslane, respectively, 13.30% (95% confidence interval 12.46–14.15) and13.25% (11.93–14.58), and the highest CG concentration (30%) caused almost complete GI. For this reason, the results obtained on lettuce could reasonably be deemed valid for purslane weed. These results confirmed in vivo the toxic effect of CG on seed germination and plant growth.

Trial 2 – Crude glycerin and defatted oilseed meal effect on lettuce seed germination

In this trial, the effect on lettuce germination of 10% CG was tested in combination with three different doses of *B. nigra* formulated DSM. The CG dose was chosen considering the LC50 value previously determined in trial 1, and GI was checked for 25 days. Germination compared to CG alone was significantly decreased with as little as 2% *B. nigra* DSM, with 3% the GI was complete at all considered times (Fig. 11). Taking into account that 3% of *B. nigra* DSM in water did not cause total GI (data not shown), it is possible to confirm in *in vivo* trials the synergistic action of CG and DSM observed at laboratory level. Furthermore, these results showed that formulation with 10% CG and 3% *B. nigra* DSM not only delays, but inhibits the lettuce seed germination, at least for 25 days.

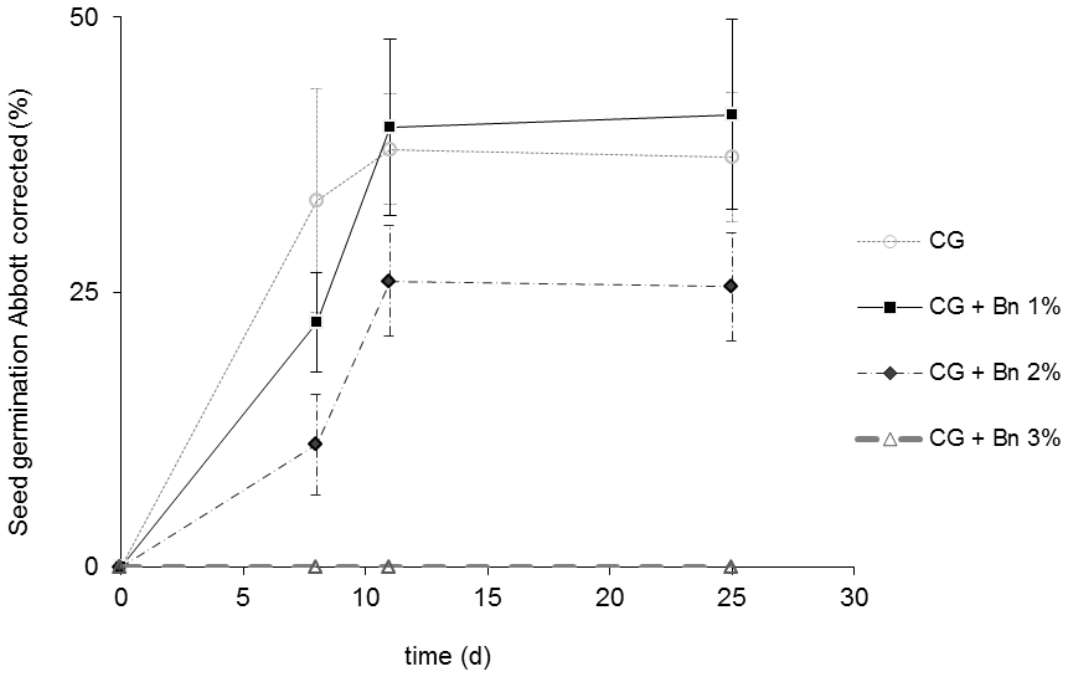

Figure 11. Lettuce germination inhibition in the presence of 10% crude glycerin (CG) solution and increasing doses of *Brassica nigra* (Bn) defatted seed meals, in pot experiment. Four pots per entry, error bar indicates standard error.

Trial 3 – Crude glycerin and defatted oilseed meal effect on cress, cucumber and sorghum seed germination

The formulations of CG and DSM after 30 days, caused a close total GI for sorghum and garden cress (93 and 97%, respectively). For cucumber, inserted in the trial as a large size seed, a significant germination delay was recorded, but the final germination rate was similar between treated and untreated pots (Fig. 12). Nevertheless, the biomass of treated cucumbers 40 days after sowing was dramatically lower than the biomass of control plants, as clearly shown in Fig. 13, suggesting a potential control of weeds despite their having larger seeds than purslane, cress or sorghum. Finally, no residual toxicity was observed during the 11 weeks of the subsequent tomato cultivation, showing no significant differences either in the number of leaves and plant height or in epigeal and hypogeal biomass.

