CONTROL OF *VERTICILLIUM DAHLIAE* CAUSING SUNFLOWER WILT USING *BRASSICA* COVER CROPS

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ABSTRACT

Since 2010, sunflower in France has been severely affected by a vascular wilt disease caused by *Verticillium dahliae*. Disease is widespread and causes significant damage up to 30% yield loss. *V. dahliae* is a soil-borne fungus living in roots able to survive in the absence of a host in the form of microsclerotia (MS). *Brassica* crops used as cover crops can naturally suppress soilborne pathogen viability. This fumigation activity has been linked to volatile isothiocyanates (ITCs) released from glucosinolates (GSLs). In this study, *Brassica* cover crops of white and brow mustard, radish, rape and rapeseed were evaluated for their ability to reduce the viability and development of *V. dahliae*. Cultivars were selected by GSL side-chain and concentration, and *V. dahliae* strain for its aggressiveness on sunflower. Biofumigation was assessed in a laboratory assay. MS and developed *V. dahliae* on growing media were exposed for 20 days to volatile compounds released by fresh or freeze ground plant tissues. The toxicity of ITCs-GSLs on *V. dahliae* was assessed by the area under fungus progress curve relating its development on the media. The five *Brassica* reduced the development and germination of *V. dahliae* by 90% (brown-mustard) to 63% (radish), and the development by 90% (rape) to 69% (white-mustard) compared to the control in absence of tissues. Aliphatic GSLs in brown mustard and rape, and indole GSLs in rape and radish may explain the strong reduction of *V. dahliae* development and viability respectively. These results indicate that *Brassica* have potential for use as cover crops for the control of soilborne disease problems and sunflower wilt.

Keywords *Verticillium dahliae*, Microsclerotia, Biofumigation, Cover crops, *Brassica* crops, glucosinolates, Sunflower.

INTRODUCTION

Verticillium dahlia Kleb. is a destructive and vascular soil-borne fungus that infects many economically major agricultural crops and ornamental plants all around the world (Schnathorst, 1981; Pegg and Brady, 2002). In France, since 2010, sunflower (*Helianthus Annuus*) has been severely affected by this pathogen causing significant damage up to 30% yield loss (Mestries and Lecomte, 2012). Symptoms of sunflower Verticillium wilt appears around flowering, first on the lower leaves and move towards upper leaves. On leaf, brown necrosis surround by a yellow halo, vascular discoloration and wilting of sunflower are observed (Wilhelm, 1956). Verticillium wilt is difficult to control because the pathogen can survive in the soil as microsclerotia (MS), its resting structure, for nearly 13 years even in the absence of a suitable host (Bruehl, 1987 ; Griffiths, 1970). Thus, MS are regarded as the primary targets to control Verticillium wilt (Hawke and Lazarovits, 1994).

Since the prohibition of effective but harmful chemical fumigants as methyl bromide, techniques to manage *V. dahliae* in sunflower are limited and not very effective. Alternative methods including sustainable disease control options for managing soilborne fungus are needed (Davis et al., 2010 ; Ochiai et al., 2007 ; Rowe and Powelson, 2002). Thus,

biofumigation, performed by the incorporation of fresh biomass from *Brassica* plants into the soil, appears as an alternative promising method (Kirkegaard et al., 1993 ; Angus et al., 1994 ; Kirkegaard et al., 1998 ; Kirkegaard et al., 2000 ; Matthiessen and Kirkegaard, 2006 ; Larkin and Griffin, 2007 ; Njoroge et al., 2008; Omirou et al., 2011). *Brassica* crops used as cover crops have disease-suppressive effects against soilborne population of fungal pathogens, nematodes and weeds (Brown and Morra, 1995, 1997 ; Buskov et al., 2002 ; Mojtahedi, 1993 ; Olivier et al., 1999 ; Sarwar et al., 1998). It is based on the high concentration of glucosinolates (GSLs) which are secondary metabolites structurally categorized as aliphatic, aromatic, and indole GSLs (Brown and Morra, 1997 ; Fahey et al., 2001 ; Omirou et al., 2011). GSLs are biologically inactive molecules, but after tissues disruption, GSL are hydrolyzed by myrosinase to volatile compounds like indoles, nitriles, thiocyanates and isothiocyanates (ITCs). Among those, ITCs have a biocidal activity and are the most toxic for soil-borne pathogens (Chew, 1988 ; Fenwick and Heaney, 1983 ; Mithen, 2001 ; Omirou et al., 2011 ; Rosa, 1997 ; Sarwar et al., 1998). However, the profile, concentration and distribution of GSLs – ITCs varies greatly within Brassica species, plant tissues and even among cultivars (Kirkegaard et al., 1998 ; Mithen, 1992).

