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Efficacy of Different Forms of Green Manure Crops to Reduce *Verticillium dahliae* in Different Soils

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ABSTRACT

The efficacy of green manure crops to reduce the number of *Verticillium dahliae* microsclerotia in different soils was investigated. Green manures tested were Indian mustard with a high glucosinolate content and sorghum-sudangrass as biocidal plants, and Indian mustard with a low glucosinolate content and rye as non-biocidal plants. The green manure plants were applied in fresh, dried, and ensilaged form. When applied as fresh plants, the glucosinolate content determining the biocidal activity of Indian mustard was only important in loam soil but not in sandy loam soil. In the latter soil, the non-biocidal rye had significantly higher efficacy than the Indian mustard. Volatiles released by fresh and dried, but not ensilaged, Indian mustard with a high glucosinolate content strongly decreased the number of living *V. dahliae* microsclerotia. When the same green manure crops were added to sandy loam and clay loam soil, the effect of the high glucosinolate content Indian mustard in fresh and dried form disappeared, whereas the ensilaged green manure crops had the highest efficacy. This effect was based on the increase of the soil microbial activity and the *Streptomyces* population size, which were negatively correlated with the number of living *V. dahliae* microsclerotia in the soil.

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1. Introduction

One of the most important soilborne diseases worldwide is *Verticillium* wilt, caused mainly by *Verticillium dahliae* Kleb. [1]. More than 400 plant species are known to be host plants of *V. dahliae* [2], and new host plant species are detected continuously. Economic losses are significant in high-value horticultural crops. A highly effective way to control Verticillium wilt is using chemical fumigants, especially methyl bromide [1]. With the phasing-out of methyl bromide in 2005 [3], the research for alternative methods to control soilborne diseases became important. Among the promising alternatives to control *V. dahliae* and other major soilborne pathogens is the use of green manures [4]. Especially interesting is the concept of biofumigation, which is based on the application of plant species that release volatile biocidal molecules after their incorporation into the soil [5]. The term biofumigation describes in the first line the use of glucosinolate (GSL) containing cruciferous species, which release isothiocyanates (ITC) during their decomposition [6]. Other plants that contain biocidal molecules are species of the genus *Sorghum* containing the glucoside dhurrin, which can be degraded in hydrogen cyanide [7]. Green manures do not affect soilborne pathogens through the generation of biocidal molecules only. They also affect the soil microbial communities by the release of easily degradable organic molecules during their breakdown. The thereby initiated stimulation of the soil microbial activity is the major driver of soil fungistasis [8]. The reduction of fungal pathogens in the soil by incorporating green manures or plant-derived products is partly caused by the increase of specific groups of antagonistic microorganisms. One such group is *Streptomyces*, whose inhibitory activity was increased after incorporating green manure crops in the soil [9].

In Europe, green manures are mainly used to cover the soils during the fall and winter seasons with the goal of reducing soil erosion and nitrate leaching [10]. In contrast, the use of green manures to control soilborne diseases is not widespread. In Switzerland, Verticillium wilt is a major problem for greenhouse vegetables grown in narrow crop frequencies of *V. dahliae* host plants such as tomato (*Solanum lycopersicon* L.), eggplant (*Solanum melongena* L.), and lettuce (*Lactuca* spp.). Horticulturists are reluctant to grow green manures in the greenhouses because it is expensive. Especially in winter, when heating adds additional costs, the greenhouses can be better used to cultivate a saleable crop, such as lettuce or corn salad (*Valerianella* spp.). Another important horticultural crop threatened by *V. dahliae* in Switzerland is strawberry (*Fragaria* x *ananassa* (Weston) Duch.), for which the major part of the production is based on the use of Frigo plants. The time gap between the end of the harvest (end of May – beginning of June) and the planting of the Frigo plants (end of June) is too short to cultivate green manure. A solution for both cases, i.e., greenhouse vegetables and strawberries, could be applying green manures in dried or ensilaged form. Drying green manures strongly reduces their weight, whereas the interest in silage is the limited loss of plant material, especially leaves.

The control of soilborne pathogens by the addition of organic or inorganic amendments is strongly influenced by the soil texture and organic matter content [11-13]. Including several soil types is, therefore, a prerequisite for the evaluation of the efficacy of green manures to reduce the number of *V. dahliae* microsclerotia in the soil. Such a reduction is negatively correlated to the severity of Verticillium wilt of several horticultural crops [14-15].

This study aims to assess the efficacy of different green manure crops to reduce the number of *V. dahliae* microsclerotia in different soils. The second aspect investigated was the influence of the conservation form of the green manure crops on their efficacy.

2. Materials and Methods

2.1. Plant Species and Soils

Biocidal plants tested were Indian mustard (*Brassica juncea* L.) with high GSL-content and sorghum-sudangrass (*Sorghum bicolor* (L.) Moench x *Sorghum sudanese* (Piper) Stapf); non-biocidal plants were Indian mustard with low GSL-content, and rye (*Secale cereal* L.) (Table **1**). Different cultivars of Indian mustard with high and low GSL-content were included to investigate the role of the GSLs of this plant species. Seeds were sown in 3 or 7 liters plastic pots (latter one only for sorghum-sudangrass) filled with a commercial, fertilizer-enriched peat substrate. The pots were placed in a greenhouse for 6 to 10 weeks until the harvest of the plant material. Growth duration

varied depending on the plant species and the growing season. Temperatures varied between 18 to 25°C, and additional light was supplemented with high-pressure sodium lamps from 6 am to 6 pm during the winter season. All species, except the high GSL-content Indian mustard cultivars, were supplemented with a 0.1 M NH_4NO_3 solution at a weekly interval starting two weeks after sowing. The Indian mustard cultivar with a high glucosinolate content was supplemented with a 0.1 M $(NH_4)_2SO_4$ solution in order to favor the formation of GSLs by a sulfur-containing fertilizer [16].

Table 1: Plant species used as green manure crops in the experiments.

Species	Latin Binomial	Cultivar	Breeder	Special Features
Indian mustard	*Brassica juncea*	ISCI-99	Triumph Italia, Livorno, Italy	High GSL-content [a]
Indian mustard	*Brassica juncea*	Arid	SKP-AAFC, Regina, Canada	Low GSL-content
Rye	*Secale cereale*	Wiandi	KWS Lochow, Bergen, Germany	Forage type
Rye	*Secale cereale*	Borfuro	Saatzucht Steinach, Steinach, Germany	Forage type
Sorghum-sudangrass	*Sorghum bicolor* x *S. sudanense*	Susu	Feldsaaten Freudenberger, Krefeld, Germany	Forage type

[a] Patalano, 2004.