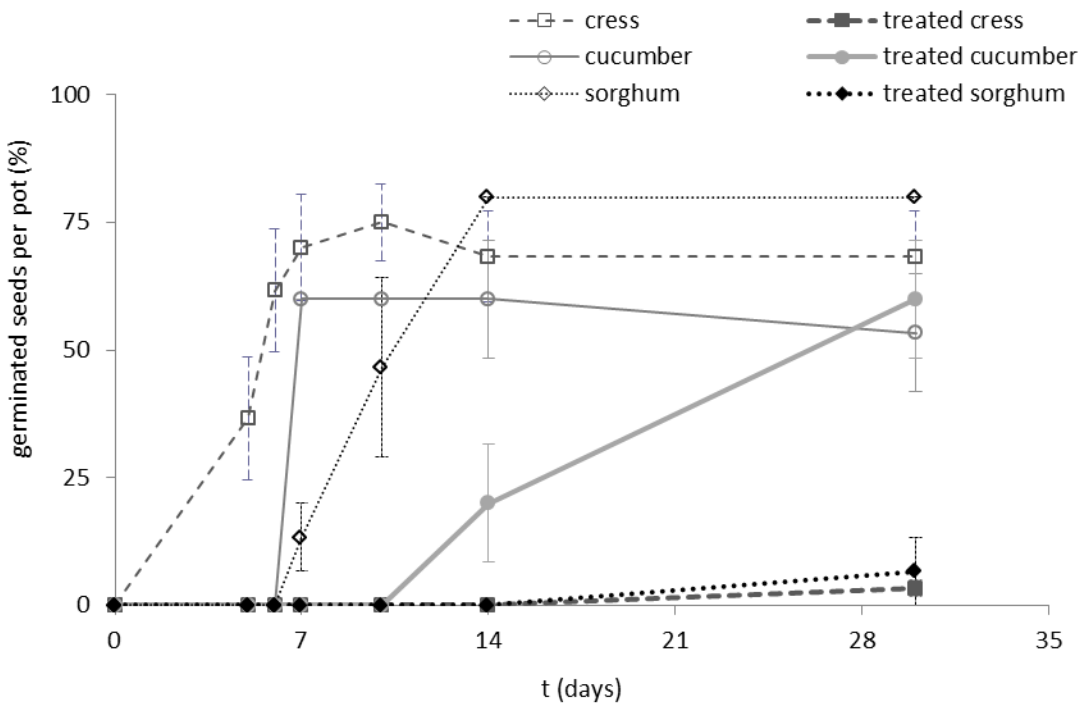

Figure 12. Time trend in germination of seeds with different sizes in greenhouse pot cultivation. Three pots per entry, error bar indicates standard error. Thin lines indicate untreated trials, thick ones indicate trials treated with a 20% crude glycerin (CG) solution containing 4% of *Brassica nigra* defatted oilseed meal (DSM).

Figure 13. Cucumber vigor 30 days after sowing in three pots treated at sowing time with crude glycerin/defatted oilseed meal formulation (top row) compared to untreated ones (bottom row).

4.4 Inhibition of black-grass seed germination through novel formulations based on defatted brassica oilseed meals and crude glycerin: results and discussion

4.4.1 Degradation products from twenty defatted oilseed meals and their efficacy in germination inhibition of lettuce seeds

The evaluation of different DSM extracts evidenced very different behavior for the effectiveness in GI, that was mainly linked to the type of GL profile more than the to the total GL content. Among the twenty tested DSMs in the preliminary *in vitro* experiment, nine of them, reported in the grey box (table 16), were considered effective according to their GI percentage.

The five DSMs chosen for further experiments are reported in bold in table 16. They were characterized from an interesting nitrogen content that ranged from 6.1±0.1% in *B. tournefortii* DSM to 7.8±0.1% in *R. rugosum* DSM, as reported in table 17. This could possibly represent an interesting integration to N from conventional fertilizer, in field conditions. In addition, a low residual oil content, never above 10%, was found in the DSMs, ranging from 5.0±0.1% to 10.4±0.0%, in *B. tournefortii* DSM and *R. rugosum* DSM, respectively. The composition and the low residual oil amount could not imply a phytotoxic effect itself (Gauvrit & Cabanne, 1993).