Few studies have investigated the potential of GSLs-containing brassicaceous cover crops for suppression of *V. dahliae*. Additionally, the role of ITC-related biofumigation often cannot be interpreted because no information is provide on the type or the concentration of GSLs present in the used biomass. Thus, predictions of the biofumigation potential of different *Brassica* species to *V. dahliae* based on GSLs concentration needs to be confirmed to evaluate the incidence of their potential biocidal activity *in vitro*. The adoption of biofumigation seems most likely to proceed if it is specified to suit the target pests and production systems. This preliminary study aimed at evaluating the potential biofumigation effects of *Brassica* cover crops on *V. dahliae* - sunflower pathosytem. The objective of this study was (1) to assess the potential biofumigation effects of five *Brassica* cover crops by following *in vitro* the relative toxicity of relevant GSLs on *V. dahliae* development and MS formation (2) to identify the most effective Brassica crops for *V. dahliae* control in future field trials.

MATERIALS AND METHODS

Production of biomass, sampling and sample preparation

Seeds of 5 different cultivars of brown mustard (*Brassica juncea* cv Etamine) (100 pl/m²), white mustard (*Sinapis alba* cv. Abraham) (100 pl/m²), radish (*Rhaphanus sativus* cv. Anaconda) (80 pl/m²), rape (*Brassica rapa* cv. Avalon) (80 pl/m²) and rapeseed (*Brassica napus* cv. Mosa) (80 pl/m²) were sown on February 2015 in 5 trays (0.6 m²; 50 cm depth) filled with potting compost into the greenhouse of INRA, Auzeville (Haute-Garonne, France). These five cultivars were selected among 22 after a field trial in 2014 for their GSL profiles and concentrations in the shoot and root tissue for each crop.

Photoperiod, temperature and air humidity were controlled in the greenhouse. A 13h photoperiod was applied from plant emergence to flowering stage with 400W High Pressure Sodium vapor lamp (SON-T AGRO, Philips). Supplemental lighting was turned off when global radiation was above 250 W/m². The temperature in the greenhouse was maintained at 13°C ± 3°C. Plants were fertilized with two applications of NPK (24 kg N, 28 kg P, 28 kg K /ha) and SO_3 (40 kg/ha) to provide nutriments for GSL synthesis in the plant. Plants were irrigated regularly to maintain adequate soil moisture until flowering. Powdery mildew (*Erysiphe cichoracearum*) was treated by triticonazole (POLYSOINS ULTRA SPRAY, Scotts France SAS) at 0.15 g /L.

The relative production of GSLs in the tissues usually reach a maximum around flowering (Sarwar and Kirkegaard, 1998). Accordingly, brown and white mustard, and radish were sampled at mid-flowering at 51 days after sawing (DAS), and rape and rapeseed at 61 DAS. Roots (RB) and shoots (SB) were sampled, washed and then separated. RB and SB of each cultivar were grinded separately using a ELIET primo mill. The mill was rinsed between each sample. Samples of a particle size <0.5 cm were either used fresh for the in-vitro assay or stored in sealed bags and immediately frozen and stored at -80 °C until processing.

Isolation and analysis of the desulfated GSL

Frozen plant materials were freeze dried and ground as fine as possible with a Tetsch MM 300 mixer mill at 30 Hz for 1 min. Aliquot of 50 mg were weighed in 2.0 ml Eppendorf tubes after witch 1ml 70% MeOH was added to the samples and boiled it for 10 min at 90°C. After boiling, samples were placed for 15 min in an ultrasonic bath and centrifuged at 6500 rpm for 10 min. The supernatant was added to 0,5ml DEAE Sephadex A-25 column. The pellet was kept, washed twice with 1ml 70% MeOH, vortex and placed in the ultrasonic bath for 15 min. After centrifugation at 6500 rpm for 10 min the supernatant was added to the same column. The column was washed twice with 1ml 70% MeOH, once with 1ml MilliQ and twice with 1ml 20mM NaOAC buffer (pH 5.5). Thereafter, 20 µl of aryl sulfatase (Sigma type H-1 of *Helix pomatia*) was added to the columns and flushed down with 50 µL NaOAc buffer (pH 5.5). The columns were covered with aluminium foil and incubated over night at room temperature. The next day, the resulting desulphoglucosinolates were elute from the column with 2 times 0,75ml MilliQ water. The elution was frozen with liquid nitrogen, freeze dried, dissolved in 1ml MilliQ water and measured on the HPLC (de Graaf et al., 2015).