The above-ground part of the plants was harvested at the tillering to beginning stem elongation stage (BBCH 22 to 31) for rye and sorghum-sudangrass and at the beginning to full flowering stage (BBCH 61 to 65) for Indian mustard. Dried plant material was obtained by drying fresh plants immediately after harvest in an oven set at 35°C for 48 h. Dry plant material was stored until utilization in tightly closed polyethylene bags in the dark at room temperatures. Ensilage plant material was prepared by cutting fresh plants in 5 cm long pieces, which were placed in a 1-liter glass jar with metal clamps and a vulcanized rubber gasket lid. The plant material was compacted manually to remove as much air as possible before closing the jar airtight. Jars were then stored until utilization in the dark at room temperature. Silage was prepared several weeks before their intended use to allow the complete fermentation of the plant material.

Soils from four sites were included in the pot trials (Table **2**). Sites Epines, Riddes, and Conthey are located in the canton Valais in the southern part of Switzerland. Site Lamothe is the location of the experimental farm of the Ecole d'Ingénieur de Purpan close to Toulouse in the southern part of France. All three Swiss soils were naturally colonized by *V. dahliae,* whereas in the soil Lamothe no *V. dahliae* could be detected. The latter soil was included in this study because it represents as soil with a quite different soil texture compared to the three Swiss soils. After collection of the soils, they were stored moist at 4°C in open polyethylene bags or air-dried at room temperature.

Table 2: Soils used in the experiments.

Site	Soil Type [a]	% Clay	% Silt	% Sand	% Organic Matter[b]	pH [c]	Soil Use
Epines	sandy loam	6	19	75	2.2	7.9	Experimental farm, open field
Riddes	loam	10	48	42	2.2	8.0	Organic grower, plastic tunnel
Conthey	sandy loam	8	20	72	2.3	7.8	Experimental farm, greenhouse
Lamothe	clay loam	32	37	31	2.4	6.6	Experimental farm, open field

[a]USDA soil texture scheme.
[b]organic matter = organic C * 1.75.
[c]Extraction in deionized water 1:2.5 (dry soil (w) : water (v)).

2.2. Soil Parameters Analysis

A dry sieving method was used to determine the number of living *V. dahliae* microsclerotia in the soil. Six weeks after sampling, air-dried soil was sieved at 0.5 mm mesh size and homogenized. Per soil sample, five 100 mg-aliquots were dry-plated [17] on Sorensen's NP-10 semi-selective medium [18]. The NP-10 plates were incubated

in darkness at 24°C for two weeks, after which soil was removed from the medium surface by adding tap water and scraping gently with a glass slide. The number of *V. dahliae* microsclerotia forming colonies was counted under a dissecting microscope.

The number of *Streptomyces* spp. in the soil was determined by dilution plating using a semi-selective double-layer agar method [8]. Per soil sample, three air-dried 10 g aliquots were analyzed. The 10 g dry soil was added to 90 ml of 0.9% sterile NaCl solution in a 250 ml Erlenmeyer flask and shaken at 175 rpm for 1 h. The soil suspension was then serially diluted, and per dilution step, 100 µl-aliquots were spread on three water agar (WA) plates. The suspension was completely covered with 5 ml of liquid starch casein agar (SCA; [19]). After solidification of the SCA, the plates were incubated in darkness at 24°C. After one week, the *Streptomyces* spp. density expressed as colony-forming units (CFU) per g dry soil was determined.

The soil microbial activity was measured with the fluorescein diacetate (FDA) hydrolysis method [20]. Per soil sample, four 5 g-aliquots of moist soil were analyzed. For wet soil samples, coarse soil particles and clearly visible parts of green manures were removed manually. For dry soil samples, the soil was sieved at a mesh size of 2 mm. All soil samples of one experiment were treated the same way. Five g soil was added to 20 ml of 60 mM potassium phosphate buffer (pH 7.6) in a 250 ml Erlenmeyer flask. Three aliquots received 0.2 ml of FDA stock solution (2 mg FDA ml^{-1} acetone), and the fourth aliquot served to determine the background absorbance. Erlenmeyer flasks were placed on a rotary shaker with a controlled temperature set at 90 rpm and 20°C. The reaction was stopped after 20 min by adding 20 ml acetone. After filtration through Whatman#1 filter paper, absorbance was measured at 490 nm with a photo-spectrometer. Soil samples were stored at 4°C until analysis for a maximum duration of 4 weeks. Before analysis, the samples were placed at room temperature for 1 h, and the buffer solution was kept at 20°C in an incubator to ensure identical conditions for all FDA analyses.

Soil electrical conductivity and soil pH were determined in an air-dried soil: deionized water extract (1:2.5, w:v), the official extraction method in Switzerland. The electrical conductivity was measured with a conductivity meter and soil pH with a pH meter.

2.3. Plant x Soil Experiment

In a pot trial, two different soil types quite common in Switzerland were mixed with fresh plant material. The two soils naturally infested with *V. dahliae* were Riddes and Epines, loam soil, and sandy loam soil, respectively (Table **2**). Plants included in the trial were the two Indian mustard cultivars ISCI-99 and Arid, with a high and a low GSL-content, respectively, and the rye cultivar Wiandi (Table **1**). As an additional treatment, the application of biocidal pellets (brand name: Biofence, Triumph Italia, Livorno, Italy) was included in this trial. The pellets are manufactured from partially defatted seed meal of Ethiopian mustard (*Brassica carninata* A. Braun) and are commercialized as organic fertilizer with a 6% organic N content [21]. These pellets contain high amounts of GSLs which are transformed to ITCs during the decomposition of the product in the soil [22]. Shredded fresh plants were mixed with the moist soils at a ratio of 9.1:1 and 12.5:1 of moist soil:fresh plant material (v:w) for Indian mustard and rye, respectively. The assumed fresh matter production was 11 kg/m^2 for Indian mustard and 8 kg/m^2 rye. The ratio of moist soil:Biofence pellets was 400:1 (v:w), which corresponds to an amount of 250 g $pellets/m^2$. For each plant or pellets x soil mix, four 640 ml aliquots were placed in a plastic pot with 0.7 liter volume. Pots were watered to the field capacity point, which was determined prior to the experiment as the weight two days after full water saturation, and then placed in a dark incubation room with the temperature ranging from 18.5 to 20.5°C. For the control treatment, bare soil was processed the same way as the plant or pellet x soil mixtures. After one week of incubation, samples for the determination of the *V. dahliae* population and soil microbial activity were taken. The trial was conducted twice, in May and September 2008.