Table 16. Main glucosinolate (GL) content, expressed as µmol g^{-1} of dry matter, of twenty Brassica defatted seed meals (DSMs) and the germination inhibition (GI) produced by the DSM extract on lettuce under *in vitro* conditions.

DSM	GL R-chain	GL Common name	GLs (µmol g^{-1})	GI (%)
Brassica nigra	**2-propenyl-GL**	**Sinigrin**	**129.0±2.5**	**100.0±0.0 b**
Brassica oleracea	4-methylsulfinylbutyl	Glucoraphanin	103.1±6.8	100.0±0.0 b
Brassica tournefortii	**3-methylsulfinylpropyl**	**Glucoiberin**	**128.5±3.5**	**100.0±0.0 b**
Eruca sativa	**4-methyltiobutyl**	**Glucoerucin**	**152.0±2.5**	**90.0±10.0 b**
Lepidium campestre	4-hydroxybenzyl	Sinalbin	139.6±0.6	100.0±0.0 b
Lepidium sativum	benzyl	Glucotropaeolin	160.3±5.4	100.0±0.0 b
Raphanus sativus	4-methylsulfinylbutyl-3-enyl	Glucoraphenin	140.0±3.3	100.0±0.0 b
Rapistrum rugosum	**3-methylsulphonylpropyl**	**Glucocheirolin**	**232.2±1.6**	**100.0±0.0 b**
Sinapis alba	**4-hydroxybenzyl**	**Sinalbin**	**187.2±1.1**	**100.0±0.0 b**
Berteroa incana	5-methylthiopentyl	Glucoberteroin	86.6*±0.4	24.0±0.0 a
Brassica rapa	but-3-enyl	Gluconapin	156.1±3.4	14.0±14.0 a
Hesperis matronalis	unknown	-	236.9*±6.1	22.0±5.8 a
Lepidium densiflorum	4-OH 3-5 dimethoxy benzyl**	-	188.4*±0.9	12.0±5.8 a
Lesquerella fendleri	3-methylsulfinylpropyl	Glucoiberin	27.2*±0.6	4.0±2.4 a
Limnanthes alba	3-methoxybenzyl	Glucolimnanthin	200.4*±2.9	14.0±4.0 a
Camelina sativa	10-methylsulfinyldecyl	Camelinin	45.8*±1.9	0.0±0.0 a
Cleome hassleriana	methyl	Glucocapparin	77.65±0.2	0.0±0.0 a
Erysimum pseudorhaeticum	Unknown	-	110.0±5.4	0.0±0.0 a
Reseda lutea	2-ramnopyranosiloxybenzyl	-	30.2*±0.1	0.0±0.0 a
Sisymbrium officinale	isopropyl	Glucoputranjivin	59.3±0.4	0.0±0.0 a

*no relative proportionality factor known for sinigrin, the coefficient was arbitrary considered equal to 1.

**GL identified in Pagnotta *et al.*, 2017 (in press).

Table 17. Defatted seed meal characterization (mean ± standard deviation).

DSM		*B. nigra*	*B. tournefortii*	*E. sativa*	*R. rugosum*	*S. alba*
Oil content	% DM	8.9±0.1	5.0±0.1	9.4±0.3	10.4±0.0	5.7±0.0
Nitrogen content	% DM	7.0 ± 0.1	6.1 ± 0.1	6.4 ± 0.1	7.8 ± 0.1	6.8 ± 0.1

Abbreviations: Defatted oilseed meal (DSM); dry matter (DM);

After preliminary GC-MS analysis of the degradation products in the ten DSM extracts applied in the *in vitro* trials, some interesting results emerged. As expected, in the experimental reaction conditions, the main degradation product of the *B. nigra* DSM extract was 2-propenyl ITC. The main products in *B. tournefortii*, *R. rugosum* and *E. sativa* DSM extracts were 3-methylsulfinylpropyl (iberin), 3-methylsulphonylpropyl (cheirolin) and 4-methyltiobutyl (erucin) ITCs, respectively. All the ITCs showed to be stable for more than 24h in a sealed bottle after the extract production, the same time spent to soak the black-grass seeds in in vitro trial (§ 3.4.6), except for those from *S. alba*. In fact, no ITCs were found in the *S. alba* extract, according to the well documented instability of sinalbin ITC in aqueous solutions which results in a quick hydrolysis to benzylic alcohols and thiocyanate ion (Agerbirk *et al*., 2012). Besides this paper, other studies had already shown an interesting effectiveness in weed containment induced by *S. alba* (Rice *et al*., 2016; Boydston *et al*., 2011), and even in the preliminary tests of this study *S. alba* DSM showed an interesting containment effect as well. In fact, in the *in vitro* trials, the active *S. alba* DSM totally inhibited the lettuce seed germination while after MYR deactivation did not shown any GI (table 18).