Verticillium dahliae isolates and inoculum production

A *V. dahliae* strain obtained from one microsclerotia (MS) was used in this study and selected for its aggressiveness among 8 strains. The strain was isolated from an infected sunflower residue in a field located in Verfeil (Haute-Garonne, France), close to the trial site, and showing severe Verticillium wilt, root dislocation and high production of MS. Inoculum was plated on Petri dishes containing potato dextrose agar (PDA, Difco) (39 g/l, 150 mg of streptomycin, pH 6) and grown at 25 ± 1 °C in the dark. For *in vitro* assay, the fungus was either used developed (DV) after 10 days growing on PDA and containing mycelium, spores and MS, or as an agar plug of MS (MSV) transplanted on PDA plate.

Evaluation of biofumigation potential of Brassica plant biomass on V. dahliae.

In vitro assay was developed to evaluate the biofumigation potential of RB, SB and a mix of root and shoot biomass (RSB), fresh or frozen, of white and brown mustard, radish, rape and rapeseed to control *V. dahliae*. The capacity of biofumigant cover crop on *V. dahliae* mycelial growth and MS germination was tested on DV and MSV fungus. For each fungus treatment, jars containing either 5 g of grinded RB, SB or RSB (4 g SB + 1g RB), fresh or frozen, were closed with inverted PDA petri containing DV or MS fungus. Each treatment were replicated 5 times corresponding to 300 jars in total. Control jars with inverted DV and MSV were prepared but no biofumigant material was added. Control were replicated 15 times. Jars were sealed with Parafilm® and incubated at 24 °C in the dark for 21 days. The radial growth of DV and MSV fungus was determined weakly.

Data analyses

Area under the fungus development progress curve (AUDPC) was calculated based on the weakly measurement of the fungus growth diameter on Petri dish. AUDPC was calculated according to the equation of Campbell and Madden (1990):

$$AUDPC = \sum_{i}^{n-1} (y_i + y_{i+1})/2 * (t_{i+1} - t_i)$$

where *n* is the number of measurement, *y* the growth diameter of the fungus, and *t* the days between each evaluation. Variables were analyzed by analysis of variance (ANOVA) using Statgraphics Plus 5.1 statistical software (Rockville, MA, USA) with replicate as a random variable. For each ANOVA, homogeneity of variance by Levene's test (confidence level of 0.95) and the normality of the residuals by the Shapiro-Wilks test (confidence level of 0.95) were conducted. When the F ratio was significant ($P < 0.05$), differences between treatment means were determined using protected least significant difference (LSD).

RESULTS

GSL concentration in Brassica crops

The GSLs profile and concentration in the shoot and root tissues of each biofumigant crops are shown in the Table 1. The contrasting GSL profiles between the five Brassica species, and within RB and SB is significant. The GSL profiles of white mustard and rape had significant aromatic GSLs with main concentration of sinalbin in SB and gluconasturtiin in BR respectively. The brown mustard GSLs profiles were dominated by sinigrin (aliphatic GSL) in BS and radish by unknown indole 16.3 (indole GSL) in RB. The GSLs profile of the rape biomass was more diverse including appreciable concentrations of glucobrassicanapin (aliphatic GSL), gluconasturtiin (aromatic GSL) and neoglucobrassicin (indole GSL) mainly in RB.

Table 1. Type and mean concentration of main glucosinolates in the shoot (SB) and root (RB) in the tissues of *Brassicas* biofumigant crop varieties