2.4. Plant form Experiment

The efficacy of three different forms of green manure crops, i.e., fresh, dried, and ensilaged plants, to reduce the *V. dahliae* in two different soil types was investigated in a pot trial. The two soils were Conthey and Lamothe, sandy loam and ~~a~~ clay loam type, respectively (Table **2**). The clay loam Lamothe was not naturally infested with *V.*

dahliae; therefore, the two soils were artificially infested with *V. dahliae* microsclerotia. Four strains of *V. dahliae* were used to produce viable microsclerotia [23]. The strains were strain Aa#2, isolated from annual wormwood (*Artemisia annua* L.) [24] strain BDE-572, isolated from eggplant, strain Vd-Michel, isolated from Japanese maple (*Acer palmatum* Thunb.) [24], and strain BDE-442, isolated from strawberry. Microsclerotia of the four strains with a size between 64 to 125 µm were mixed with acid-washed quartz sand and stored in the dark at room temperature until use.

The plants included in the trial were Indian mustard cultivars ISCI-99 and Arid with a high and a low GSL-content, respectively, rye cultivar Borfuro and sorghum-sudangrass cultivar Susu (Table **1**). An additional treatment was the application of the methyl-ITC generating soil fumigant dazomet. For the calculation of the amount of material to be mixed with the soils, the assumption was based on a quantity of fresh matter of 5 kg/m^2 and incorporation depth of 15 cm, i.e., the average depth between a rotavator (10 cm) and a spading machine (20 cm). Shredded fresh and ensilaged plants were mixed with the air-dried soils previously sieved at 4 mm mesh size at a ratio of 30:1 of air-dried soil:fresh plant material (v:w). As the dry matter content of the dried plants was six times the fresh and ensilaged plants, a ratio of dried plant material:air-dried soil of 180:1 was applied to add the same amount of dry organic matter to the soils. The ratio for dazomet, applied as Basamid (Syngenta, Basel, Switzerland), was 2500:1 (v:w), which corresponds to an amount of 60 g granules/m^2, the maximum amount authorized in Switzerland. Before adding the plant material or dazomet, the soils were inoculated with *V. dahliae* by mixing 20 g of microsclerotia/sand mixture with 0.8 liters of soil for 1 min in a concrete mixer. The 0.8 liters of inoculated soil were then mixed with the corresponding amount of plant material of dazomet for 3 min. For each plant material or dazomet x inoculated soil mix, four 200 ml aliquots were placed in a plastic pot with 0.25-liter volume. Pots were watered to the field capacity point by adding 50 or 60 ml of tap water to the pots with soil from Conthey or Lamothe, respectively. Additional 10 ml water was added to the treatments with dried plant material to compensate for the lower quantity of water of this plant material form. For the control treatment, bare soil was processed the same way as the plant or dazomet x soil mixtures. The pots were placed in a dark incubation room with a temperature range from 19 to 20°C. The weight of each pot was measured at the beginning of the trial, and after one week, water was added to achieve the initial soil moisture status for each pot. After two weeks of incubation, samples were taken to determine the *V. dahliae* population, *Streptomyces* population, soil microbial activity, soil electrical conductivity, and soil pH. The trial was conducted twice, in April and May 2015.

2.5. Fumigant Experiment.

The direct effect of the volatiles generated by the plant material or dazomet was tested in a jar trial. The same amount of shredded fresh ensilaged or dried plant material or dazomet as added to the pots in the plant form experiment was placed on the bottom of a 0.5-liter glass jar. This quantity was based on the assumption of an average soil porosity of 50% [25]. Therefore, the pore volume in the pot trial, with 0.8 liters of soil per treatment, corresponding to 400 ml, whereas the volume in the jar corresponded to 420 ml (500 ml – 80 ml occupied by four Petri dishes, see below). Glass jars were equipped with metal clamps and a vulcanized rubber gasket to achieve complete airtightness. A metallic grid was put on the plant material (Fig. **1A**), on which four with *V. dahliae* inoculated plastic Petri dishes (5 cm diameter) without lids were placed. Each jar received 2 x 2 types of Petri dishes to test the effect on the germination of microsclerotia and on the growth of mycelium. For each of the four replicates, the Petri dishes were placed in a different position of a predefined order (Fig. **1B**) to avoid a possible gradient of the volatiles distribution in the jar.

For the germination test, a suspension of *V. dahliae* microsclerotia from strain Aa#2 in sterile deionized water was sprayed with an airbrush (REVELL 39199, Bünde, Germany) on Petri dishes containing NP-10 medium. The compressor of the airbrush was surface sterilized with 70% ethanol and placed on a sterile bank to avoid the aspiration of fungal spores suspended in the laboratory's air. Immediately after spraying the microsclerotia suspension, 50 to 60 microsclerotia per petri dish were marked with a green dot on the bottom of the dish (Fig. **2**). The mycelium growth test was effectuated with strain Aa#2 grown on PDA (potato dextrose agar) medium in the dark at 24°C. From an actively growing colony, a 6 mm diameter agar plug was taken and placed in the middle of a PDA containing a Petri dish (Fig. **2**). After placing the four Petri dishes in the jar, 55 ml of tap water was added to cover the material on the bottom. The jar was then closed hermetically to avoid any losses of the volatiles formed

by the materials. The jars were incubated in the dark at 19°C for 24 h. The Petri dishes were then removed from the jar, the lid was added, and they were incubated in the dark at 24°C. After one and two weeks of incubation, the percentage of germination and the mycelium diameter were assessed for the germination and growth test, respectively. The trial was conducted twice, in March and April 2015.

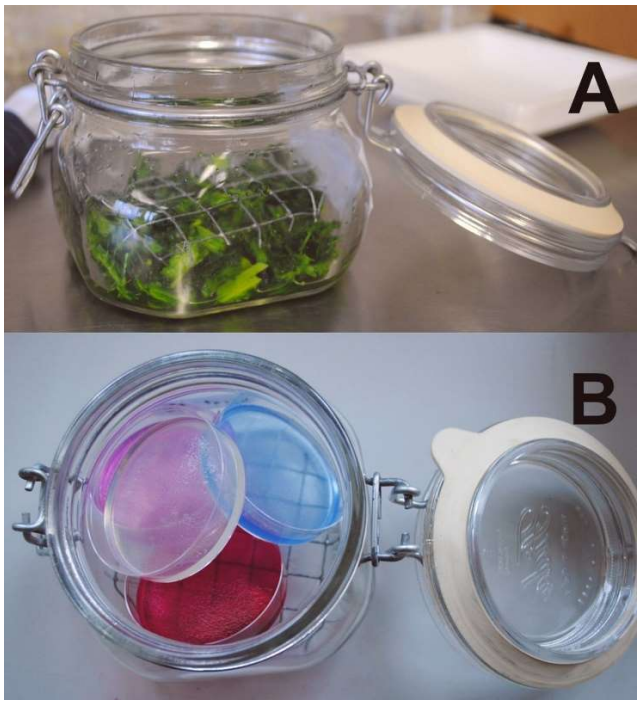

Figure 1: A glass jar with 0.5-liter volume is equipped with metal clamps and a vulcanized rubber gasket containing a metallic grid placed over shredded fresh plant material (**A**). Petri dishes (5 cm diameter) were placed in different positions of a predefined order (**B**), indicated by different colored agar (for demonstration purposes only).