Table 18. Interaction between factors and their relevance in black-grass germination and development in the glasshouse trial.

DSM	GI%	Main degradation product	Retention time
A *Brassica nigra*	100.0±0.0	2-propenyl-isothiocyanate-ITC	5.33
A *Brassica tournefortii*	100.0±0.0	3-methylsulfinylpropyl-ITC	18.74
A *Eruca sativa*	90.0±1.0	4-methyltiobutyl-ITC	15.03
A *Rapistrum rugosum*	100.0±0.0	3-methylsulphonylpropyl-ITC	18.28
A *Sinapis alba*	100.0±0.0	-	-
D *Brassica nigra*	6.7±6.7	-	-
D *Brassica tournefortii*	0.0±0.0	-	-
D *Eruca sativa*	0.0±0.0	-	-
D *Rapistrum rugosum*	0.0±0.0	-	-
D *Sinapis alba*	0.0±0.0	-	-

Abbreviations: Defatted oilseed meal (DSM); Activated Myrosinase (A); Deactivated Myrosinase (D); GI (germination inhibition); ITC (isothiocyanate).

As reported in Borek *et al*. (2005), when *S. alba* DSM are watered there is the release of 4-hydroxybenzyl-ITC that quickly hydrolyzes to parahydroxybenzyl alcohol and SCN^- in presence of alkaline pH values, this compound is probably responsible for the observed phytoxicity. At the same time, there is a light effect on the subsequent development of the sprouts treated with deactivated DSMs, on which a reduction of root and epicotyl lengths was observed. This activity could be due to other compounds that could be involved in phytotoxic effect (fig. 14).