	Glucosinolate concentration (µmol.g^{-1} dry weight tissue)									
	White Mustard		Brown Mustard		Rape		Rapeseed		Radish	
	SB	RB	SB	RB	SB	RB	SB	RB	SB	RB
Aliphatic										
Sinigrin	1,3	0,0	42,1	3,6	0,0	0,0	0,0	0,0	0,0	0,0
Glucoerucin	3,0	0,1	0,0	0,0	0,0	0,6	0,0	1,1	0,0	0,0
Glucoraphanin	0,2	0,0	0,0	0,0	0,0	0,0	0,1	0,0	0,0	0,0
Gluconapin	0,0	0,0	0,0	0,0	0,0	0,0	0,2	0,1	0,0	0,0
Progoitrin	0,0	0,0	0,0	0,0	0,9	2,6	0,1	0,3	0,0	0,0
Glucobrassicanapin	0,0	0,0	0,0	0,0	1,9	4,2	0,3	0,2	0,0	0,0
Aromatic										
Gluconapoleiferin	0,0	0,1	0,0	0,0	1,6	3,0	0,0	0,1	0,0	0,0
Gluconasturtiin	0,1	2,8	0,0	2,4	0,0	13,4	0,0	15,2	0,0	0,0
Glucotropaeolin	2,8	0,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Sinalbin	15,1	4,0	0,0	0,0	0,0	0,0	0,0	0,1	0,0	0,0
indole										
4-hydroxyglucobrassicin	0,0	0,1	0,0	0,1	0,5	1,0	0,0	0,1	0,0	0,0
Glucobrassicin	0,0	0,1	0,2	0,0	0,7	0,8	0,7	0,4	5,2	0,1
4-methoxyglucobrassicin	0,1	0,2	0,0	0,0	0,5	1,1	0,1	0,2	0,3	0,2
Neoglucobrassicin	0,0	0,4	0,1	0,4	1,5	4,9	0,8	2,2	0,0	0,0
Unknown indole 16.3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	7,4	41,6

Biofumigation potential of plant biomass on V. dahliae development

Results from the *in vitro* assays to evaluate the incidence of different biofumigant crops showed that the *V. dahliae* development or germination, measured by the AUDPC, was significantly ($P < 0.05$) affected both for the fungus developed (DV) or the plug of microsclerotia (MSV) compared with the nonamended control (Fig. 1A and B). The AUDPC

was reduced by 63 to 90 % according to the species and a stronger impact on MSV fungus than DV was observed.

The biofumigant potential of the five *Brassica* toward *V. dahliae* differed according to the fungus stage (DV /MSV) (Fig. 1). From DV fungus, brown mustard and rape were more effective and radish was less effective to reduce mycelial growth of *V. dahliae* (Fig. 1A). From MSV, rape and radish were more effective than white mustard to reduce the MS germination of *V. dahliae* (Fig. 1B).

Regarding the type of biomass, there was no significant ($P > 0.05$) difference of RB, SB or RSB on fungus development for the different cover crop except for SB of brown mustard and RB of rape in DV, and for RB of the radish in MSV that reduced significantly ($P < 0.05$) the progression of the fungus. Because there was no significant effect of the cover crop biomass conditioning (fresh or frozen) on fungus development (DV and MSV), statistical analyses were performed on pooled data.

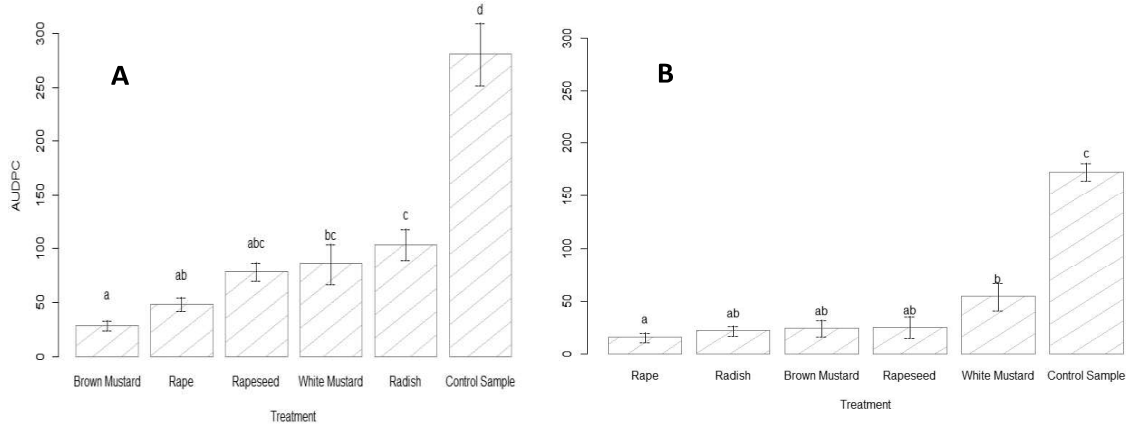

Figure 1. Effect of cover crop types and non-amended control on *V. dahliae* development and germination as measured by the area under the fungus development progress curve (AUDPC) for pooled data from conditioning and type of biomass from the fungus developed (DV) (**A**) and the plug of microsclerotia (MSV) (**B**). Within figures, means followed by letters are significantly different from one another based on LSD ($P < 0.05$).