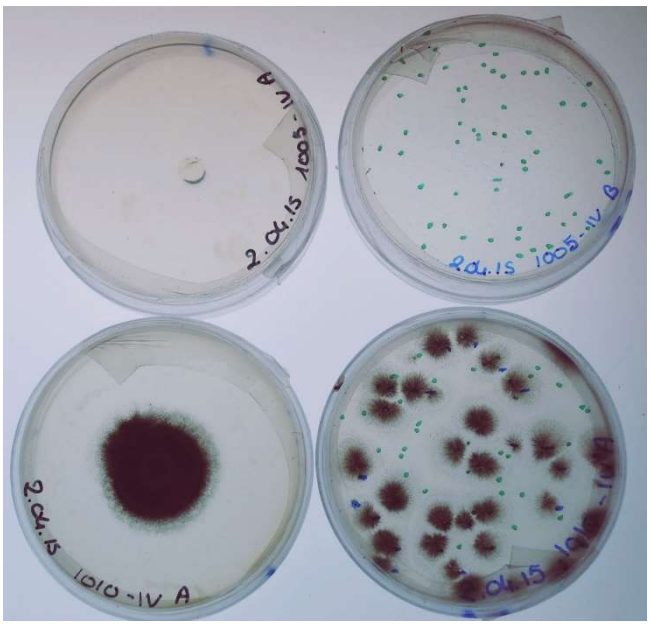

Figure 2: Petri dishes (5 cm diameter) for mycelium growth test on PDA medium (left side) and microsclerotia germination test on NP-10 medium (right side) of *Verticillium dahliae*. Efficacy was assessed by measuring mycelium diameter or counting the number of germinated microsclerotia. Latter ones were marked with a green dot immediately after their deposition on the agar. The top treatment with high efficacy is Indian mustard ISCI-99 in dry form, the bottom treatment with a low efficacy is Indian mustard Arid in ensilaged form, both after two weeks of incubation at 24°C.

2.6. Data Analysis

For the plant x soil and the plant form experiments, the number of living *V. dahliae* microsclerotia in the soil was transformed into a relative number of microsclerotia with the control treatment set to the value of 1. For this transformation, which allows a better comparison of the soil effect than the absolute number of living microsclerotia, the average value of the control treatments calculated for each trial separately was set to 1, thereby maintaining the variance in the control treatment. For the fumigant experience, non-transformed data of the number of living microsclerotia were used for statistical analysis.

Data were analyzed using SigmaPlot 13 (Systat Software, Inc., San Jose, CA). Analysis of variance (ANOVA) was used for all trials, and in cases of significant differences at $P < 0.05$, multiple comparisons were run using the Tukey test. In the plant x soil experiment and the plant form experiment, the influence of the soil parameters on the relative number of living microsclerotia was analyzed with linear or stepwise regression analysis.

3. Results

3.1. Plant x Soil Experiment

Adding green manures and biocidal pellets to the two soils Epines and Riddes, resulted in significantly different numbers of living *V. dahliae* microsclerotia ($P < 0.001$). All three green manures resulted in a significant reduction of living microsclerotia in both soils, whereas the effect of the biocidal pellets was not significantly different from the control (Fig. **3A**). However, the efficacy of the treatments was strongly influenced by the soil type (interaction treatments x soil: $P < 0.001$). In the sandy loam soil Riddes, the Indian mustard cultivars significantly reduced the

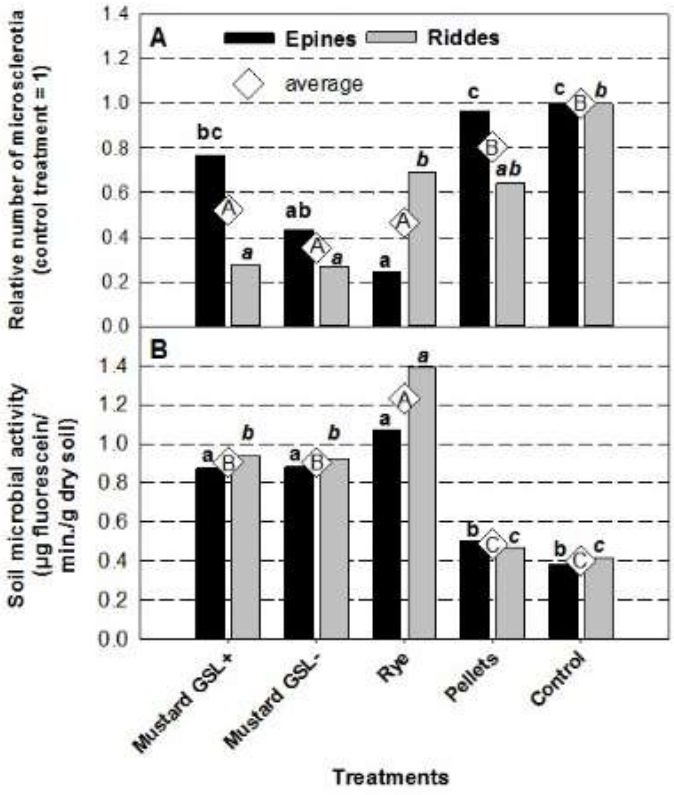

Figure 3: The relative number of living *Verticillium dahliae* microsclerotia (**A**) and soil microbial activity (**B**) of Indian mustard with a high glucosinolate content (GSL+, cv. ISCI-99) and a low glucosinolate content (GSL-, cv Arid), rye cultivar Wiandi, and biocidal Biofence pellets in the sandy loam soil Epines and the loam soil Riddes. Treatments with different letters are significantly different (Tukey-test, $P = 5\%$). Upper-case letters concern average values over both soils, lower-case normal letters soil Epines, lower-case italic letters soil Riddes. Data presented are the means of two trials.

number of living *V. dahliae* microsclerotia independently from their GSL content. However, rye as non-*Brassica* species did not significantly reduce the number of microsclerotia compared to the control. In contrast, in the sandy soil, Epines, rye, and the low GSL-content Indian mustard cultivar Arid significantly reduced the number of living *V. dahliae* microsclerotia. In contrast, the high GSL-content mustard ISCI-99 and the biocidal pellets were not significantly different from the control. The green manures significantly increased the microbial activity compared to the pellets and the control ($P < 0.001$). Rye had a more substantial effect than the two Indian mustard cultivars (Fig. **3B**).