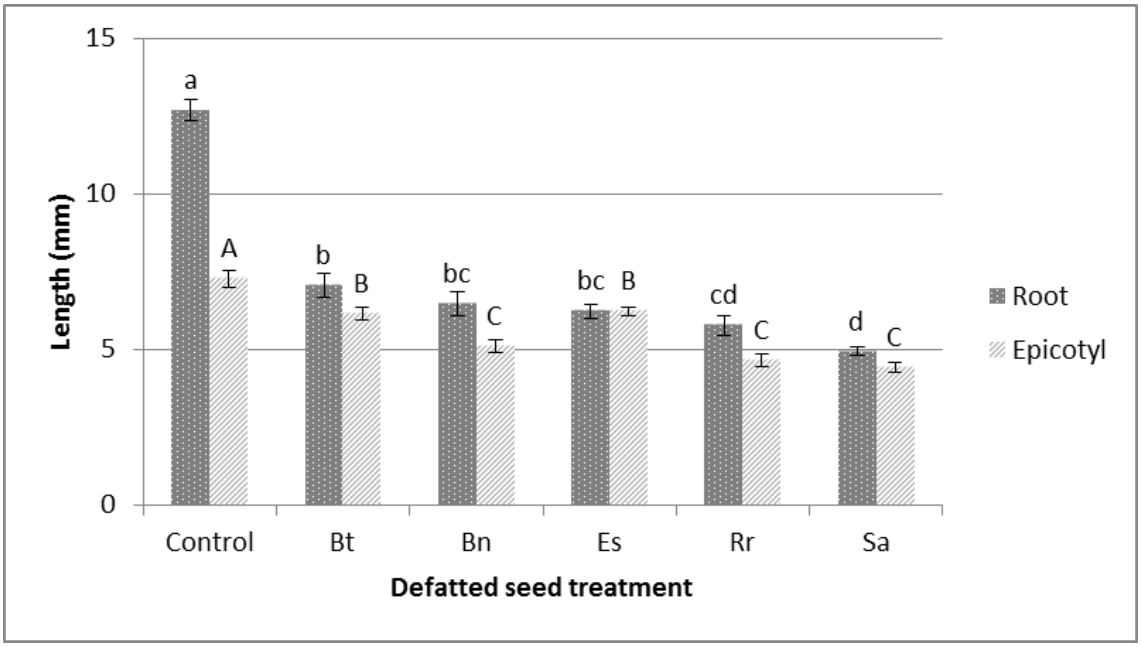

Fig. 14. *In vitro* experiment, lettuce root and epicotyl length after the application of the extracts from the deactivated DSMs: *Brassica tournefortii* (Bt); *Brassica nigra* (Bn); *Eruca sativa* (Es); *Rapistrum rugosum* (Rr); *Sinapis alba* (Sa). Different letters indicate a significant difference between treatments applying ANOVA and Tukey's test ($P<0.05$) on measured values. Uppercase letters refer to the Epicotyl lengths, lower case letters refer to the root lengths.

The *in vivo* trials confirmed an interesting efficacy of DSMs formulated with CG in controlling lettuce seed germination (fig. 15): from 95% of *B. nigra* and *E. sativa* treatments, to 80% of *S. alba*, even the CG applied alone showed a GI of 78%. In this trial, only the addition of *B. nigra* and *E. sativa* DSMs to the formulation determined a statistically consistent germination reduction compared to the CG applied alone, whilst the other DSM had a slighter effect. In addition, all treatments, CG alone included, showed a dramatic reduction in dry biomass yield, from 20 to 100 times lower if compared to the biomass yield in the control pots (fig. 15).

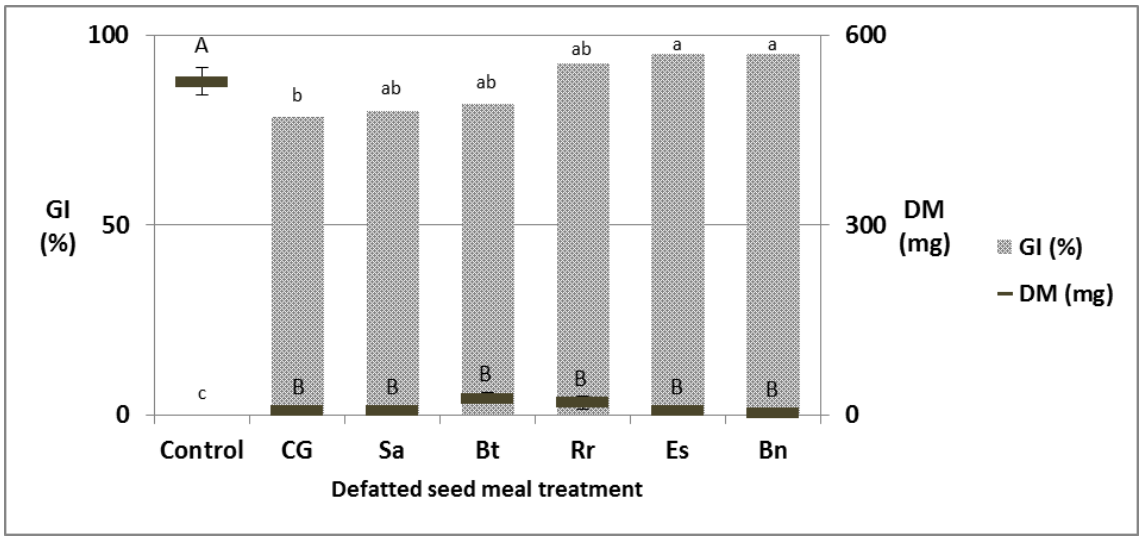

Fig. 15. Glasshouse experiment, showing application of different defatted oilseed meals 2.7 g L^{-1} of soil, applied in formulation with a solution of crude glycerin (CG) at 10% (9.2 ml L^{-1} of soil) in lettuce pots. Germination inhibition (GI) and biomass dry matter production (DM) are expressed as mean per pot. Different letters indicate a significant difference between treatments applying ANOVA and Tukey's test ($P < 0.05$) on GI and DM. Uppercase letters refer to the dry matter production, lower case letters refer to the GI. Abbreviations: *Brassica tournefortii* (Bt); *Brassica nigra* (Bn); *Eruca sativa* (Es); *Rapistrum rugosum* (Rr); *Sinapis alba* (Sa).