DISCUSSION

The aim of using cover crop to manage soilborne diseases is mainly the elimination of the inoculum source to reduce yield losses caused by pathogens infections. In this study, assessing the potential of *Brassica* for their biofumigation potential toward *V. dahliae* mycelial growth and the ability of microsclerotia to germinate was evaluated *in vitro*. Optimizing this method for control of *V. dahliae* requires knowledges of the type and concentration of the different GSLs occurring in the respective plant tissues. Therefore, the relative toxicity of ITCs released by their precursor GSLs in root and shoot biomass of white and brown mustard, radish, rape and rapeseed toward *V. dahliae* was tested.

In this study, the five cover crop treatments resulted in statistically significant ($P < 0.05$) reduction of the fungus development from *V. dahliae* developed (DV) and from microsclerotia (MSV) compared with the non-amended control. The toxicity of biological

compounds induced by the grinded biomass and more specifically the ITC-liberating GSLs in the tissues of *Brassica* crops towards *V. dahliae* was confirmed, which is in accordance with other studies testing *in vitro* toxicity of ITCs to other soilborne fungi (Manici et al., 1997 ; Sarwar et al., 1998 ; Smith and Kirkegaard, 2002,). However, the toxicity of ITC showed contrasted effect depending on whether the fungus was mycelial developed or from microsclerotia, which has not been investigated in the literature before. From DV, the mycelial growth of *V. dahliae* was significantly reduced with brown mustard and rape. From MSV, rape, brown mustard and radish blocked the germination of MS.

Brown mustard produced amounts of 2-propenyl ITC from sinigrin GSL (Kirkegaard and Sarwar, 1998; Morra and Kirkegaard, 2002) and the toxicity of this aliphatic GSL could have a significant biofumigation potential as confirmed towards *V. dahliae* and other soilborne pathogens (Angus et al., 1994; Mayton et al., 1996 ; Olivier et al., 1999 ; Smolinska and Horbowicz, 1999 ; Larkin and Griffin, 2007 ; Neubauer et al., 2014). For the DV treatment, the radish could be rated as a poor biofumigation crops as concluded by Neubauer et al., (2014) who evaluated the biofumigation potential of the culture by the aliphatic and aromatic GSL concentrations. However, the radish blocked significantly the germination of MS, more than the brown mustard. The high concentration of unknown indole 16.3 (indole GSL) could be involved but the biofumigant potential of indole GSL has not been studied against soilborne pathogens but more toward plant-parasitic nematodes (Ruanpanun et al., 2010 ; Kruger et al., 2013). Contrary to the radish and brown mustard, the sensitivity of the fungus toward rape biomass was equivalent in DV and MSV who reduced the viable MS by 90 % and *V. dahliae* growth by 83 % compared with the control. Despite the total concentration of aliphatic GSL (10.2 $\mu mol.g^{-1}$ DW) and indole GSL (10.9 $\mu mol.g^{-1}$ DW) was significantly lower than brown mustard (45.6 $\mu mol.g^{-1}$ DW) and radish (54.8 $\mu mol.g^{-1}$ DW) respectively, the rape biomass released a wider diversity of GSLs with aliphatic, aromatic and indole GSLs whereas those were specifics in one type of GSL. This could thus be involved in the high biofumigant potential of the rape, and the high concentration of one type of GSLs would not be predominant to reduce or suppress the development of *V. dahliae*. Regarding the incidence of white mustard and rapeseed to reduce the pathogen growth compared to the other, their profile predominant in gluconasturtiin and sinalbin (aromatic) GSL could explained this lower toxicity due to their lower volatility (Sarward et al., 1998). Although their biofumigation potential has been demonstrated before, aromatic GSLs released from these *Brassica* crops did not seems as effective for biofumigation as those from brown mustard, rape and radish.

CONCLUSION

These results demonstrate that *Brassica* cover crop are able to reduce *V. dahliae* growth and microsclerotia germination. Moreover, the importance of identifying which GSLs type release the most toxic hydrolysis products toward the pathogen was underline. Inhibition of fungi development by grinded cover crops containing sinigrin (aliphatic), unknown indole 16.3 (indole) or a wider diversity of GSLs hydrolysis in *Brassica* tissue was superior to aromatic GSL, suggesting an important role for these compounds in the pest suppression potential of some *Brassica*. The variation in toxicity of different GSLs - ITCs to the fungi suggests there is significant scope to enhance the biofumigation potential of these crops by selecting those which produce a wider diversity of GSLs precursors to the most toxic ITCs such as rape to suppress Verticillium wilt in sunflower fields.

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