The soil microbial activity was positively correlated with the efficacy to reduce the number of *V. dahliae* microsclerotia (Table **3**). Over both soils, the microbial activity explained a 26% (adjusted r^2-value) reduction in the number of living microsclerotia. In the sandy soil Epines, the effect of the soil microbial activity was more important with 36% than in the sandy loam soil Riddes with 18%.

Table 3: Linear regression between soil microbial activity (sma) as independent factor, and relative number of *V. dahliae* microsclerotia (rel_Vd_MS) as dependent factor of the plant x soil experiment. Data from both trials were bulked for analysis.

	Equation	number	adj. r^2-value	*P*-value
Both soils	rel_Vd_MS = 109.8 - (59.7*sma)	80	0.263	< 0.001
Epines	rel_Vd_MS = 128.2 - (80.7*sma)	40	0.358	< 0.001
Riddes	rel_Vd_MS = 95.8 - (46.3*sma)	40	0.181	0.004

3.2. Fumigant Experiment

The effect of the volatiles released by the different forms of green manure plants was highly significant on both the microsclerotia germination and the mycelium growth after 7 days ($P < 0.001$) and 14 days ($P < 0.001$). The Indian mustard cultivar ISCI-99 in the dried form completely reduced microsclerotia germination and mycelium growth even after 14 days (Fig. **4**). As a fresh plant, ISCI-99 reduced by 90% the microsclerotia germination and by more than 70% the mycelium growth 14 days after treatment. The dry form of the sorghum-sudangrass and, less pronounced, of the Indian mustard cultivar Arid significantly reduced the microsclerotia germination 7 days after treatment, but not after 14 days. Both treatments did not affect mycelium growth. On both dates, the soil fumigant dazomet had a significantly higher effect on the microsclerotia germination but not on mycelium growth than the control treatment. All other treatments were not significantly different from the control.

3.3. Plant form Experiment

The treatments resulted in significant differences for the parameters relative number of living *V. dahliae* microsclerotia, soil microbial activity, soil electrical conductivity, and *Streptomyces* population (all at $P < 0.001$), only the soil pH was not influenced. The soil type had a significant effect on parameters relative number of living *V. dahliae* microsclerotia, soil microbial activity, soil electrical conductivity, soil pH (all at $P < 0.001$), and *Streptomyces* population ($P = 0.002$). Interactions between treatments and soil type occurred for the parameters relative number of living *V. dahliae* microsclerotia ($P = 0.017$) and soil microbial activity ($P < 0.001$). The average reduction or the relative number of living *V. dahliae* microsclerotia was 26.5% and 61.4% in the soil Conthey and Lamothe, respectively. The higher effect in soil Lamothe was expressed by a significant reduction of the microsclerotia by all treatments with the exception of the fresh form of the Indian mustard cultivars Arid and ISCI-99 (Fig. **5A**).

In contrast, in soil Conthey, only ensilaged rye and dazomet significantly affected the number of living microsclerotia. The soil microbial activity was significantly decreased in both soils by the fumigant dazomet (Fig. **5B**). In contrast, all green manures except fresh sorghum-sudangrass significantly increased the microbial activity in soil Conthey. In soil Lamothe, where the soil microbial activity in control was 83% higher than in soil Conthey, the influence of the plant material was less pronounced; 7 out of 12 treatments had a significant effect. The

treatments did not affect the soil pH and only slightly affected electrical conductivity (data not shown). For the latter parameter, only dried rye did increase the electric conductivity significantly compared to the control. The *Streptomyces* population was significantly increased, mainly by the ensilaged form of the green manures, i.e., the Indian mustard cultivar Arid, rye, and sorghum-sudangrass silage (Fig. **5C**). The two other treatments with a significantly positive effect on *Streptomyces* were the dried Indian mustard cultivar ISCI-99 and dried rye. No effect of the fumigant dazomet on the *Streptomyces* population was measured. The two parameters, soil pH and *Streptomyces* population, explain 18% (adjusted r^2-value) of the efficacy of the treatments (Table **4**). When dazomet, which knowingly acts by the generation of the toxic volatile molecule methyl-ITC, was excluded from the backward stepwise regression analysis, the soil microbial activity was the third parameter to explain the efficacy, with 32% of the effect explained by a combination of the three parameters soil microbial activity, soil pH and *Streptomyces*-population.