4.4.2 Effectiveness of defatted oilseed meal extracts in black-grass germination inhibition: in vitro trials

The *in vitro* trials evidenced strong differences among DSM extracts for their efficacy in inhibiting black-grass germination (fig. 16). In fact, after 15 days *S. alba*, *R. rugosum* and *E. sativa* showed no significant differences compared to the untreated control showing a GI of 0%, 12% and 20%, respectively. *Brassica nigra*, instead, totally inhibited the seed germination (GI = 100%), confirming even on Black-grass high activity observed on lettuce.

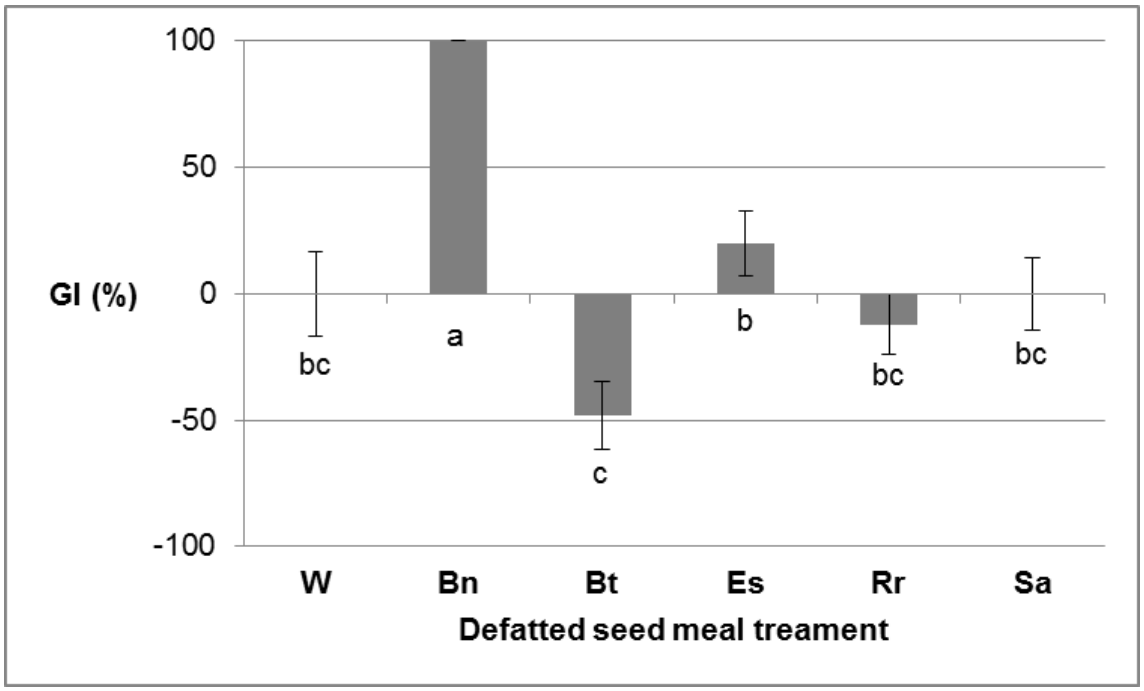

Fig. 16. *In vitro* experiment, black-grass Germination inhibition (%) after the application of defatted seed meal extracts (15 mg ml^{-1}) expressed as mean per Petri dish. Different letters indicate a significant difference between treatments applying ANOVA and Tukey's test ($P < 0.05$) on GI percentages. Abbreviations: *Brassica tournefortii* (Bt); *Brassica nigra* (Bn); *Eruca sativa* (Es); *Rapistrum rugosum* (Rr); *Sinapis alba* (Sa).

An unexpected result was related to the *B. tournefortii* DSM extract which, in Petri conditions, showed a GI of -48%. It is not yet clear which is the interaction that determined this surprising result. In the literature for instance, it is reported that *Phelipanche ramosa* germination, a major parasitic weed of *Brassica napus*, is strongly and specifically triggered by ITCs (Auger *et al*., 2012), and recent studies have provided evidence for a link between indole GLs and indole-3-acetic acid (Bak *et al*, 2001; Zhao *et al*., 2002). Other experiments showed how biostimulant effect of *Brassicaceae* extracts occurs particularly at a concentration near to 0.1-1 mg L^{-1} (Rivera *et al*., 2010). New experiments are currently ongoing for a better understanding of the involved processes.