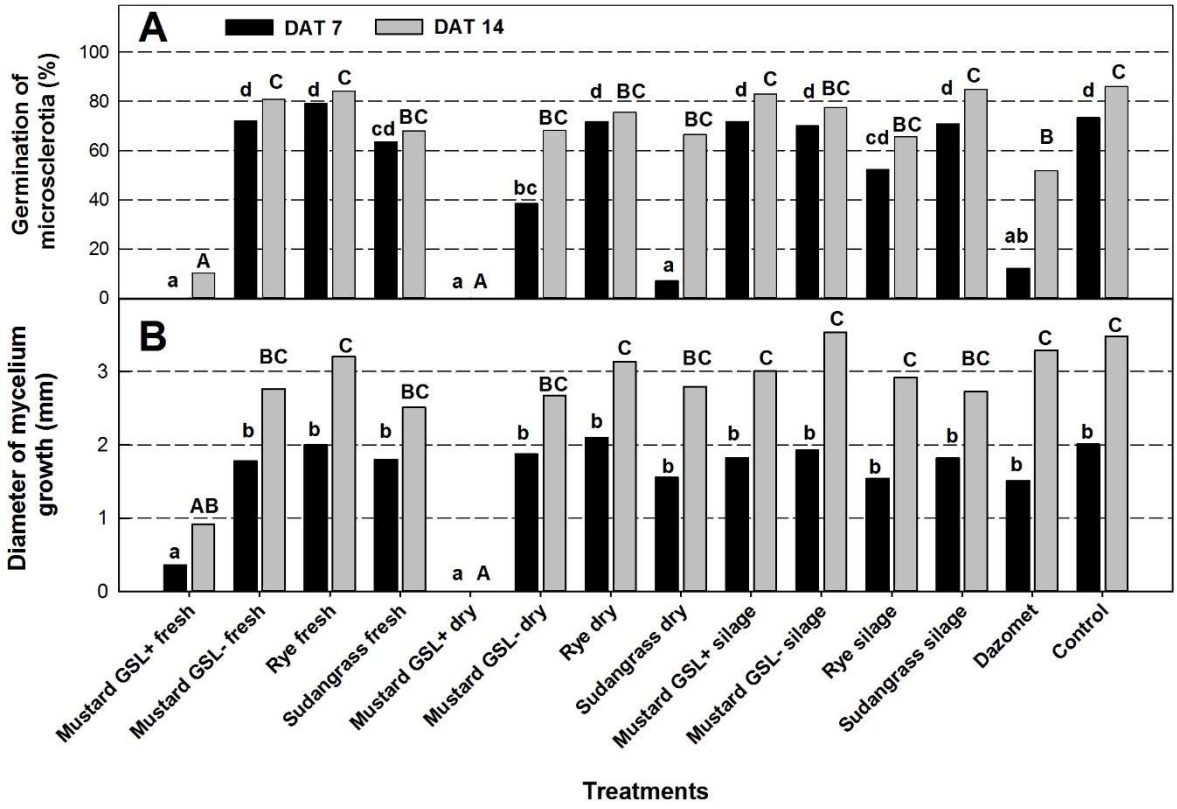

Figure 4: Effect on the germination of microsclerotia (**A**) and the mycelium growth (**B**) of volatiles, which are generated by Indian mustard with a high glucosinolate content (GSL+, cv. ISCI-99) and a low glucosinolate content (GSL-, cv Arid), rye (cv. Borfuro, sorghum-sudangrass (cv. Susu), and the soil fumigant dazomet (product Basamid). The plants were applied in fresh, dried, and ensilaged form. Data were assessed 7 and 14 days after treatment started (DAT). Treatments with different letters are significantly different (Tukey-test, *P* = 5%). Lower-case letters concern DAT 7, upper-case letters DAT 14. Data presented are the means of two trials.

4. Discussion

One of the prerequisites for a successful application of biofumigation is the incorporation of a sufficient amount of GSLs in the soil to generate a level of ITCs high enough to kill the inoculum of soilborne pathogens [6]. Therefore, the use of cultivars with a high GSL-content is recommended [11, 26]. The biofumigation effect of reducing the number of living *V. dahliae* microsclerotia was partially demonstrated in the plant x soil experiment. In the loam soil Riddes, no significant difference between the two Indian mustard cultivars ISCI-99 with a high and Arid with a low GSL-content occurred, but both were more efficient than rye. In contrast, in the sandy loam soil Epines, the efficacy of the Indian mustard cultivar ISCI-99 with a high GSL-content was significantly lower than the efficacy of rye and not significantly different from the untreated control. The two soils have very similar organic

matter contents and pH values but a quite different soil texture, mainly concerning the percentage of sand and silt. The lower effect of the high GSL-content Indian mustard ISCI-99 and the biofumigation pellets in the soil Epines might result from the more coarse texture of this sandy loam (75% sand, 6% clay). In soils with a high fraction of sand and a low fraction of clay, water retention is lower and larger pores are rapidly filled with air [27]. Such a situation increases the risks of ITC-losses by volatilization or leaching [5] Studies on the influence of the soil type on the efficacy of aliphatic ITCs, such as 2-propenyl ITC generated by sinigrin, the major GSL in Indian mustard cultivars [28] showed in the first line the negative correlation between soil organic matter content and biological activity of 2-propenyl ITC [11, 13].

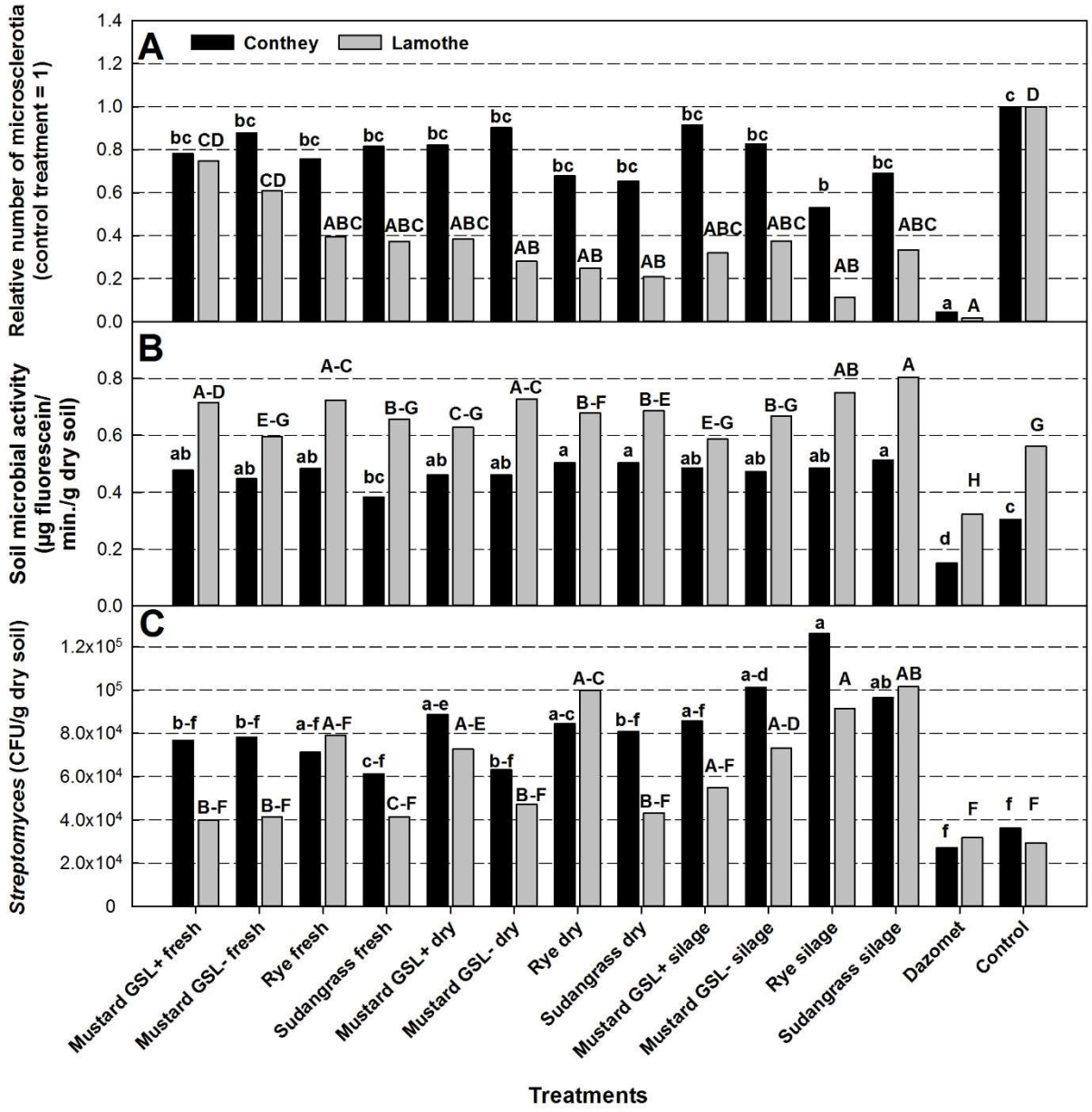

Figure 5: Effect on the relative number of living *V. dahliae* microsclerotia (A), on the soil microbial activity (B), and the soil *Streptomyces* populations (C) of Indian mustard with a high glucosinolate content (GSL+, cv. ISCI-99) and a low glucosinolate content (GSL-, cv Arid), rye (cv. Borfuro, sorghum-sudangrass (cv. Susu), and the soil fumigant dazomet (product Basamid) in the sandy loam soil (Conthey) and the clay loam soil (Lamothe). The plants were applied in fresh, dried, and ensilaged form. The parameters were measured 14 days after plant material and dazomet were added to the soils. For all three parameters, significant differences between the two soils existed. Treatments with different letters are significantly different (Tukey-test, P = 5%). Lower-case letters concern soil Conthey, upper-case letters soil Lamothe. Data presented are the means of two trials.

Table 4: Multiple linear regression of the plant form experiment based on a backward stepwise regression analysis including soil microbial activity (sma), soil pH (pH), electrical conductivity (EC), absolute *Streptomyces* population (Str.) and log10 *Streptomyces* population (log Str.) as independent factors and relative number of *V. dahliae* microsclerotia (rel_Vd_MS) as dependent factor. Data from both trials were bulked for analysis.

Equation	n	adj. r^2-value	*P*-value
With dazomet			
rel_Vd_MS dazomet = 2.15 + (0.39*pH) - (0.00000137*Str.)	224	0.182	< 0.001
Without dazomet			
rel_Vd_MS = 1.31 - (0.58*sma) + (0.333*pH) - (0.00000192*Str.)	208	0.322	< 0.001

Additionally to the organic matter, an influence of the soil type was reported to be responsible for reducing the lethal effect of 2-propenyl ITC on white-fringed weevil (*Naupactus leucoloma*) larvae [11]. The efficacy was slightly higher, i. e., lower LD_{95} concentration, with sandy soil compared to loamy soil. However, the insect larvae were not exposed to the ITC in but above the soil, i.e., in the headspace of the flask in which the soil was placed. In such a case, the higher efficacy over a sandy soil means more ITC accumulated in the headspace, which means that more ITC losses occurred from the sandy soil, which would fit with the lower efficacy in the sandy loam soil in the plant x soil experiment. No suppression on *V. dahliae* microsclerotia with biofumigation was reported from a field trial in soil with a very high sand fraction (94.9%). In this soil, the incorporation of a *B. juncea* green manure supplemented with a high amount of broccoli (*Brassica oleracea* L.) and also the chemical fumigation with metam-sodium resulted in no difference compared to the non-amended control treatment, whereas adding a large amount of chitin reduced the number of microsclerotia [29] significantly.

Adding easily degradable organic matter in the form of fresh green manure plants strongly stimulated in both soils of the plant x soil experiment the soil microbial activity, which is the major factor causing soil fungistasis [8]. Soil microbial activity expressed as FDA hydrolysis was associated with lower development of Verticillium wilt of potatoes, which was reduced by incorporating three green manures [30]. Among a series of microbiological, chemical, and enzymatic parameters, FDA hydrolysis was one of the most valuable features to identify the suppressiveness of organic amendments [31]. In the plant x soil experiment, the soil microbial activity expressed as FDA hydrolysis explained by 36% the effect of the organic amendments to reduce the number of *V. dahliae* microsclerotia in the sandy loam soil Epines, and to a lower extent of 20% in the loamy soil Riddes. This difference might be explained by a stronger effect of the volatiles generated by the high GSL-content Indian mustard ISCI-99 in the loamy soil Riddes. The 2-propenyl ITC generated by ISCI-99 is not only toxic to *V. dahliae*, but was also reported to affect negatively beneficial microorganisms such as ammonium and nitrite-oxidizing bacteria [32]. ITCs have unspecific toxicity [33], which could explain the lower importance of the soil microbial activity on the efficacy in the soil Riddes where ISCI-99 had the highest efficacy.

In both soils, incorporating fresh green manures increased soil microbial activity compared to non-amended soil. The incorporation of fresh Indian mustard plants was reported to increase the soil bacterial and fungal densities [34]. The increase of the bacterial density caused by the mustard incorporation was exceptionally high one week after incorporation. After the incorporation of a mixture of fresh wooly pod vetch (*Vicia dasycarpa* Ten.) and oats (*Avena sativa* L.), the soil microbial activity was increased compared to the non-amended control, with again the strongest FDA activity one week after incorporation [35]. In both studies, the increase of the microbial activity had no longer-lasting effect on the aimed pathogens *Rhizoctonia solani*, *Fusarium oxysporum,* and *Pythium aphanidermatum*, which might be caused by their high saprophytic capabilities to survive on the decomposing green manure material [31]. In contrast, *V. dahliae,* with very low saprophytic capacities [1], makes this pathogen more vulnerable to control methods based on the amendment of the soil with easily degradable organic matter. Incorporating sudangrass (*Sorghum vulgare* var. *sudanese*) or corn (*Zea mays* L.) resulted in a strongly reduced Verticillium wilt incidence and an increased yield of potato [36].

Another specific group of soil microorganisms stimulated by the incorporation of several green manure crops are *Streptomyces* [9]. Species of this genus are antagonistic to *V. dahliae* [37]. The antagonistic effect of

Streptomyces is based on their production of a range of enzymes capable of degrading the cell wall of fungal pathogens and on the production of antifungal compounds [38]. The production of antibiotics by *Streptomyces* is influenced by the carbon compounds used as a substrate of these saprophytic bacteria [39]. Organic amendments such as rapeseed seed meal are suited for the production of antibiotics by *Streptomyces thermoviolaceus* [40] and increase the *Streptomyces* densities in the soil [41]. In the plant form experiment, the *Streptomyces* population was positively correlated to the efficacy in reducing the number of *V. dahliae* microsclerotia. Among the three forms of green manures, silage resulted in the highest number of *Streptomyces* in the soil. Rye silage with the highest *Streptomyces* population was the only green manure treatment with a significant efficacy against *V. dahliae* in the soil Conthey. During the silage process, sugars are transformed into lactic acid under anaerobic conditions by lactic acid bacteria naturally occurring on the ensilaged plant material [42]. This process increases the digestibility of green plants for ruminants, and, as the first step of the digestion system of ruminants is based on microbial fermentation, it most probably also stimulates the growth of soil microorganisms. Next to an increase in the number of *Streptomyces*, the ensilaged forms might also have affected the production of antifungal compounds. The ability to inhibit the growth of *Pythium ultimum* and *Rhizoctonia solani* by a strain of *Streptomyces lydicus* was influenced by different sugars [43]. A high nutrient input also results in a more inhibitory *Streptomyces* community [39], which might help to explain the highest efficacy of silage as a form in which green manures were applied.