4.4.3 Effectiveness of defatted oilseed meals formulated with glycerin in black-grass germination and growth inhibition: in vivo trials

The application of the DSM formulated with and without CG in semi-controlled conditions in greenhouse added some new information for a practical application of Brassica DSMs in weed control. The CG used in the trials had a glycerol content of 80% and a residual water content 14%. The remaining components were mainly inorganic (ash 4%) and hazardous compounds were found only at very low traces (D'Avino *et al.*, 2015[a]). In this experiment, *B. nigra* (5.5 g/L of soil) formulated with a solution of CG at 10% (9.2 ml/L of soil) completely inhibited the germination and the subsequent seedlings development until 4 weeks after the treatment. Even the *B. nigra*, applied without CG, at the highest dose showed a GI of 97.9% after 4 weeks. Furthermore, with regard to the GI, *Bn* applied both with and without CG showed a very clear dose/response effect on GI percentage (Fig. 17), unlike the other DSMs.

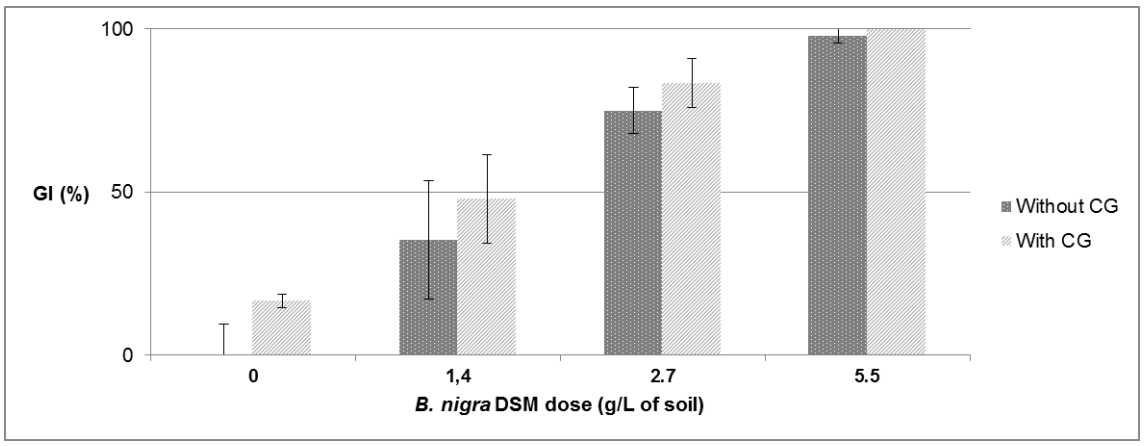

Fig. 17. Glasshouse experiment, showing the effect of applying *Brassica nigra* defatted oilseed meal (DSM) formulated at three different doses (mg/pot), with and without crude glycerin (CG) on black-grass (*Alopecurus myosuroides* Huds.) germination inhibition (GI%). Black-grass germination inhibition (GI) is expressed as mean per pot.

Whilst the effectiveness of the *B. nigra* DSM was clearly observed in our experiments, the high phytotoxic action of allyl-ITC compared to other ITCs has been reported in other publications. Oleszek (1987) and Bialy *et al*. (1990), for instance, demonstrated that germinating seeds exposed to either pure allyl-ITC or the pulverized leaves of *B. juncea* or *B. nigra*, could be inhibited or, the overall seedling growth had been stunted. The effectiveness of *B. nigra* DSM could be linked also to the high volatility of its main GL degradation product. On the other hand, different DSMs could increase their effectiveness in GI through a formulation optimization, aimed at improving the active compounds release.

Although *in vitro* trials *B. tournefortii* DSM showed to determine, an increased germination of black-grass , this effect was not confirmed in the subsequent greenhouse trials. In fact, in pot conditions none of the three tested doses of *B. tournefortii* DSM increased the number of black-grass germinated seeds, nor height or dry weight of plants. Other studies are ongoing for a better understanding of the processes involved in this phenomenon.

Even if the other DSMs did not show such a dose/effect response, probably because of the reduced effectiveness in these specific assay conditions, some meaningful results clearly emerged from the factorial ANOVA applied to the GI, HRED and DMRED measurements. Firstly, the black-grass seed germination was inhibited by a complex interaction of factors. In fact, a statistically significant effect due to the DSM and CG factors, if considered separately, was observed. Furthermore, the GI was significantly affected by the interactions between i) '*DSM'* x '*DSM concentration',* and ii) '*DSM concentration'* x '*CG'*. Regarding the growth parameter HRED and DMRED, if compared to the untreated control, the '*CG'* was the unique factor which had a significant effect on plant growth (Tab. 19).