In contrast, the fumigant experiment clearly showed that the generation of toxic volatile molecules was not part of the suppressiveness of the ensilaged green manures. The very high effect of the volatiles released by the high GSL-content Indian mustard ISCI-99 in fresh and dried form was entirely canceled by the ensilage process of the same cultivar. One consequence of this process is a pH between 4.2 and 5.0 of the silages used in the fumigant and plant form experiments. The pH value influences the outcome of the GSL hydrolysis of the *Brassica* tissues [33]. At a low pH, non-volatile nitriles are generated in place of volatile ITCs. Drying as the other conservation form did not affect the efficacy of the volatiles released by the green manures. With a drying method similar to the one used in our experiments, GSL contents of different cruciferous species were reduced by less than 40%, and myrosinase activity was maintained at a sufficient level [22]. The dried form of the high GSL Indian mustard ISCI-99 had a more consistent efficacy compared to the fresh form. Dry low GSL Indian mustard Arid and sorghum-sudangrass had even a significantly higher efficacy against *V. dahliae* microsclerotia one week after application than their fresh form. A possible reason for the increased efficacy of the dry form might be the finer shredding grade of the dried plant material and no losses of volatiles during the shredding process, which eventually occurred for the fresh plants.

The low efficacy of dazomet in the fumigant experiment was in complete contrast to the very high efficacy of this chemical substance in both soils in the plant form experiment. This was explained by an additional fumigant experiment, in which one g of a soil mixture from Conthey and Lamothe was added to the dazomet before water was applied. This favored the hydrolysis of the dazomet to methyl ITC and resulted in a 100% efficacy to inhibit *V. dahliae* microsclerotia germination and mycelium growth (data not shown).

Our experiments included rye as non-biofumigant green manure, i.e., a plant species not releasing toxic volatiles. However, a small effect of volatiles generated by macerated rye plants on the growth of *Rhizoctonia solani* and *Pythium ultimum* was reported by [44]. But the initial suppressive effect of rye was not lasting, similar to results of the fumigant experiment, where after one week, no effect of rye on the microsclerotia and mycelium of *V. dahliae* occurred.

The effect of the toxic volatiles generated by the high GSL Indian mustard ISCI-99 in fresh or dried form appeared ineffective when the plant material was mixed with the soils. In the clay loam soil Lamothe, where nearly all treatments had a significant efficacy, fresh ISCI-99 resulted in the lowest efficacy, which was not significantly different from the control. Neubauer *et al*. [13] reported that the 2-propenyl ITC released by *B. juncea* reduced the number of living microsclerotia from 69 to 81% when the plant material was added to sterile quartz sand, but in naturally infected soil, the reduction was only 10 to 66%, which was considered as an insufficient level of control. Two factors were considered responsible for this lower efficacy: organic matter content of the soils [42, 13] and microbial degradation of the ITC [45-46]. The latter factor was probably not important in the plant form experience, as both soils used were not treated with ITC generating plants or products before they were collected.

But the organic matter content of the two soils Conthey and Lamothe, of 2.3 and 2.4%, respectively, were both above the organic carbon content of 1% (which corresponds to 1.75% organic matter) considered as critical for a successful application of biofumigation [13].

In the plant form experiment, the efficacy of the green manures was strongly affected by the soil type, as this was clearly demonstrated with one and ten treatments significantly different from the control in the soil Conthey and Lamothe, respectively. The mechanisms of suppressiveness to a specific soilborne pathogen can be quite different between soils and be based on different biological factors, such as mycoparasitism by fungi or antibiosis by actinomycetes [47]. But also, the composition of a specific group of soil microorganisms can be influenced by the soil type. The soil type more strongly influenced the diversity of fluorescent pseudomonads than the plant species cultivated on the soils [48]. The bacterial diversity in six soils was most influenced by the pH [49], and pH is considered as the main factor influencing bacterial community structures on a worldwide scale [50]. In the plant form experiment, the two soils had, next to a different soil texture, a pH value difference of 1.2. In this experiment, the efficacy in reducing the number of *V. dahliae* microsclerotia was negatively correlated to the pH value. Therefore, the lower pH of the soil Lamothe was most probably an important factor that caused the lower survival ability of *V. dahliae* microsclerotia in this soil. The other factor, the *Streptomyces* population, was positively correlated to reducing the *V. dahliae* population in the plant form experiment.

In contrast to soil pH can the size and/or composition of the soil *Streptomyces* populations be influenced by the addition of organic amendments [9, 51]. This aspect has to be taken into account for the application of organic amendments, especially when the soil health status is the priority [52]. Ensilaged green manures are especially well suited for this purpose. Not only have they a positive effect on the *Streptomyces* populations, but also are their application time and site-independent. This is a key factor for their use on prime horticultural soils, especially for greenhouse or peri-urban production sites. Applying green manures on a different site from where it was produced also avoids the multiplication effect on soilborne pathogens through the green manure crop [53].

The production and storage of dried or ensilaged green manures, however, causes additional costs. The economic aspects of the use of green manures have to be considered, and not always the most efficient treatment is also the most profitable [54]. The economic advantages of green manures concern not only the soil health status. Green manures can also improve the water infiltration rate [55] or reduce nitrate leaching [10]. The choice of biocidal vs. non-biocidal is an important question as the toxic molecules released by biocidal green manures are non-specific and can also affect beneficial soil organisms [56]. On the other hand, could the delayed N mineralization after incorporating biocidal *Brassicaceae* [57] be advantageous when substantial amounts of green manures are incorporated. Therefore, the decision of which kind and which form of green manures to apply depends on multiple factors that are different for each specific grower's situation.

5. Conclusion

In conclusion, the effect of green manure crops to reduce the number of *V. dahliae* microsclerotia is influenced by the soil texture, plant species, and conservation form. Biofumigant green manures such as Indian mustard with a high GSL content can be used in soils with a low sand fraction in fresh or dried form, but not as silage. In contrast, non-biocidal green manures are less influenced by soil texture. Next to their stimulation of the general soil microbial activity, they increase the soil *Streptomyces* populations, especially when applied in ensilaged form.

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