Table 19. Interaction between factors and their relevance in black-grass germination and development in the glasshouse trial.

Factorial ANOVA P value	**GI**	**HRED**	**DMRED**
DSM	**<0.001**	>0.05	<0.01
DSM concentration	>0.05	<0.05	<0.05
Crude Glycerin	**<0.001**	**<0.001**	**<0.001**
DSM x DSM concentration	**<0.001**	>0.05	>0.05
DSM x Crude Glycerin	**<0.001**	<0.05	>0.05
DSM concentration x Crude Glycerin	>0.05	>0.05	>0.05
DSM x DSM concentration x Crude Glycerin	>0.05	>0.05	>0.05

Abbreviations: defatted oilseed meals (DSM); germination inhibition (GI); height reduction (HRED); dry matter biomass reduction (DMRED).

The greenhouse trial showed how the presence of DSM in the treatments affected mainly the early stages of black-grass seed development. In fact, the interactions between type of *'DSM'*, *'DSM concentration'* and presence or absence of *'CG'*, which affect the seed germination, resulted very manifold. Once the seedlings emerged, the subsequent growth (plant height and biomass) was mostly affected by the presence of CG in the formulations. These results confirmed the limited persistence of the effect of GL degradation products in the soil and consequently the safety of the application of *Brassicaceae* DSMs in weed control. The concern in using CG is based upon the presence of methanol and sodium residues used as catalysts in the biodiesel production process. In general, CG is considered a safe product that can be used, for instance, as an energy-rich feed component, from 10 to 15%, in animal diets (Alexander *et al*., 2010).
Since the *B. nigra* DSM, the most effective tested meal, showed a total GI at same concentration and conditions as the other DSMs, higher concentrations were not investigated. As previously reported, the effectiveness of the DSMs depends on the optimization of the formulation, and under different experimental conditions (e.g. higher concentration, different pH, mode of distribution or formulations), it could greatly increase.

5. CONCLUSIONS

During the three year of research in the field of the exploitation of the potential of minor Brassica oilseed crops some interesting results were achieved.

In particular:

i) The introduction of winter brassica oilseed crops such as camelina in crop rotation with cereals, have shown a positive impact on subsequent crop seed yield, but more knowledge about its management and its impact on soil physical-chemical and biological properties must be investigated. Camelina has shown to be potentially well adaptable, even with low environmental input techniques to cultivation in the Po Valley pedo-climatic conditions. As for other minor oleaginous a varietal selection would be useful for higher and more stable yields that could permit the reduction of the gap between potential and effective yields. *Camelina sativa* showed a good adaptability in autumnal sowing in the Po Valley in a low input condition cropping system and the potential applications in a biorefinery chain linked to the territory is of deep interest given its high added value oil and oilseed cake residue for different industrial uses.

ii) Until now, the commercial know-how in nematode control products from Brassicas has been limited pretty much to allyl-ITC, especially through *B. carinata* DSM. Among different Brassica DSMs, the best results in the reduction of *M. incognita* infestation, regardless the inoculation severity, were achieved by *Eruca sativa* (rocket), *Barbarea verna* (land cress) and *Brassica nigra* (black mustard), whereas the other species gave either alternate results or similar results to the untreated and sunflower DSM controls. These first results open new perspectives for innovative bioactive molecules in biofumigation applied in cropping systems where nematode suppression is a critical factor.

ii). The synergic effect of the formulation based on Brassica DSMs and CG on seed germination was shown both in *in vitro* and *in vivo* as glasshouse trials. Though, both *in vitro* and *in vivo* results confirmed the effectiveness of the formulations, especially those based on *B. nigra*, even on black-grass seeds, further research needs to be done in order to optimize the product and to reach the most effective release of active compounds.

The potential of Brassica derived DSMs in weed control has to be consider with great attention for different reasons: the proposed formulations are completely bio-based

products that could be considered not only for conventional farming but even applicable both in integrated pest management and organic farming (where at moment no herbicides are allowed); they present a combined effect both on weeds and on soil borne pests and diseases; formulated Brassicaceae DSMs could also represent an interesting integration to fertilizers with their good nitrogen content.

All these aspects, applied within a virtuous biofumigant cropping system, make the use of DSMs in agriculture an interesting novel technological proposal that need to be investigated before the application at full field level.

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